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GRAS Notification

***Dunaliella bardawil* Powder**
Food Usage Conditions for General Recognition of Safety

Volume 1 of 2

Submitted for:

Nikken Sohonsha Corporation (Nikken)
1-32 Asahira, Fukuju-cho, Gifu-Pref
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Submitted by:

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July 30, 2010

COVER LETTER

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REGULATORY ASSOCIATES, LTD.

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Sterling, Virginia 20166

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August 2, 2010

Robert L. Martin, Ph.D.
Office of Food Additive Safety (HFS-255)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

VIA: Courier

RE: GRAS Notification (GRN) for *Dunaliella bardawil* powder

Dear Dr. Martin:

Phoenix Regulatory Associates, Ltd. (Phoenix) is submitting the enclosed GRAS notification on behalf of Nikken Sohonsha Corporation (Nikken). In December 2008, Nikken submitted a GRN for this ingredient (GRN-276) which was withdrawn voluntarily in March 2009. In this new submission, Nikken has provided substantive information that responds to all the comments provided by FDA scientific reviewers.

Nikken's *Dunaliella bardawil* alga is grown with high levels of extracelluar salt and under extreme light and temperature conditions. Under these 'stress' conditions, the alga produces higher levels of β-carotene and glycerol than when subject to low salt concentration and normal light and temperature conditions. Other constituents of the alga are starch, protein, and lipids. The β-carotene produced as a constituent of the Nikken alga is composed of two stereoisomers, all-*trans* and 9-*cis*, with a trace amount of other mono-*cis* and di-*cis* β-carotene stereoisomers. In producing Nikken *Dunaliella bardawil* powder, the alga is **not** subject to biotechnological procedures. Moreover, no toxic metabolites or toxins have been identified for the genus *Dunaliella* in over 100 years of investigation. In addition, Nikken alga does not concentrate metals, such as Pb, As, or Cd, nor does it concentrate Iodine.

In accordance with the Food and Drug Administration's (FDA) proposed policy [62 FR 18938-18964; April 17, 1997], Nikken is voluntarily notifying the agency that it convened a Panel of Experts to review the data and information available relevant to this notification. Nikken concurs with the Expert Panel's conclusion based on scientific procedures that Dunaliella *bardawil* powder substance is "generally recognized as safe" (GRAS) for the intended uses. Therefore, in accordance with Section 201(s) of the Federal Food, Drug, and Cosmetic Act, Nikken has concluded that its food substance is exempt from the food additive premarket provisions of the Act.

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In addition, Nikken certifies that the intended uses of *Dunaliella bardawil* powder are similar to those of other food algal substances and that its alga ingredient which contains β-carotene is not intended to impart color.

If additional information or clarification is needed as you undertake your review, please feel free to contact me.

We look forward to your feedback.

Sincerely,

(b) (6)

C. K. Gund, Ph.D.
President

Enclosures: Three (3) copies of GRAS Notification for *Dunaliella bardawil* powder

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Dunaliella bardawil

Food Usage Conditions for General Recognition of Safety

for

Nikken Sohonsha Corporation

1-32 Asahira, Fukuju-cho
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July 30, 2010

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ATTACHMENTS

I. GRAS EXEMPTION CLAIM

A. Claim of Exemption From the Requirement for Premarket Approval Pursuant to Proposed 21 CFR 170.36(c)(1)[1]

Nikken Sohonsha Corporation ("Nikken") *Dunaliella bardawil* alga (hereinafter referred to as *Dunaliella bardawil* powder), meeting the specifications described below, has been determined to be Generally Recognized As Safe (GRAS), in accordance with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act*. This determination, which is based on scientific procedures as described in the following sections, was made by experts qualified by scientific training and experience. The subject evaluation accurately reflects the conditions of the ingredient's intended use in foods.

In addition, *Dunaliella bardawil* powder is similar to that of other GRAS substances, such as brown algae (21 CFR, 184.1120), red algae (21CFR 184.1121) and *Spirulina* (GRN 127).

Signed:

(b) (6)

July 30, 2010

Date

B. Name and Address of Notifier

Nikken Sohonsha Corporation
1-32 Asahira, Fukuju-cho
Hashima-city, Gifu-Pref.
JAPAN 501-6255

As the notifier, Nikken accepts responsibility for the GRAS determination that has been made for *Dunaliella bardawil* powder as described in the subject notification; consequently, *Dunaliella bardawil* powder meeting the conditions described herein is exempt from pre-market approval requirements for food ingredients.

[1] See 62 FR 18938 (17 April 1997) or http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/ucm083058.htm.

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C. Common Name and Identity of the Notified Substance

Dunaliella bardawil alga, also known as *Dunaliella bardawil* powder; also see Section III.B.

D. Conditions of Intended Use in Food

Dunaliella bardawil powder is intended to be added as a food ingredient to the following food categories at 100 ppm per serving level: cheese, bread and rolls, mayonnaise, cookies, crackers, tofu and soybean fermentation products as discussed in Section IV.B. Because of the naturally occurring β-carotene content within the *Dunaliella bardawil* powder, the subject ingredient functions as a nutrient supplement as defined in 21 CFR 170.3(o)(20). The maximum daily consumption of the *Dunaliella bardawil* powder will provide up to 71 mg/person/day.

E. Basis for the GRAS Determination

Pursuant to 21 CFR § 170.30, *Dunaliella bardawil* powder has been determined to be GRAS on the basis of scientific procedures as discussed in the detailed description provided below.

F. Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the US Food and Drug Administration (FDA) upon request or will be available for review and copying at reasonable times at the offices of Phoenix Regulatory Associates, Ltd., 21525 Ridgetop Circle, Suite 240, Sterling, VA 20166.

II. BACKGROUND

A. Objective

At the request of Phoenix Regulatory Associates, Ltd. ("Phoenix") and on behalf of Nikken, GRAS Associates, LLC ("GA") has undertaken an independent safety evaluation of Nikken's *Dunaliella bardawil* alga (henceforth "*Dunaliella bardawil* powder"). The purpose of the evaluation is to ascertain whether the intended food uses of *Dunaliella bardawil* powder at the levels specified are generally recognized as safe, i.e., GRAS.

B. Foreword

GA obtained information needed to enable the GRAS assessment to be undertaken which included: the safety/toxicity information describing the microcellular alga, *Dunaliella bardawil, as well as Dunaliella bardawil* powder, the history of use of *Dunaliella bardawil* powder in food; compositional details, specifications, and method of preparation of food grade *Dunaliella bardawil* powder. GA also reviewed the most recent published literature related to β-carotene, a major constituent of the *Dunaliella bardawil* powder, to ensure that there were no safety concerns related to the ingestion of this natural carotenoid at the specified low levels in which it would be incorporated into the proposed food categories.

Safety/toxicity studies performed with laboratory animals, along with available clinical studies, were provided. Nikken was also asked to supply present and proposed food use information. Determining the quantity of *Dunaliella bardawil* powder that can be safely consumed on a daily basis, i.e., the so-called "dose" or use levels, is critical in the determination of safe exposure levels for *Dunaliella bardawil* powder when consumed as a food ingredient. The composite safety/toxicity studies in concert with anticipated exposure information constitute the two critical pieces of information that form the basis of the GRAS evaluation.

The safety/toxicity studies, consumption/exposure information and other related documentation were augmented with an independent search of the scientific and regulatory literature.[2] Based upon the composite information, a GRAS assessment based primarily on available safety information with supportive information provided by common use in food was undertaken. Those references that were deemed pertinent to the objective at hand are listed in Section VIII.

[2] Literature databases were searched periodically. The last search conducted in July 2010 included Toxline®, Medline® and Google Scholar®.

C. FDA Regulatory Framework and Existing Regulations of *Dunaliella bardawil* Powder

Dunaliella bardawil powder is readily available in dietary supplements in the US marketplace. At least two other dietary supplement products produced from *Dunaliella bardawil* were being marketed in the US that contain α- and β-carotene when Nikken submitted its notification for the use of its product. Nikken's NDI document (Report 410) was for Nikken's *Dunaliella* Dried Powder with the usage level at 100 mg in milk 1-3 times per day as a source of its natural β-carotene. FDA issued its dietary supplement acknowledgement letter August 15, 2007 [Attachment 1].

As is the case with many other dietary supplement products sole in the US, including one brand of spirulina, the label for Nikken's US dietary supplement contains the cautionary statements that the product should be kept out of the reach of children, and women who are pregnant or nursing should consult with their physicians before taking this product.

In addition to the FDA-authorized use of *Dunaliella bardawil* powder in dietary supplements, FDA regulates one of its important components, β-carotene, as an affirmed GRAS substance for use as a nutrient (21 CFR 184.1245) based on a review by the Select Committee on GRAS Substances (SCOGS, 1979) and as a color additive (21 CFR 73.95). The food grade specifications for the US are published in *Food Chemicals Codex* ("FCC"). These specifications do not specify any particular synthetic or natural source as long as the FCC identity and purity specifications are met.

In accordance with FDA regulation of foods, however, dietary supplements cannot legally be added to conventional foods. Such ingredients must undergo pre-market approval by FDA as food additives or, alternatively, the ingredients to be incorporated into conventional foods must be determined to be generally recognized as safe (GRAS). The authority to make GRAS determinations is not restricted to FDA. In fact, GRAS determinations may be provided by experts who are qualified by scientific training and experience to evaluate the safety of food and food ingredients under the intended conditions of use.[3]

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[3] See 21 CFR 170.3(i)(3). [3] See 21 CFR 170.3(i)(3).
[3] See Federal Register 62 April 17, 1997, 18937; or http://www.
fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/ucm083058.htm.

In 1997, FDA altered the GRAS determination process by eliminating the formal GRAS petitioning process. At that time, the petitioning process was replaced with a notification procedure.[4] While outlining the necessary content to be considered in making a GRAS determination, FDA encouraged that such determinations be provided to FDA in the form of a notification. However, notifying FDA of such determinations is strictly voluntary.

D. Additional Regulatory Background for *Dunaliella bardawil* Powder

Besides the recent approval for use in dietary supplements in the US (Attachment 1), Nikken has provided the following information:

> In Japan, Nikken's *Dunaliella bardawil* powder is currently incorporated in noodles (0.03%), breads (0.1%), soybean pastes (0.05%), soybean sauce (0.05%), traditional Japanese confectionery (0.1%), and a Western-type confectionery (0.1%) similar to sponge cake that is sold in Japan only.
>
> Nikken's *Dunaliella bardawil* powder was approved by the Japanese Health Food and Nutrition Food Association in 1988, and since the latter part of 1988, Nikken has been selling the subject powder for use as a health food supplement in various countries, such as Japan, Korean, Canada, Indonesia, Thailand, Singapore, and India.
>
> Beginning in November 1995, Nikken supplied hard capsules of its *Dunaliella bardawil* powder containing natural β-carotene as a health food supplement product in Japan, Indonesia, Thailand, India, Singapore, and the Republic of China.
>
> In 1993, Nikken gained approval as a feed additive in Japan and began selling its *Dunaliella bardawil* powder for animal feed for fish, cows, and chickens.
>
> Since 1988 Nikken has marketed approximately 55,900 kgs and has never had to recall its powder nor has it received any adverse events associated with its powder.

In addition, a review has been conducted by the Joint Expert Committee on Food Additives ("JECFA") on applications submitted to establish ADIs for natural β-carotene of *Dunaliella* algae using different preparations, such as oil extract, dehydrated algal carotene and equivalents; however, not all the literature at hand was reviewed. At that time, the major issue was natural versus synthetic β-carotene. The committee considered the data to be inadequate to establish an ADI for the dehydrated algal carotene preparations or for the vegetable oil extracts of β-carotene from *Dunaliella salina* and concluded that there is no history of use of *Dunaliella* algae neither as food nor for comparing different species of *Dunaliella* algae (WHO, 1993). JECFA has not conducted any other reviews of the biomass of *Dunaliella bardawil* since this review in 1993, and since then, a variety of new studies was published and is discussed in this notification.

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[4] See Federal Register 62 April 17, 1997, 18937; or http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/ucm083058.htm.

III. CHEMISTRY, HISTORY, TAXONOMY MICROBIOLOGY AND MANUFACTURE OF *Dunaliella bardawil* Powder

A. Chemistry, History, Taxonomy and Classification

The halophilic, photosynthetic green algae, *Dunaliella bardawil,* has been known and studied for 100 years (Oren, 2005). This genus includes several ill-defined unicellular species that live in a wide range of marine habitats from seawater to fresh water (Brock, 1975; Polle, Tran, and Ben-Amotz, 2009; Zamir, 1992; Oren, 2005; and Butcher, 1959). Usually algae of the genus *Dunaliella* are consistently found in sea water and evaporation salt ponds which contain greater than 2 M salt and greater than 1 M magnesium (Ben-Amotz, 2009 and Brock, 1975). This organism is one of the 'most halotolerant eukaryotic' organism known, showing a remarkable degree of adaptation to a variety of salt concentrations, from as low as 0.1 M to salt saturation of >5 M (González, Gómez, and Polle, 2009; Borowitzka 1981, and Borowitzka, 1986). There have been many decades of research and development of aquaculture systems, principally in Australia and Israel, to commercialize *Dunaliella bardawil,* rich in carotenoids for use as an animal and human food source (Rao, 2009).

The subject powder consists of the dehydrated biomass of the alga *Dunaliella bardawil* and, therefore, is a mixture which largely consists of protein, carbohydrate, fat, minerals and carotenoids. Further description of the isolation and cultivation of the cells as well as their unique characteristics is provided below. The parameters on growth conditions for the algae are explained in the manufacturing procedure later in this Section. More details of the composition are given in the product specifications that follow.

The microalgae taxonomy and phylogeny are the following:

Kingdom:	Plantae
Phylum:	Chlorophtya
Class:	Chlorophyceae
Order:	Chlamydomonadales*
Family:	Dunaliellaceae
Genus:	*Dunaliella*
Species/strain	*D.bardawil*

* The National Center for Biotechnology Information has revised the name of the Order for this alga in its database.

Dunaliella bardawil has been classified and assigned the following numbers

Integrated Taxonomic Information System Serial number 189432[5]

The ATCC #30861 culture listing is included at Attachment 2. #LB 2538 UTEX Culture Collection, USA listing is shown below as published with no information provided for either the category, "Collection" or "Also Know As". Neither of these listings use the same media as is used to cultivate and grow Nikken's *Dunaliella bardawil* powder in Israel.

UTEX Number:	LB 2538
Class:	Chlorophyceae
Strain:	*Dunaliella bardawil*
Media:	2X Erdschreiber's Medium
Origin:	Bardawil Lagoon, North Sinai, Israel
Description of Location:	salt pond
Type Culture:	No
Collection:	
Isolation:	M. Avron, A. Ben-Amotz (1976)
Isolator Number:	ATCC 30861
Deposition:	R. Adams (1980-2)
Relatives:	ATCC 30861
Also Known As:	
Notes:	patented organism for production of glycerol, carotenes, and protein-rich material (Avron & Ben-Amotz U.S. Pat. 4,199,895)

B. Common or Usual Name

The common or usual name for the subject material is *Dunaliella bardawil* alga or *Dunaliella bardawil* powder.

[5] Accessed at www.itis.gov.

C. Microbiology of *Dunaliella bardawil*

1. Isolation and Identification of *Dunaliella bardawil* Species and Strains

In 1976 Professor Ben-Amotz, as a student at the Weizmann Institute of Science (WIS), Israel, collected reddish water samples from the Bardawil lagoon, Egypt and isolated the alga species, *Dunaliella bardawil.* Bardawil lagoon is located at 31°09'N, 33°08'E on the southeastern Mediterranean coast and is separated from the sea by a long narrow sand bar (Abd Ellan and Hussein, 2009). When the physical limnology of the lagoon was studied in 2004, the investigators found that in summer the air and water temperatures as well as the evaporation rate were high (27.0°C and 24.6°C, 6.0 mm/day, respectively). Salinity range also varied greatly and from the 1970s to 2006, the annual variation of maximum water salinity ranged from over 95% in some isolated basins in the 1970s to approximately 70% in 2004 (Abd Ellan, and Hussein, 2009).

Typical microbiological procedures were used to isolate and characterize this species, such as streaking the cultures brought back from the Bardawil lagoon on salt (8%) medium agar plates and incubating them under culture room quartz tungsten 30,000 Lux light. Colonies were isolated and purified using aseptic conditions by streaking the colonies repeatedly until an isolated cell was obtained. The relevant isolated cell then was studied using a variety of techniques, analytical and chemical as well as light and electron microscopy. The cultures are maintained over the years through cultivation indoors and outdoors by using high salt concentration (close to saturation (35%), high light irradiation (direct solar light, >32,000 Lux) and nitrogen deficiency (0.1 mM KNO_3 or NH_4Cl). No induced genomic techniques were used to isolate or produce this algal species.

2. Physiological and Biochemical Description of *Dunaliella bardawil* Cells

The alga identified contains unicellular motile cells, with a single cell being about 15 to 19 μm long and 10 to 14 μm in diameter. The cell shape is oval or elliptical and has a fine elastic periplast with no rigid cell wall (U.S. Patent 4,199,895, 1980). This characteristic allows rapid changes in the cell volume in response to extra cellular changes in osmotic pressure (Ben-Amotz, 2009). There are two flagella, 1.5 to 2 times the length of the cell, emerging along one edge of the long cell axis (U.S. Patent 4,199,895, 1980). When the alga is placed in high salt concentration (>4 M), it loses its flagella and the cells round to form a rigid cyst (Ben-Amotz, 2009 and Loeblich, 1969). Using electron microscopy, one large chloroplast is observed and it occupies about ½ of the cell volume. This chloroplast is often arranged in a cup-shape around the nucleus. A single median pyrenoid embedded in the basal portion of the chloroplast is surrounded

by starch granules (U.S. Patent 4,199,895, 1980). The cell also contains "interconnected and oppressed membranes thylakoids, within which the highly pigmented photosynthetic apparatus is located" (Jeffrey and Egeland, 2009). The cell volume is 200 to 1000 μm^3. The cells reproduce vegetatively by longitudinal division of the motile cells (U. S. Patent 4,199,895, 1980).

Cells of the species *Dunaliella bardawil* can change their colors from green to red depending on the growth conditions. When cells are growing in nutrient replete media at a salinity of about 1.0 M NaCl and at about 1/10 or less of sunlight intensity, cells have a green color due to the high intracellular concentration of chlorophyll. In contrast, when cells of the *D. bardawil* are exposed to an environment that has high light intensity as equivalent to solar irradiation (110,000 Lux), high salinity of >2 M, nutrients such as nitrogen, phosphate and sulfate below 0.5 mM, and extremes of temperature <5°C, >33°C, massive quantities of β-carotene can be synthesized (Jeffrey and Egeland, 2009). *D. bardawil* cells accumulate the β-carotene in lipid globules in the stroma of chloroplasts. The amount of the carotenoids can be up to 12% of the algal dry weight (Ben-Amotz, 2009). The high amounts of β-carotene present in the cells result in cells appearing orange-reddish.

However, by allowing the high β-carotene containing *D. bardawil* to replicate and divide under growing conditions of low salinity (~0.5 M), low light intensity (200 Lux) and moderate temperature (~25°C), the accumulation of β-carotene is reversible. With each replication, the concentration of the β-carotene reduces by ½ until the organism becomes green, and the level of β-carotene is less than 1% (Ben-Amotz, Katz, and Avron, 1982).

The exact mechanisms of how cells of *D. bardawil* sense changes in growth conditions and react to them in triggering carotenoid accumulation are unknown. It is believed that the regulatory mechanisms leading to accumulation of β-carotene in cells of *D. bardawil* are similar to those of higher plants and other photosynthetic organisms (Lamers, et al., 2008; Ye, Jiang, and Wu 2008; Jin and Polle 2009; and Ben-Amotz, Lers, and Avron, 1988).

When cells of *D. bardawil* are placed under stress conditions, the β-carotene produced is composed of two stereoisomers, *all-trans* and *9-cis,* with a trace amount of other *mono-cis* and *di-cis* β-carotene stereoisomers (Ben-Amotz, 2009). Using this strain, total β-carotene and the ratio of all-*trans* to 9-*cis* stereo isomers are dependent on the light intensity during the division cycle. The authors conclude that the isomerization reaction takes place early in the biosynthesis of carotene and probably before the step forming the all-*trans* isomer; however, the isomerization step that produces the 9-*cis*-β-carotene is not yet defined (Ben-Amotz, 2004).

In addition to β-carotene, glycerol may accumulate in the cell when cells are exposed to high levels of extracellular salt concentration. The glycerol "concentration is directly proportional to the extracellular salt concentration and is sufficient to account for all the required cytoplasmic and most of the chloroplastic osmotic pressures" (Ben-Amotz, 2009). The osmotic cycle responds to high salinity by enhancement of photosynthesis through diversion of carbon and energy resources in the production of glycerol (Ben-Amotz, 2009). Other constituents, such as starch, protein, and lipids, have been identified as components of the cell.

Algae of the genus *Dunaliella* have been studied for more than 100 years. There have been no published articles throughout the entire last century that identified the production of any toxic metabolites or toxins (Ben-Amotz and Avron, 1990). On the contrary, in hypersaline environments algae of the genus *Dunaliella* play an important role as the major primary producers on which animals such as brine shrimp feed. No reports on any toxins in the foodchain of hypersaline habitats are known.

In addition, the European Commission Scientific Committee for Food indicated that the unicellular alga, that are classified as food sources and which are species of *Chlorophyceae* do not produce toxins (European Commission 1998). Finally, the lack of toxicity is well presented in a detailed study by Mokady, Abramovici and Cogan, 1989 and described in a chapter by Mokady, 1992.

3. Isolation of Nikken *Dunaliella bardawil* Strain

Organisms, such as microalgae, exist in fluctuating environments. As a consequence, they have the inherent basic genetic flexibility to acclimate to short-term changes in the environment. In addition, cells may adapt to specific environments over a time period of hundreds of generations (Rebound and Bell, 1997; Collins and Bell 2004; and Collins, Sultemeyer and Bell 2006). In the case of *Dunaliella bardawil*, over more than two decades and thousands of generations, the original strain has adapted to Nikken's pond environment in Eilat by producing higher amounts of β-carotene but when removed from that environment, can reverse accumulation of this chemical and the cell returns to the increased production of chlorophyll as discussed above.

In 1978 the above species (ATCC 30861) was transferred after ten years of basic studies at the WIS from Rehovot to Eilat where it was inoculated in open ponds of NBT Ltd. Natural selection has been initiated on the ATCC 30861 species by the extremely harsh environmental conditions (temperature below 5°C and above 40°C, continuous high irradiance all year round at maximum solar illumination (120,000 Lux), and high salt concentration up to saturation of >35%). These

stress environmental conditions, typical in the area of Eilat, selected a strain from the original *D. bardawil* (ATCC 30861) that has faster motility, and has higher resistance to irradiation, temperatures and salinity by producing excess of β-carotene and glycerol. No techniques of artificially induced mutations were used to produce this strain of *Dunaliella bardawil.*

This new strain produces an increased concentration of the β-carotene and of the *9-cis* isomer of β-carotene compared to that of the strain isolated from the Bardawil lagoon. The table below shows the differences between these two strains, but in all other characteristics Nikken's *Dunaliella bardawil* is comparable to the earlier strain, and both are classified under species identified by ATCC 30861.

Table 1. Identification and Comparison of *Dunaliella bardawil* Strains
(Nikken Study)

Feature	*Dunaliella bardawil* Amotz & Avron, ATCC 30861	*Dunaliella bardawil* Eilat ATCC 30861
Dimensions, width x length (μm)	5 x 15	3 x 10*; 8 x 25*
Pigments, chlorophylls	*a & b*	*a & b*
Pigments, carotenoids	Violaxanthin, neoxanthin, lutein, zeaxanthin, α-carotene, β-carotene	Violaxanthin, neoxanthin, lutein, zeaxanthin, α-carotene, β-carotene, α & β cryptoxanthin, ζ-carotene
Pigments, β-carotene (%)	0.2-10.0	0.5-12.0
Stereoisomers of β-carotene Max ratio of 9-*cis*/all-*trans*	1.0	1.3
Cell-Protein, minimum content under N starvation (% per dry weight)	28	18
Cell-Starch, maximum under N starvation (% per dry weight)	43	61
Phosphate storage	not determined	polyphosphates
Motility	slow-medium	Medium-fast

* Cell transition twice a year—in autumn and in spring.

The carotenoid profile found in the strains *D. bardawil* (ATCC 30861) is the same as found in various fruits and vegetables (Ben-Amotz et al., 1988). However, after analyzing common fruits and vegetables, the author found that the 9-*cis* to all-*trans* β-carotene ratio averaged only 0.2 g/g in higher plants compared to the average of ~1 g/g in *Dunaliella bardawil* (Ben-Amotz et al., 1988).

D. Manufacturing Procedure

1. Production of *Dunaliella bardawil* Powder, (Ben-Amotz, 2004 and Ben-Amotz and Avron, 1990)

The mode of cultivation of Nikken's *Dunaliella bardawil* used by NBT Ltd. is referred to as intensive cultivation. The ponds at Eilat where *Dunaliella bardawil* grow are manmade, usually oblong, lined, constructed 'shallow raceways' varying in size. One of the larger ponds has a surface area of approximately 3,000 m^2. Long arm, slow revolution paddle wheels are used to provide circulation for the ponds. Attachment 3 shows a diagram where the size of a pond (Nos. 1–6 and 19–26) is 150–170 meters long and 10–20 meters wide and about 3,000 square meters in area. The other ponds shown in the diagram are used for test cultures, etc.

The alga is grown in several ponds through an autotrophic, photosynthetic environment using salt water supplemented with carbon dioxide and food grade nutrients, nitrate, and phosphate. The ponds are inoculated by culture liquid transfer and dilution 1:3 (V/V). The inoculate from the laboratory cultivation is grown in the same medium as is used in large scale algal production, namely, seawater, nitrate (or ammonia) and phosphate. pH is maintained by CO_2/air. The water for the ponds comes from the Red Sea, and recycled salt water originates from the Red Sea. The addition of each is determined by the desired concentration of salt required per pond, per day. The salt concentration of the ponds varies from 1.5 M in the winter to above 3 M in the summer. The environmental conditions, including the salinity concentration, pH, total dissolved carbon and light penetration by depth, are computer controlled. The ponds are maintained at the desired salinity by using Red Sea water which is approximately 4% salt. Nutrients are supplied by nitrogen in the form of KNO_3 or NH_4Cl at deficient level of up to 0.5 mM while phosphate is maintained by adding H_3PO_4 at 0.5 mM.

During the growth phase which can be 1-7 days, cell number, cell volume, chlorophyll, β-carotene, pH, nutrients' concentration, salinity, etc., are measured and monitored daily using well-known published classical chemical analytical methods. The growth cycle can vary depending on the season and growth conditions, e.g., one division rate can be from 24 to 168 hours. The algae are harvested when the carotene to chlorophyll ratio is above 6.

The growth of the algae is conducted under good agricultural practices and no pesticides or other harmful chemicals are used in its production. The sea water is analyzed once a week both when it is pumped into the ponds and when it is discarded for recycling or back to the Red Sea. These reports are sent to the Marine and Coastal Environment Division, Ministry of Environment, State of Israel, as part of the Permit given to NBT Ltd. Eilat. A copy of one of the reports

is provided [Attachment 4]. Using an independent laboratory NBT periodically tests the powder for pesticides, including herbicides, and heavy metals [Attachment 5]. All values tested are below the specific tests' detection levels.

Nikken's Dunaliella bardawil is cultivated in nitrogen deficient culture medium at high salinity under unscreened solar irradiation conditions that eliminate most predators, common bacteria and other algae.

β-Carotene is analyzed daily by spectrophotometric means during the growth period, and when it reaches 5-10 mg/L, the liquid containing *Dunaliella* is pumped and filtered through a 100 μm filter to remove foreign matter and then conveyed through elevated balance tanks 10 meters above the centrifugation room so that a constant air free flow to the four centrifuges is provided. The liquid-containing *Dunaliella* is centrifuged, using Westphalia Separators AG (Germany), at 4,200 rpm, 13 ~ 15 m^3/h to produce 1,200 L paste. 1,200 m^3 is recorded throughout the manufacturing process as a lot.

After the first centrifugation, 1,200 liters of paste are sterilized by APV AnhydroAS (Denmark) CTHE372 Tubular Heat Exchangers by heating to 85°C for 30 minutes and then cooled down to 10°C by a jacketed water chiller (Antoni S.P.A. Water Chiller AE200 (Italy) (4°C). In the desalination step about 600 L of paste are diluted to 4,000 L with water and stirred using a bar with propellers for 15 minutes in a 7000 L tank. After stirring, a second centrifugation step at 4,200 rpm, 10 ~ 13 m^3 per hour removes the water/salt mixture which results in a mixture of 400 L the washed paste. A second sterilization step is conducted at this point using the Tubular Heat Exchangers, and a quantity of 100 ~ 150 L/h mixture is heated to 80°C. Once the mixture reaches 80°C, the product is vaporized and dried using a spray drier APV Anhydro .ISR135:'60 (Denmark). The flow rate in the drier is 100 ~ 150 L with the entrance temperature of 190°C and the exhaust exit temperature at 90°C. This step produces 50 kg/h *Dunaliella bardawil* powder. The diagrams of the manufacturing process and facility are provided at Attachment 6.

The powder is wrapped in 12.5 kg vacuum polyethylene bags (80 cm x 60 cm x 78 microns) (Plastobarr N 80). These bags are then placed in cardboard cartons (52 cm x 52 cm x 43 cm (h)) which holds 4 bags (50 kg each carton). Each carton is stamped with the lot number. The cartons are shipped to the Japan facility where they are stored in a dark, cool, temperature-controlled warehouse separate from other stored products. The resulting product meets food grade specifications. NBT certifies that it is in compliance with good agricultural practices and with CGMPs (21 CFR Part 110).

Table 2 reveals the composition and specifications of three lots manufactured at different times. In addition, three different lots were analyzed for metal impurities (heavy metals (as Pb), mercury and arsenic). The results show that heavymetals (as Pb) (QL 5 ppm) and mercury (QL 0.01 ppm) were not detected at the limits of

detection for the methods used while arsenic as As_2O_3 was detected at not more than 0.4 ppm. Nikken also updated the specifications based on FDA comments by having three separate lots analyzed for iodine, lead and cadmium.
The results of these analyses were: iodine, less than 0.3 ppm in all lots; lead, less than 1.2 ppm in all lots; and cadmium, 0.06 ppm in all lots [Table 3]. There is no evidence of bioaccumulation of any of these elements by this organism and an analysis of the growing medium indicates that the levels of arsenic, cadmium, iodine, lead and mercury are all less than 1 ppm in the growing medium. Copies of the laboratory reports referred to in this paragraph are included in Attachment 7.

Table 2. *Dunaliella bardawil* Powder Composition and Specifications

Examination Items[a]	Lot #0705 Manufacturing Date: May/10/2007		Lot # 0710 Manufacturing Date: Oct/19/2007		Lot # 0802 Manufacturing Date: Feb/4/2008		Final Specification
Properties	Orange-brownish color. Typical *Dunaliella* Algae smell.	Orange-brownish color. Typical *Dunaliella* Algae smell.	Orange-brownish color. Typical *Dunaliella* Algae smell.	Orange-brownish color. Typical *Dunaliella* Algae smell.	Orange-brownish color. Typical *Dunaliella* Algae smell.	Orange-brownish color. Typical *Dunaliella* Algae smell.	Orange-brownish color. Typical *Dunaliella* Algae smell.
Moisture[1]	2.8	3.4	3.7	4.4	2.6	3.3	< 6%
Ash[2]	6.4	6.3	6.5	6.4	6.5	6.4	< 20%
Protein[3]	24.1	23.5	24.8	24.1	23.5	22.9	< 30%
Fat[4]	19.5	20.8	23.9	22.8	21.2	23.1	< 30%
Carbohydrate[5]	47.2	46.0	41.1	42.3	46.2	44.3	< 50%
Total Carotene[6]	7.89	7.91	7.21	7.34	7.89	7.81	5 ≤ 10%
β-carotene[7]	7.52	7.47	6.88	6.95	7.59	7.45	5 ≤ 10%
β-carotene inside Ratio of 9-*cis* in β-carotene[8]	31	32	33	32	35	34	30 ≤ 50%
Sodium Chloride[9]	2.2	2.1	2.1	2.2	2.3	2.4	< 3%
Heavy Metals (Pb)[10]*	< 5 ppm	< 5 ppm	< 5 ppm	< 5 ppm	< 5 ppm	< 5 ppm	< 5 ppm
Arsenic (As)[11]	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm
Total Aerobic Mesophiles[12]	≤ 3,000/g	≤ 3,000/g	≤ 3,000/g	≤ 3,000/g	≤ 3,000/g	≤ 3,000/g	≤ 3,000/g
E-coli[13]	Negative/g	Negative/g	Negative/g	Negative/g	Negative/g	Negative/g	Negative/g
Staphylococcus aureus[14]	Negative/0.02 g	Negative/0.02 g	Negative/0.02 g	Negative/0.02 g	Negative/0.02 g	Negative/0.02 g	Negative/0.02 g
Molds[15]	Negative/0.1 g	Negative/0.1 g	Negative/0.1 g	Negative/0.1 g	Negative/0.1 g	Negative/0.1 g	Negative/0.1 g
Clostridium perfringens[16]	Negative/0.1 g	Negative/0.1 g	Negative/0.1 g	Negative/0.1 g	Negative/0.1 g	Negative/0.1 g	Negative/0.1 g
Salmonella[17]	Negative/20 g	Negative/20 g	Negative/20 g	Negative/20 g	Negative/20 g	Negative/20 g	Negative/20 g
Pseudomonas aeruginosa[18]	Negative/0.02 g	Negative/0.02 g	Negative/0.02 g	Negative/0.02 g	Negative/0.02 g	Negative/0.02 g	Negative/0.02 g

[a] Methods for these determinations are listed in Attachment 8.

* Heavy metals (Pb) will be removed as a specification and the updated Pb specification is found in Table 3.

Table 3. *Dunaliella bardawil* Powder Supplemental Specifications [Attachment 7]*

Examination Items	Lot #0909 Manufacturing Date: Aug/12/2009		Lot # 1001 Manufacturing Date: Jan/22/2010		Lot # 1004 Manufacturing Date: Apr/6/2010		Final Specification
Iodine	0.28ppm	0.26ppm	0.28ppm	0.26ppm	0.28ppm	0.25ppm	< 0.5ppm
Lead (Pb)	1.12ppm	1.10ppm	1.11ppm	1.12ppm	1.12ppm	1.09ppm	< 2ppm
Cadmium	0.06ppm	0.06ppm	0.06ppm	0.06ppm	0.06ppm	0.06ppm	< 0.4ppm

*Method: Inductively coupled plasma mass spectrometry

E. Stability Data

Nikken has conducted shelf-life stability on *Dunaliella bardawil* powder. Three different lots contained in sealed vacuum opaque polyethylene aluminum bags were stored in a room where temperature varied from 15-30°C and the humidity range was 30-70% for two years. No appreciable changes in the various compositions of the subject material were seen. The data are summarized in Table 4.

Table 4. Stability Test Data for Expiry Date *Dunaliella bardawil* Powder

Examination Items[a]	Powder Lot# 0301008		Powder Lot# 0408319		Powder Lot# 0501001		Final Specification
	Starting Day	After 2 years	Starting Day	After 2 years	Starting Day	After 2 years	
Properties	Orange-brownish color. Typical *Dunaliella* Algae smell	Orange-brownish color. Typical *Dunaliella* Algae smell	Orange-brownish color. Typical *Dunaliella* Algae smell	Orange-brownish color. Typical *Dunaliella* Algae smell	Orange-brownish color. Typical *Dunaliella* Algae smell	Orange-brownish color. Typical *Dunaliella* Algae smell	Orange-brownish color. Typical *Dunaliella* Algae smell
Moisture	3.6	3.4	3.1	2.8	2.7	2.7	< 6%
Ash	6.4	6.4	6.5	6.4	6.4	6.4	< 20%
Protein	25.3	25.5	23.3	23.4	24.8	24.5	< 30%
Fat	25.1	25.8	23.6	23.3	21.3	21.7	< 30%
Carbohydrate	39.6	38.9	43.5	44.1	44.8	44.7	< 50%
Total Carotene	8.55	8.28	8.03	8.22	7.69	7.72	5 ≤ 10%
β-carotene	7.95	7.87	7.54	7.63	7.07	7.15	5 ≤ 10%
β-carotene inside Ratio of 9-*cis* in β-carotene	34	34	31	33	31	31	30 ≤ 50%
Sodium Chloride	2.5	2.2	2.2	2.4	2.5	2.4	< 3%
Heavy Metals (Pb)	< 5 ppm	< 5 ppm	< 5 ppm	< 5 ppm	< 5 ppm	< 5 ppm	< 5 ppm
Arsenic (As)	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm
Total Aerobic Mesophiles	≤ 3,000/g	≤ 3,000/g	≤ 3,000/g	≤ 3,000/g	≤ 3,000/g	≤ 3,000/g	≤ 3,000/g
E-coli	Negative/g	Negative/g	Negative/g	Negative/g	Negative/g	Negative/g	Negative/g
Staphylococcus aureus	Negative/ 0.02 g	Negative/ 0.02 g	Negative/ 0.02 g	Negative/ 0.02 g	Negative/ 0.02 g	Negative/ 0.02 g	Negative/ 0.02 g
Molds	Negative/ 0.1 g	Negative/ 0.1 g	Negative/ 0.1 g	Negative/ 0.1 g	Negative/ 0.1 g	Negative/ 0.1 g	Negative/ 0.1 g
Clostridium perfringens	Negative/ 0.1 g	Negative/ 0.1 g	Negative/ 0.1 g	Negative/ 0.1 g	Negative/ 0.1 g	Negative/ 0.1 g	Negative/ 0.1 g
Salmonella	Negative/ 20 g	Negative/ 20 g	Negative/ 20 g	Negative/ 20 g	Negative/ 20 g	Negative/ 20 g	Negative/ 20 g
Pseudomonas aeruginosa	Negative/ 0.02 g	Negative/ 0.02 g	Negative/ 0.02 g	Negative/ 0.02 g	Negative/ 0.02 g	Negative/ 0.02 g	Negative/ 0.02 g

[a] Methods for these determinations are listed in Attachment 8.

The data presented demonstrate that the *Dunaliella bardawil* powder is growing under good agricultural conditions and processed under Current Good Manufacturing Practices (21 CFR Part 110).

IV. INTENDED DIETARY USES

A. Intended Food Uses

Nikken intends to market *Dunaliella bardawil* powder with its naturally occurring β-carotene content as a nutrient supplement as defined in 21 CFR 170.3(o)(20) for use within selected foods, including bread, rolls, cookies, crackers, cheese, mayonnaise, tofu and fermented soybean products. Maximum use levels proposed for each food category are listed in Table 5.

The intended addition to designated foods at these proposed use levels as a food ingredient is similar in nature to spirulina (FDA, 2003). A major component in Nikken's *Dunaliella bardawil* powder, β-carotene which is present at a level of approximately 8%, is authorized by FDA as both a color additive (21 CFR 73.95) and a GRAS affirmed nutrient supplement (21 CFR 184.1245). With the latter functionality, the β-carotene is added to food products for the nutrient advantages and not for its potential to impart color. Nikken states that the addition of its *Dunaliella bardawil* powder to the various food products occurs at sufficiently low levels that the β-carotene will not impart a significant color to the resulting food products. Furthermore, the subject ingredient is not intended to function as a color additive. Therefore, the incorporation of *Dunaliella bardawil* powder with its naturally occurring β-carotene falls outside of the definition of color additive (21 CFR 70.3(g)).

B. Estimated Daily Intake

Based on the proposed food uses of Nikken's *Dunaliella bardawil* powder, a point estimate for dietary intake was calculated using FDA guidelines (FDA, 2006). USDA survey data were used to estimate mean levels of consumption from the chosen food categories. Therefore, based on the use of the FDA guidelines and USDA survey data for per capita consumption, a level of twice the mean was calculated as an overestimate for the 90th percentile total consumption of these foods. This estimate indicates that a high consumer of foods containing *Dunaliella bardawil* powder is not likely to consume more than 0.071 grams per day of the proposed ingredient or 6 mg of β-carotene per day. (See Table 5.)

This exposure of β-carotene from *Dunaliella bardawil* powder is similar to the level (3-6 mg/day) the Food and Nutrition Board estimated from a well-balanced diet containing daily consumption of 5 servings of fruit and vegetables (FNB, 2000). It is less than the level based on the ADI of 5 mg/kg/day established by JECFA (WHO, 1975). Furthermore, this exposure compares favorably to the estimated consumption of 37 mg/person/day from all dietary sources that was not of concern when previously reviewed by FDA (SCOGS, 1979).

As indicated in the 2006 FDA guidance, these proposed values of *Dunaliella bardawil* powder and the β-carotene component of the powder should be viewed as overestimates of the 90th percentile consumption.

Table 5. Proposed Consumption of Nikken's *Dunaliella bardawil* Powder

Food Category	USDA Category	Use Level grams per 100 grams (Percent by Weight)	USDA Mean Grams of Food Consumed	Mean Additive Consumed (Grams Users only)	Mean x 2 Additive Consumed (Grams Users Only)	Source Number, Page Number
Low fat cheese, cheese spreads, cottage and cream cheese	Total Cheese	0.01	16	0.0016	0.0032	Reference 1, Table 9.4 p. 29
Mayonnaise	Table Fats	0.01	4	0.0004	0.0008	Reference 1, Table 9.6, p. 31
Bread Rolls	Yeast breads and rolls	0.01	50	0.0050	0.0100	Reference 1, Table 9.1, p. 26
Tofu[a]	Meat	0.01	197	0.0197	0.0394	Reference 1, Table 9.5, p. 30
Fermented soybean products (soy sauce, miso, soy paste)	Total soy based sauces	0.01	11[b]	0.0011	0.0022	Reference 2, p 197
Cookies	Cakes, cookies, pastries, pies	0.01	38	0.0038	0.0076	Reference 1, Table 9.1 p. 26
Crackers	Crackers, popcorn, pretzels, corn chips	0.01	38	0.0038	0.0076	Reference 1, Table 9.1 p. 26
	Total *Dunaliella bardawil* powder consumed			0.0354	0.0708	
	Total ß-carotene consumed			0.0028	0.0057	

[a] Overestimation due to lack of appropriate food category in survey
[b] Mean not found for per capita consumption; value used is for eaters only

Reference 1: DATA TABLES: Results from the USDA's
1994-96 Continuing Survey of Food Intakes by Individuals and
1994-96 Diet and Health Knowledge Survey Table Set 10
Food Surveys Research Group, Beltsville Human Nutrition Research Center

Reference 2: Foods Commonly Eaten in the United States
Quantities Consumed Per Eating Occasion and in a Day, 1994-96
Helen Smiciklas-Wright, Diane C. Mitchell, Sharon J. Mickle, Annetta J. Cook, Joseph D. Goldman
http://www.ars.usda.gov/SP2UserFiles/Place/12355000/pdf/Portion.pdf.

C. Intake of Trace Elements from *Dunaliella bardawil* Powder

The daily intake of certain trace elements from use of Nikken *Dunaliella bardawil* powder is summarized in Table 6. The analysis in Table 6 assumes that the consumption of the Nikken product is 71 mg/person/day.

Table 6. Calculated Dietary Intake of Certain Trace Elements from Consumption of Nikken *Dunaliella bardawil* Powder

Element	Level in Nikken Product[a]	Dietary Contribution[b] (ng/person/day)	Background Dietary Load (µg/person/day)	Reference for Background Dietary Load
Arsenic	<0.5 ppm	35.5	8.3 to 14	Yost et. al., 1998
Cadmium	<0.4 ppm	28.4	30	http://www.eco-usa.net/toxics/chemicals/index.shtml
Iodine	< 0.5 ppm	35.5	138 to 353	Murray et al., 2008
Lead	<2 ppm	142	60	ATSDR, 2007
Mercury	<0.01 ppm[c]	0.71	3	http://www.eco-usa.net/toxics/chemicals/index.shtml

[a] Specification levels from Table 2 and Table 3.
[b] Assume 71 mg/person/day consumption of Nikken product.
[c] Used analytical limit of detection for mercury cited on page 10.

D. Other Information on Human Exposure to *Dunaliella bardawil* Powder: Use as Food Ingredient and Dietary Supplement

Nikken sales figures for 2008 indicate that more than 4.5 million capsules of Nikken *Dunaliella bardawil* powder were sold in Japan, Indonesia, Thailand, Taiwan and India for use as a dietary supplement. In addition, powdered product was sold for use in conventional foods as well as a dietary supplement in these designated countries and in South Korea. The total amount of *Dunaliella bardawil* powder sold by Nikken for total food uses in 2008 exceeded 1900 kg and in 2009 exceeded 1200 kg. In addition, over the past twenty plus years Nikken has sold approximately 55,900 kgs and has not received any reports of adverse events, including any associated with allergenicity, nor have there been any adverse events reported in the literature.

V. SAFETY DATA FOR *DUNALIELLA bardawil* POWDER

A. *Dunaliella bardawil* Powder Safety Considerations

The safety of Nikken's *Dunaliella bardawil* powder (hereinafter in this section referred to under each subsection initially as *Dunaliella bardawil* powder and subsequently then as "the powder "or" the dried algal powder" has been demonstrated by a considerable number of toxicology and clinical studies. Many of these studies were of complex design with several test materials although most included at least one group of animals given a crude dried algal powder. Some of the studies were focused on the efficacy of the major component, β-carotene, and many studies used several test materials that contained varying levels of β-carotene. In addition, many of these studies were not available for review by JECFA in 1993 (WHO, 1993), and this will be discussed more fully subsequently. Those studies available to the Expert Panel that were not considered by JECFA are highlighted in the discussions to follow.

One issue that arose in the interpretation of the studies is the amount of salt present in the test sample that was carried over from the brine pits where the algae were grown. It is common practice now to wash *Dunaliella bardawil* algae before drying to remove some of the excess salt. This was not the case with early manufacturing samples with which at least one of the safety studies were conducted. It is important to note that the Nikken product contains 2-3% salt, while the test sample in the Mokady study discussed below contained 24.7% salt.

1. Acute Toxicity Studies for *Dunaliella bardawil* Powder

Two unpublished acute studies addressing the toxicity of *Dunaliella bardawil* powder given as single doses to mice or rats have been conducted. Both studies were reviewed by JECFA. No lethality was seen within 14 days after administration, and no clinical signs of toxicity were found in either study.

The powder was nontoxic and not lethal when administered by oral gavage to male and female Crj:CD-1 (ICR) Charles River mice (10/sex/group) at 2.5 g/kg, 5.0 g/kg and 10 g/kg (Aruga, 1987) [Attachment 9]. There were no test article-related changes in body weight, and no visible abnormalities were observed in mice at the post-mortem examination. The study was performed in compliance with Japanese Good Laboratory Practice Standard for Safety Studies on Drugs.

Male Sprague-Dawley rats were administered the powder by oral gavage at a dose of 5 g/kg and were observed for the subsequent 14 days (Lock, 1985). There were neither mortalities nor overt signs of toxicity, and all the rats gained weight during the observation period.

2. Reproductive and Developmental Studies for *Dunaliella bardawil* Powder

Reproductive and developmental studies have been conducted in laboratory animals with *Dunaliella bardawil* powder and are summarized below. There do not appear to be any adverse effects of the powder on reproduction or developmental parameters in rats. The study by Mokady et al. (1989) is the most toxicologically comprehensive study because it was a multigenerational study, and the F_0 generation was maintained on the diet for one year and then full histopathology was done. The primary purpose of this study was to examine the powder as a dietary supplement, so very high doses were used.

Mokady et al. (1989) studied the effect of dried algal powder on the reproduction of Charles River CD strain rats. The powder contained 24.7% sodium chloride. Three groups of 30 animals (10 males and 20 females/group) were provided diets with 0, 5% or 10% of the dehydrated test article. The dietary composition of the diets for all three groups was adjusted so that all diets contained 18% protein. The author estimated that the 5% group consumed 5 g/kg bw algal powder as weanlings and 10 g/kg bw as adults, and the 10% group consumed twice that amount. The rats were followed through four generations. Rats (5/sex/group) of the F_0-generation were kept on their allotted diets for one year, and 5 male and 5 female rats were studied for general toxicological effects. Reproductive performance for all generations was recorded. Clinical observations, survival, and weekly physical exams were conducted. At the end of the feeding period F_0 at one year of age, F_1 and F_2 at 5 months, and F_3 at weaning), 5 rats/sex of each group were used to assess hematological and blood chemistry parameters.

Rats (5/sex) of the F_0 generation were kept on the diets for 1 year and were studied for general toxicological effects. Organ weights were measured in both dose groups, and histopathology was done on tissues from the high dose group. There were no significant differences in general appearance, growth and behavior, or for reproductive parameters including frequency of pregnancy, litter size, pup weights and survival data in any generation. The only effect of the powder on organ weights increased relative kidney weight in the F_0 generation, statistically significant–in males but not in females, in both the 5% and 10% groups. The author discounted this effect due to the high salt content of the algae used.

The F_0 generation animals had no meaningful differences in blood chemistry and hematology parameters. The only differences in histopathology observed were a decrease in some chronic inflammations, a slightly higher frequency of metaplasia of the renal pelvis epithelium with ectopic nephrocalcinosis in the renal papillae, and an increased frequency of focal bronchopneumonia in rats fed 10% algae when compared with the controls. The latter effect was attributed to

the powdery nature of the algal diet. **The author attributed the effects in the kidney to the high salt content of the unwashed test material and cited similar effects of salt administration in the literature. Due to the absence of adverse effects in this study, the authors concluded that the results were indicative of safety for human consumption at levels of 10-20 g per day of the powder.**

A reproductive study was also conducted in mice (Nagasawa *et al.,* 1989a). Male and female SHN/Mei mice were divided into three groups. The control received AIN-76TM diet containing 2.2 X 10^{-4}% retinyl palmitate. The two treated groups were provided with a vitamin A-deficient AIN-76TM diet supplemented with spray dried powder of Nikken's *Dunaliella bardawil* and *D. salina* extract oily solution; the final concentrations of β-carotene in the diets were 0.51 mg/kg and 300 mg/kg diet, respectively. Based on levels indicated in older publications, the carotene content of the algal powder was likely 5% (Ben-Amotz et al., 1989). Therefore, the estimated level of algal powder in the diet of the high dose group is estimated to be 6% and the maximum dose to the animals is estimated as 12 g/kg bw/day.[6] Animals were mated, and on the day of parturition (day 0 of lactation), the number of pups was adjusted to 6-7 within each group through day 20 of lactation. Reproductive parameters measured included delivery interval, mother's weight at parturition, still-birth rate, number of still-born pups, litter size, average pups' weight on days 0, 12 and 20, pups' growth rates, and rearing rates on days 12 and 20. Body weights, estrus cycle, and plasma prolactin levels were assessed in the dams.

Both test groups were apparently lower in delivery interval and still-birth rate, and higher in litter size than the control, while the differences were statistically marginal. Rearing rate was significantly higher in the *Dunaliella* groups than in controls on both days 12 and 20. Little differences were observed among groups in the other parameters. Body weights were increased in males of the *Dunaliella* groups, while there was very little difference in weights of the females. There were no statistical differences in the age of vaginal opening, the pattern of estrus cycle or plasma prolactin levels (Nagasawa et al., 1989a). JECFA did not include this study in its review.

[6] Study authors did not indicate level of algal powder in the diet nor did they calculate animal doses of algal powder. The concentration of β-carotene in algal powder was assumed to be 5% based on contemporaneous information about composition of algal extracts. Conversion of ppm in mouse diet to mg/kg bw was done by dividing by a factor of 5 as recommended by Auletta, 2002 (Table 2.29, page 105). All samples of algal extracts studied by Nagasawa (1989, a,b,c, 1991) were produced by the same production facility in Israel that is the source of current Nikken material.

Although the latter study described in this subsection was designed to test the ability of the natural β-carotene contained in the algal powder to substitute for dietary vitamin A as compared to the control group given retinol, there was an absence of adverse reproductive and developmental effects from the ingestion of the powder at high doses in mice as there was in rats (Mokady et al., 1989).

3. Subacute and Subchronic Studies for *Dunaliella bardawil* Powder

While the one year study conducted on the F_0 generation in the study (Mokady et al., 1989) discussed in Section V.A.2 is the animal study with the longest duration of dietary exposure, a number of other studies were conducted in rats or mice where Nikken's *Dunaliella bardawil* powder or the algal powder from another source was administered orally from 2 weeks to 6 months in duration.

a. 2-Week Studies

Shaish et al. (2006) showed that the algal powder of *Dunaliella bardawil* increased plasma HDL-cholesterol in fibrate treated mice. Six-month-old transgenic male mice (apoA-I C57Bl/6) were fed the powder for two weeks in two separate studies. In the first study, 24 mice divided into 4 groups received either saline, fibrate (30 µg/g bw), or the powder of approximately 4 g/kg diet (approximately 16 mg 9-cis β-carotene/mouse/day), or fibrate plus *D. bardawil* powder. The second experiment was set up like the first, except the mice receiving the powder were given approximately 57 nmol of 9-cis β-carotene/day. Results showed that the combination treatment of fibrate and the powder amplified the effect of the drug on HDL-cholesterol levels.

The effect of a powder from a different source (an authenticated indigenous strain of *D. bardawil* isolated from the Sambar lake of Rajasthan, India) on CCl_4-induced toxicity was studied by Vanitha et al. (2007). Albino Wistar rats were administered either 2.5 or 5.0 g powder/kg body weight each day for 14 days. Animals were tested for CCl_4-induced liver toxicity by measuring biochemical markers in the blood and liver. Administration of the powder decreased the deleterious effect of CCl_4, as evidenced by the decreased level of liver marker enzymes. The results also showed a protective effect of the powder against DNA strand breaks in hepatocytes, as measured by single cell gel electrophoresis. Liver histopathology was better with the powder treatment, supporting the protective action on hepatic oxidative injury.

Neither of these recent studies were available at the time of the JECFA review.

b. 4-Week Study

In an unpublished study directed by T. Furuhashi (1989) and reviewed by JECFA, *Dunaliella* bardawil powder was suspended in aqueous CMC-Na and was administered by oral gavage at 0, 0.5 and 2.5 g/kg bw for 28 days in Sprague Dawley rats (5/sex/group). The ash or salt content for this test sample was not given. The control was 0.5% aqueous CMC-Na solution (10 mL/kg).

Animals were administered their specific dose level *via* gavage daily for 28 days and were observed for general signs, body weights, food consumption, urinalysis, ophthalmic examination, and hematology and blood chemistry parameters. After 28 days of dosing, gross and histopathology examinations were performed [Attachment 10]. JECFA summarized the results of this study as follows:

> There were no deaths or abnormal general signs in any group. No toxicological changes were observed for body weights, food consumption, and ophthalmological examination or in hematological, blood chemistry or urinalysis parameters. The authors concluded that necropsy, absolute organ weights, and histopathological examinations showed no effects related to the test article, however, in high dose males, JECFA observed that a small, statistically significant increase in the ratio of kidney to body weight but not in absolute kidney weight. JECFA also cited slight changes in a few animals in the histopathology of the thymus and kidneys of high dose animals although some of these changes were also noted in control animals (WHO, 1993).

The authors concluded that the powder at the 2.5 g/kg dose level had no toxic effects. The study was performed in compliance with Japanese Good Laboratory Practice Standard for Safety Studies on Drugs.

c. Subchronic Studies (≥ 10 Week Dietary Exposure)

Subchronic studies with oral administration have been conducted in mice and rats.

In a study conducted to evaluate the efficacy of prefeeding *Dunaliella bardawil* powder in ameliorating acid-induced enteritis in a rat model, female Sprague-Dawley (11/group) rats were fed powder diets containing β-carotene for 10 weeks (Lavy et al., 2003). The effects of β-carotene were evaluated by changes in myeloperoxidase activity, histology and histomorphometry. Feeding the powder at a dose of 20 g/kg bw in the diet (containing 5% β-carotene, i.e., for a dose of 1 g/kg bw) with an approximate equal amount of all-*trans* and 9-*cis* isomers) resulted in suppressed mucosal myeloperoxidase in rats. β-carotene also prevented acetic acid-induced histopathologic changes. Therefore,

β-carotene in a rat model is a prophylactic dietary measure that can reduce the effects of acetic acid enteritis (Lavy et al., 2003). No toxicological evaluation was done in the study. JECFA did not include this study in its review.

The effects of spray-dried *Dunaliella bardawil* containing β-carotene on mammary growth and endocrine parameters were examined in mice (Nagasawa et al., 1989b). For 100 days between 20 and 120 days of age, a high tumor strain of SHN/Mei mice received a vitamin A-deficient synthetic standard diet supplemented with synthetic all-trans ß-carotene, and the control animals received the normal synthetic diet adequate in vitamin A. The concentration of ß-carotene in both test diets was 0.55 mg/kg of diet. Assuming that the algal abstract contained 5% ß-carotene based on published information on contemporaneously available material, the level of algal powder in the diet is 11 mg/kg of the diet and the corresponding dose of algal extract to the animal would be approximately 2.2 mg/kg bw/day which is equivalent to 132 mg/person/day in humans as previously noted. (See footnote 6.) The ingestion of the *Dunaliella bardawil*-containing diet showed no deleterious effects on mammary gland and uterine growth or mammatrophic hormone secretion. Puberty and body growth were accelerated by *Dunaliella bardawil* containing β-carotene compared to the synthetic all-trans ß-carotene. The authors concluded based on this study and the study discussed previously in V.A.2 (Nagasawa et al., 1989a), that in mice, several reproductive parameters and body growth are improved by β-carotene from the algal powder.

Two other studies were done in the same high mammary tumor strain of SHN/Mei mice. Both studies were designed to study the influence of dietary algal powder on the development and progression of mammary tumors and neither study employed any systematic toxicological evaluation. In the first study (Nagasawa et al., 1989c), unspecified levels of algal powder were added to the diet of 2 month old virgin females to provide a β-carotene level of 5.1×10^{-5}% for 4-9 months (animals were sacrificed 3 weeks after tumors appeared). Tumorigenisis was significantly inhibited compared to a control group but there was no effect on estrous cycle, prolactin levels or endocrine organ weights. In the second study (Nagasawa et al., 1991), three groups of mice received algal powder to provide a β-carotene level of 5.1×10^{-5}% for three different 3 month periods (1-4, 4-7 and 7-10 months of age, respectively). Tumor incidence was higher in mice given the algal powder during months 1-4, while reduced in the groups that received algal powder during months 4-7 and 7-10. The level of algal powder in the diet for both studies was calculated as 10.2 mg/kg of diet for corresponding dose of algal powder to animals of approximately 2 mg/kg bw/day. (see footnote 6.) The authors did not mention any deleterious effect in either study. JECFA did not include either study in its review.

In an unpublished study which was reviewed by JECFA (which is not currently publicly available), male and female weanling Sprague-Dawley rats (10/sex/group) were provided powdered diets containing 0 or 10% of a test

material described by JECFA as "algal ß-carotene powder" (source of test material not cited) for 12 weeks (Majnarich, 1988). Body weight gain and food intake were recorded at intervals up to and at termination when the animals were autopsied. Weights of heart, lungs, liver, kidneys, spleen, gonads and adrenals were determined at autopsy, and blood was collected for determination of serum glucose, AST, ALT, alkaline phosphatase, uric acid, BUN, triglycerides and cholesterol. One male rat in the treated group died during the study from "non-specific problems" not related to treatment.

There were no significant differences observed for food intake or body weight gain between treated and untreated animals of either sex, and there were no treatment-related differences in organ weights. Except for one treated male with elevated AST and ALT levels, no significant differences were observed in any of the clinical biochemical parameters between treated and untreated animals of either sex. No histopathological examination was performed.

4. Mutagenicity and Genotoxicity Studies Dunaliella bardawil Powder

Three mutagenicity and genotoxicity studies were conducted on dried *Dunaliella bardawil* from Nikken and other sources. None of the studies demonstrated any adverse activity. No studies published after 1993 were reviewed by JECFA. These studies are summarized in Table 7.

Table 7. *Dunaliella bardawil* Mutagenicity and Genotoxicity Studies

Study	*Dunaliella* strain	Test Article/ Concentration	Test System	Result
Aruga et al., 1988 [Attachment 11]	*Dunaliella bardawil* powder	Algal Paste/312.5, 625, 1250, 2500 and 5000 µg/plate. Calculated β-carotene content was not described.	Ames Test using *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2uvrA (With and without S9 activation)	*Dunaliella bardawil* algal paste is not considered mutagenic under conditions of study either in the presence or absence of S9 exogenous for metabolic activation.
Umegaki et al., 1994	*Dunaliella bardawil* (uncertain whether Nikken's powder)	Dry powder of either 0.5% of diet (equivalent to 50 mg/kg β-carotene) or a 4% of diet group (equivalent to 400 mg/kg β-carotene) in male mice.	Chromosomal Damage spontaneous or induced by X-Ray (analyzed from blood sample reticulocytes)	Decrease of micronuclei in reticulocytes with ingestion of *Dunaliella bardawil* suggests protective effect of β-carotene against DNA damage.
Vanitha et al., 2007	*Dunaliella bardawil (not Nikken's* powder)	Oral gavage of 2.5 or 5.0 g/kg body weight to Albino rats (equivalent to 50 and 100 mg of β-carotene/kg) for 14 days.	Comet Assay	*Dunaliella bardawil* treatment did not induce DNA damage and protected against DNA strand breaks in hepatocytes.

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5 Clinical Studies on Nikken *Dunaliella bardawil* Powder

Several published clinical studies have been conducted on Nikken *Dunaliella bardawil* powder since the JECFA review in 1993. Although the studies results were related primarily to β-carotene component of the powder, they also show that there are no adverse events associated with ingesting the powder itself at high levels over a significant period of time. The two most significant studies conducted are the most recent studies where the highest dose of the powder and longest duration were employed.

Morinobu et al., (1994) studied the changes in blood β-carotene levels during long-term administration of the powder in healthy males. Subjects consumed capsules of 365 mg of the powder containing 20 mg of β-carotene and 96 mg of "minerals" three times daily (1095 mg algae, 60 mg β-carotene daily) for 44 weeks. The β-carotene as derived from the powder contained about 50% all-*trans* β-carotene and 50% 9-*cis* β-carotene. Results showed that the all-*trans* β-carotene in the powder had a higher bioavailability than the 9-*cis* β-carotene, even though a small amount of the 9-*cis* form was detected on the blood cells and plasma. The authors did not report any adverse events due to the administration of the powder itself.

Tamai et al., (1996) compared the bioavailability of synthetic all-*trans* β-carotene with naturally occurring β-carotene derived from *Dunaliella bardawil* powder, which contained 50% all-*trans* β-carotene and 50% 9-*cis* β-carotene. Subjects were given total doses of either 60 mg of synthetic all-*trans* β-carotene or 365 mg capsules of the powder which contained 60 mg of β-carotene 3 times per day for 44 weeks. Plasma levels of all-*trans* were higher in the group that received synthetic β-carotene than that of the group that received β-carotene from the powder. In addition, the plasma 9-*cis* levels were higher in the synthetic group than the powder group. This suggests that all-*trans* β-carotene may isomerize to the 9-*cis* form after intake in humans. Bioavailability was considered adequate in both groups. No adverse events were observed by the authors during the study period for the ingestion of the powder or the concentration of β-carotene.

All the clinical studies conducted on Nikken *Dunaliella bardawil* powder are summarized in Table 8. No adverse events were noted by any of the investigators of these published studies. The most notable of the clinical studies are those where more than a gram of Nikken *Dunaliella bardawil* powder was consumed for 44 weeks without ill effects discussed immediately above (Morinobu et al., 1994; Tamai et al., 1996).

Table 8. Clinical Studies on Nikken *Dunaliella bardawil* Powder

Author	Title	Subjects	Dose and Duration	Parameter(s) Tested	Results
Morinobu et al., 1994	Changes in β-Carotene Levels by Long-Term Administration of Natural β-Carotene Derived from *Dunaliella bardawil* in Humans	20 males, age 20-25 years. 10 males in β-carotene group, 10 males in placebo group	Three capsules daily containing 20 mg β-carotene preparation (10 mg all-*trans* and 10 mg 9-*cis*) from *Dunaliella* powder (capsules of 365 mg each) for 44 weeks.	β-carotene concentration was determined in the plasma, red blood cells, platelets and mononuclear cells (MN).	All-*trans* β-carotene present at higher levels in plasma, platelets and MN than 9-*cis* β-carotene. Bioavailability of *Dunaliella bardawil* preferential for all-*trans* β-carotene. No side effects or toxicities occurred during study period.
Levy et al., 1995	Effect of Dietary Supplementation of Different β-Carotene Isomers on Lipoprotein Oxidative Modification	14 males, age 20-30 years	A single 300 mg dose (study one) or three daily doses (study two) totaling 180 mg/day of either synthetic all-*trans* β-carotene or β-carotene from *Dunaliella* (biomass not reported) for 14 days.	Plasma LDL content and the susceptibility of LDL to oxidation (*in vitro*) were tested.	Plasma LDL was elevated in both groups, as well as a reduction in LDL oxidation. The inhibitory effect on oxidized LDL may reduce atherosclerosis. No adverse events were reported by the authors.
Ben-Amotz, and Levy, 1996	Bioavailability of a natural isomer mixture compared with synthetic all-trans-β-Carotene in human serum	60 males, age 20-30 years	Diet supplemented with 20 mg β-carotene twice daily, synthetic or from *Dunaliella* powder (250 mg per capsule) for 14 days.	Serum Analysis to determine distribution of carotenoids, carotenoids isomers, retinol and vitamin E.	All-*trans* β-carotene supplementation resulted in a higher concentration of oxidized products in the serum, suggesting that 9-*cis* β-carotene is a better *in vivo* antioxidant. The authors did not report any adverse events in this study.
Tamai et al., 1996	Bioavailability of Synthesized and Naturally-occurring β-Carotene in Humans	30 males, age not specified	3 times daily of either 20mg All-*trans* β-carotene, 20 mg β-carotene from 365 mg *Dunaliella* powder or placebo for 44 weeks (60 mg β-carotene daily),	β-carotene and retinol concentrations were measured in the plasma and the CD4/CD8 ratio determined.	All-*trans* treatment resulted higher serum levels of All-*trans* β-carotene and 9-*cis* β-carotene compared to *Dunaliella* treatment. This suggests that isomerization of all-*trans* to 9-*cis* form may occur. No adverse events were reported.

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Author	Title	Subjects	Dose and Duration	Parameter(s) Tested	Results
Ben-Amotz et al., 1998	Effect of natural β-Carotene supplementation in children exposed to radiation from the Chernobyl accident	262 children from Chernobyl contaminated nuclear area. 99 were selected for β-carotene intake, age 8-16 years.	Daily supplementation of 40 mg β-carotene from *Dunaliella* (2 capsules of 250 mg each, 20 mg β-carotene each) for 3 months.	Blood serum analysis of conjugated dienes (indicative of oxidated blood lipids), total carotenoids, retinol and α-tocopherol.	Levels of conjugated dienes decreased in the sera after β-carotene supplementation without affecting other compounds tested. β-carotene may act as a lipophilic antioxidant. No adverse events due to β-carotene consumption were reported.
Neuman et al., 1999	Prevention of exercise-induced asthma by a natural isomer mixture of β-Carotene	38 patients (26 males and 12 females) with exercise induced asthma (EIA), age 8-33 years.	Oral, daily supplementation of 64 mg β-carotene derived from *Dunaliella* powder (250 mg per capsule, 22 mg β-carotene per capsule) or placebo for one week.	Pulmonary function (forced expiratory volume) before and after exercise was assessed before and after treatment.	Reduction in forced expiratory volume in patients receiving placebo vs. β-carotene post exercise. 53% of patients receiving β-carotene were protected against EIA. No adverse events were reported.
Levy et al., 2000	Dietary Supplementation of a Natural Isomer Mixture of β-Carotene Inhibits Oxidation of LDL Derived from Patients with Diabetes mellitus	20 subjects with diabetes mellitus and 20 age and sex-matched control subjects.	3 capsules daily of 250 mg *Dunaliella* powder containing 20 mg of β-carotene each.	LDL oxidation measured by malondialdehyde lipid peroxides and conjugated dienes generation induced by $CuSo_4$-induced oxidation.	LDL susceptibility was higher in diabetes patients than controls, which was reduced by β-carotene supplementation. The authors did not report any adverse events during the study period.
Shaish et al., 2006	9-cis β-Carotene-rich powder of the alga *Dunaliella* Bardawil increases plasma HDL-cholesterol in fibrate-treated patients	Trial 1: 20 fibrate-treated men with HDL levels below 40 mg/dl, age 24-65. Trial 2: 22 fibrate-treated patients (20 men, 2 women), age 34-67.	Trial 1: 60 mg β-carotene from *Dunaliella* for 6 weeks. Trial 2: 11 patients received 60 mg β-carotene *Dunaliella* from and 11 patients received placebo for 6 weeks. All patients continued fibrate treatment. *Dunaliella* biomass not reported.	Blood serum lipid profile was assessed.	After 6 weeks, plasma HDL increased in Trial 1 by 24.5% and in Trial 2 by 12.7%. β-carotene seemed to improve the function of fibrate in increasing plasma HDL. No adverse events due to β-carotene administration were reported.
Rotenstreich et al., 2009	Treatment of a Retinal Dystrophy, Fundus Albipunctatus, with Oral 9-Cis Beta-Carotene	Seven patients with fundus albipunctatus	90 days of an unspecified dose of a powder rich in 9-cis-β-carotene	Patients were tested for improvements in retinal function	Patients consistently showed significant improvements in retinal function after 90 days of daily oral treatment.

6. Absorption, Distribution, Metabolism and Excretion (ADME) Studies on the Nutritional Aspects of *Dunaliella bardawil* Powder

Several nutritional studies have been done on algal biomass to assess the protein value of *Dunaliella bardawil* as reviewed by Mokady, 1992. *Dunaliella* protein was found to be of low nutritional quality. No studies have identified any unusual metabolism of the fat, protein and carbohydrate which primarily make up the biomass of *Dunaliella bardawil*.

B. β-Carotene Safety

Because β-carotene is a major component of *Dunaliella bardawil* powder which provides the nutrient supplement functionality intended in finished foods, GA reviewed the literature to determine whether there were any studies published since the 1993 JECFA report which might raise safety concerns. A brief summary is provided below. GA concludes that there is no safety concern raised from the incremental increase in ingestion of β-carotene (3-6 mg/kg/day) as a component of Nikken's powder based on the ADI of 5 mg/kg/day established by JECFA (WHO, 1975) and furthermore, this exposure compares favorably to the estimated consumption of 37 mg/person/day from all dietary sources that was not of concern when previously reviewed by FDA (SCOGS, 1979).

In 1998, the European Commission Scientific Committee for Food accepted β-carotene from *Dunaliella* on the basis of the review of the following studies:

- *Dunaliella salina* belongs to the *Chlorophyceae*, and the unicellular alga, like 25 other species of *Chlorophyceae* which are classified as food sources, does not produce toxins.
- Acute Toxicity - LD_{50} of a preparation containing 30% ß-carotene is >20 g/kg bw in the mouse.
- Short-Term Toxicity - Several studies, lasting between two and eight weeks, have been conducted on rats and chickens with a powder of the alga *Dunaliella bardawil* (which was reported to be identical to the species *Dunaliella salina*) without signs of toxicity up to the equivalent of 0.1% ß-carotene in the diet.
- Multigeneration Reproduction Study - No toxicity has been assigned to a powder of *Dunaliella bardawil* ingested by the rat at dietary levels up to 10% in the diet (equivalent to 0.2% ß-carotene).
- Genotoxicity - The algal ß-carotene was unable to induce gene mutation or chromosomal aberrations in two Ames tests and one chromosome aberration assay in human lymphocytes.

In a comprehensive scientific review of synthetic β-carotene published in 1999, the available toxicology data were reviewed (Woutersen et al., 1999). Synthetic β-carotene was found to be largely neither mutagenic nor genotoxic in *Salmonella, bacillus, E. coli*, mouse mammary cells, bone marrow cells from mice and Chinese hamsters. One study found an increase in chromosome aberrations in cells from mice administered 27 mg/kg but not 2.7 mg/kg. Lack of toxicological effects on reproductive capacity was observed in rats. In several developmental studies, lack of dose related effects was seen in rats and rabbits. Adequate no effect levels were seen in multiple subchronic and chronic studies in several species.

In their review of the dietary necessity of β-carotene and the related safety issues, the Food and Nutrition Board of the National Academy of Sciences concluded as follows:

> Based on these considerations, the existing recommendation for consumption of five or more servings of fruits and vegetables per day is supported because this would provide 3 to 6 mg/day of β-carotene. A UL has not been set for β-carotene or carotenoids. Instead, it is concluded that β-carotene supplements are not advisable for the general population. This conclusion is based on a totality of evidence that includes several large-scale randomized trials of supplemental β-carotene. These trials indicate a lack of evidence of overall benefit on total cancer or cardiovascular disease and possible harm in certain subgroups such as current smokers or asbestos exposed subjects. This advisement does not pertain to the possible use of supplemental β-carotene as a provitamin A source or for the prevention of vitamin A deficiency in populations with inadequate vitamin A nutriture or in patients suffering from erythropoietic protoporphyria (FNB, 2000).

In addition, GA reviewed a subchronic toxicity study and several mutagenic studies which were testing the β-carotene isolated from *Dunaliella bardawil*. The results of the 90-day study in F344/DuCrj rats concluded that based on growth suppression, NOAELs were estimated to be 1.25% (696 mg/kg bw/day) for males and 5% (2879 mg/kg bw/day) for females. However, because of increases in serum calcium in the lowest group in both sexes, a NOEL could not be determined in this study (Kuroiwa et al., 2006).

Several genetic toxicology studies showed that β-carotene isolated from *Dunaliella* bardawil does not exhibit mutagenic or clastogenic effects under a variety of conditions (Ma et .al., 1998; Cifone, 1987; Jagannath, 1987; Ivett 1987; and Young, 1987).

There were two published clinical studies conducted in humans with β-carotene from *Dunaliella* sources (Jensen et al., 1985; Jensen et al, 1987) and, as noted in the discussion of safety of the powder itself, the clinical studies showed that neither the powder nor β-carotene raised safety issues.

VI. DISCUSSION OF SAFETY INFORMATION and GRAS CRITERIA

A. Discussion of GRAS Criteria

FDA defines "safe" or "safety" as it applies to food ingredients as

> "...reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use. It is impossible in the present state of scientific knowledge to establish with complete certainty the absolute harmlessness of the use of any substance."[7]

Amplification is provided in that the determination of safety is to include probable consumption of the substance in question, the cumulative effect of the substance, and appropriate safety factors. It is FDA's operational definition of safety that serves as the framework against which this evaluation is provided.

Furthermore, in discussing GRAS criteria, FDA notes that

> "...General recognition of safety requires common knowledge about the substance throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food."
>
> "General recognition of safety through experience based on common use in food prior to January 1, 1958, shall be based solely on food use of the substance prior to January 1, 1958, and shall ordinarily be based upon generally available data and information."[8]

FDA discusses in more detail what is meant by the requirement of general knowledge and acceptance of pertinent information within the scientific community, i.e., the so-called "common knowledge element," in terms of the two following component elements:[9]

- Data and information relied upon to establish safety must be generally available, and this is most commonly established by utilizing published, peer-reviewed scientific journals; and

There must be a basis to conclude that there is consensus (but not unanimity) among qualified scientists about the safety of the substance for its intended use, and this is established by relying upon secondary scientific literature such as published review articles, textbooks, or

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7 See 21 CFR 170.3(i).
8 See 21 CFR 170.30(a).
9 See Footnote 4.

compendia, or by obtaining opinions of expert panels or opinions from authoritative bodies, such as JECFA and the National Academy of Sciences.

The apparent imprecision of the terms "appreciable", "at the time" and "reasonable certainty" demonstrates that the FDA recognizes the impossibility of providing absolute safety (Lu,1988; Renwick,1990).

B. Safety Evaluation of Related Algae and Microorganisms as Food Sources

Brown and red algae have GRAS status in the US as flavoring substances, as noted in 21 CFR 184.1120 and 21 CFR 184.1121, respectively. These ingredients were affirmed as GRAS on the basis of reviews conducted by the Select Committee on GRAS Substances (SCOGS, 1973). The affirmation was based on information that is sufficient to support the low use levels needed in food to impart flavor.

More recently, FDA has not objected to a GRAS notification (GRN 147) on the dried biomass of spirulina (*Arthrospira platensis*) for use in certain foods at levels of 0.5-3.0 grams per serving (FDA, 2003). This action establishes that FDA recognizes the acceptability of the addition to foods of algae whose safety has been demonstrated. FDA based its determination on studies in animals, along with several published studies on humans, that addressed safety and potential beneficial effects of spirulina. No adverse effects were noted in oral feeding studies in rats, pigs, mice and hamsters, nor were any adverse side events reported in the clinical studies. The lack of objection by FDA was also predicated on the notifier's adherence to good manufacturing practices and the establishment of adequate food grade specifications, including limits on various contaminants, such as heavy metals, pesticides, rodent hairs, insect fragments, and bacterial contamination.

The European Commission has proposed criteria for the safety of microorganisms for use as a food source (European Commission, 2003). The Commission suggested criteria supporting an approach of a Qualified Presumption of Safety (QPS) based on reasonable evidence. Among the criteria suggested was the assurance that pathogenic forms of the organisms do not exist or cannot be formed and that no harmful metabolites are produced or cannot be eliminated in processing.

C. Discussion of *Dunaliella bardawil Powder* Safety Studies

The Expert Panel has considered the entire compilation of chemical, toxicological and clinical evidence related to the safety of Nikken's *Dunaliella bardawil* powder as a food ingredient. The Panel has also considered previous reviews and regulatory determinations, such as those described in Section VI.B, by expert bodies and regulatory agencies on *Dunaliella* and related forms of algae. Key safety information considered by the Panel is summarized below.

1. Consideration of JECFA Review

JECFA last reviewed the safety of dried biomass of *Dunaliella bardawil* in 1993. They concluded that there were insufficient data to establish an ADI from consumption in food (WHO, 1993). It is apparent that JECFA reviewed the available information merely on toxicological principles using laboratory animal data. Over the past 17 years, more data on *Dunaliella bardawil* powder have become available. In addition, the conventional wisdom has evolved to consider other information, such as the harmlessness of many sources of biomass and lack of harmful metabolites in the review of natural products, including spirulina (European Commission 1998, 2003; FDA, 2003).

2. Consideration of Composite Safety Information on *Dunaliella bardawil* Powder Containing β-Carotene

The Panel reviewed the cumulative available safety data to determine if consumption of 71 mg per person per day (1.2 mg/kg bw/day for a 60 kg adult) of the dried biomass of *Dunaliella bardawil* as manufactured by Nikken is safe.

The most compelling information addressing the safety of *Dunaliella bardawil* powder relates to the composition of the dried biomass which is largely carbohydrate, protein, and fat and which contains a significant amount of β-carotene. There has been much research spanning many decades on *Dunaliella bardawil* itself that has established it to be a non-pathogenic organism; furthermore, it is not known to form toxic metabolites or toxins (Ben-Amotz and Avron, 1990). The organism has been selected due to the fact that it readily synthesizes large quantities of β-carotene under stress conditions after the growth phase in culture. The organism was isolated decades ago using traditional genetic selection techniques and is not a product of advanced biotechnological manipulation. Based on well documented basic chemistry information, the Panel agrees that there is a "qualified presumption of safety" for the dried biomass of *Dunaliella bardawil,* as defined by the criteria under development by the European Commission (2003).

Several published clinical studies have been done on Nikken *Dunaliella bardawil* powder since the JECFA review. In all nine studies summarized in Table 8, no adverse events were noted. The studies of the greatest significance based on duration and dose are the clinical studies where healthy human volunteers consumed 1.1 g daily of the Nikken *Dunaliella bardawil* powder over a 44 week period (Morinobu et al., 1994; Tamai et al., 1996). The test material was well tolerated by the participants, and no adverse events were noted. Moreover, Nikken has been marketing its product in several different countries as a health food supplement for over twenty years and has not received reported adverse events---including allergenicity---nor has it had to recall its product. There also have not been any adverse events related to this powder reported in the published literature.

Three mutagenicity and genotoxicity studies have been conducted on *Dunaliella bardawil*, including one for the subject powder, all of which support the conclusion that no adverse effects were exerted on genetic mechanisms. The absence of activity on genetic mechanisms is indicative of a low likelihood that any constituent of *Dunaliella bardawil* powder is carcinogenic or teratogenic.

The most comprehensive animal toxicology evaluation comes from a 4-generation reproductive study where the F_0-generation was maintained on the diet for one year (Mokady et al., 1989). However, a NOEL was not identified in this study, as animals of the F_0-generation maintained on diets containing 5% and 10% dried algal powder containing ß-carotene showed increased kidney weights and slight renal pathological changes. In addition, there was focal bronchopneumonia in lungs at the higher level. The study authors are of the opinion that the increase in kidney weight and the low incidence of renal changes (metaplastic changes in the pelvic epithelium and nephrocalcinosis) were likely due to the high salt content of the test material. JECFA reported that this was not clearly established (WHO, 1993). However, the authors noted that salt content was apparently found to be a problem by other investigators since the kidney effects seen were typical findings from studies on salt.

The salt content of the test sample was 24.7% salt. This is much higher than modern samples which have been washed before spray drying. The Nikken product typically is about 6% ash and 2-3% salt. The Panel is of the opinion that the high ash in the Mokady sample is likely mostly salt from the brine pits where the algae were grown. The Panel investigated the literature on the effects of salt and determined that high salt diets in rats lead to epithelial hyperplasia (Meneely et al., 1953), including in renal pelvic epithelium (Lalich et al., 1974), and increases in kidney weight (Yamori et al., 1983).

The authors also indicated that focal bronchopneumonia observed in the lungs at the higher dose level was likely associated with inhalation of powdery diets, although both low-dose and control rats also received powdered diets without showing similar effects. However, the Panel believes that the lung effects are likely due to inhalation of the diet and are irrelevant because the use of *Dunaliella bardawil* powder in human food at much lower levels is not expected to present an inhalation exposure.

The Panel notes that there are two other unpublished rat studies employing high salt levels in the diets for 4 weeks (Furuhashi, 1989) and 12 weeks (Majnarich, 1988), respectively. Although the ash, mineral or salt contents were not reported for either test samples in these studies, since the studies were done contemporaneously with the Mokady study, the Panel believes it is likely that these samples were also high in salt. In the 4-week study, the Panel notes that no changes were detected in absolute kidney weight but that a statistically significant increase in kidney-to-body-weight ratio was seen in high-dose males. Slight kidney changes were seen by histopathology in only a few animals, and the incidence was not clearly higher than that seen in the untreated control group.

A report of a 12-week study was not available for Panel review, but JECFA indicated that no kidney effects were seen (WHO, 1993). In view of the low salt content of the Nikken product and the low consumption proposed for the ingredient, the Panel is not concerned about the kidney effect seen in the rat studies conducted at very high doses.

For the evaluation of the safety of a novel food substance for human consumption, it is necessary to consider the amount of the substance which the human population may consume and to use this value as one of the parameters for safety assessment. In addition, as suggested by a committee of the European Commission (2003), the Panel believes that there is a "qualified presumption of safety" based on the research on *Dunaliella bardawil* powder indicating no concerns based on the information on the genetics of the organism or its chemical composition. Based on the food uses proposed by Nikken, the human consumption of dehydrated *D. bardawil* will not exceed 0.071 grams per day which is equivalent to an average consumption of 1.2 mg/kg bw/day for a 60 kg adult. In the previously mentioned multigeneration study (Mokady 1989), a much greater amount was employed. The daily dose of algae in the rats fed the 10% dietary level of algae was 10 g/kg bw/day in the weanlings and 20 g/kg bw/day in the adult animals.

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If the Mokady study were to be repeated with better control of salt levels in the test sample and better diet preparation to avoid dust, in view of the fact that no other adverse effects were seen, the NOEL would likely be in the range of 2-20 g/kg bw/day due to the fact that no other effects were noted in the study. If this were the case, the margin of safety to the calculated EDI (0.071 g/day, or 1.2 mg/kg/day for a 60 kg person) would be in the range of 28-280 fold to the projected NOEL in the Mokady study. It should be noted that the estimated consumption level of 0.071 grams/person/day was calculated by methodology that FDA guidelines indicate overestimates the 90th percentile exposure. The consumption level calculated by assuming consumers eat the mean quantity of each food type Nikken proposes is 0.035 g/day. More sophisticated statistical methods would likely show that the 90th percentile consumption level is somewhere between 0.035 and 0.071 g/day. The Panel agrees that this level of consumption of algal biomass is safe.

Other aspects of the Nikken product have been reviewed for safety considerations. The Panel reviewed the manufacturing process and purity specifications and agrees that the Nikken product is suitable food grade. The Panel has reviewed the calculated consumption of certain trace elements in the Nikken product and has concluded that the levels are very low, and the level of consumption of these elements from maximum consumption Nikken product would be at least 2 orders of magnitude lower than background levels in the diet. The Panel also notes that there is no indication in the literature that *Dunaliella bardawil* bioaccumulates any element including iodine, arsenic and other heavy metals. Nikken reports that they have not received any complaints of possible allergenicity to any of their products currently used in foods and dietary supplements on the market world-wide, nor have there been such reports in the literature.

The Panel is aware that Nikken includes cautionary statements on the labels of their US dietary supplement products to "keep out of the reach of children," and that women who are pregnant or nursing should consult their physicians before taking its product.

This action prompts the question as to possible safety concerns associated with the designated significant subpopulations. The Panel notes that Nikken does not include this cautionary statement regarding children or women who may become pregnant or are nursing on its *Dunaliella powder* dietary supplement products that it markets outside of the US. In addition, Nikken has had no adverse events reported for over 20+ years with these products. Although the objective of a clinical trial using children 8-16 years of age was not to demonstrate the safety of the powder itself, the results revealed no adverse events when 500 mg/day of *Dunaliella* powder was ingested over a 3 month period (Ben-Amotz et. al., 1998).

Furthermore, review of laboratory animal data in concert with the clinical studies revealed to the Panel no indications of any safety problems for children or for pregnant or lactating women. Finally, the Panel recognizes that consumption from the use of the subject product is likely to be several fold **lower** from its use in conventional foods than is commonly consumed as a dietary supplement.

The firm's decision to retain the cautionary statement on US dietary supplement products appears to reflect a preference to err on the side of caution and general industry practice in the US. The Panel is satisfied that there are no specific or known safety concerns associated with the intended food uses of the subject *Dunaliella* powder.

Furthermore, the Panel has concluded that sufficient safety data are available on *Dunaliella* to consider the low level of consumption proposed for Nikken's *Dunaliella bardawil* powder to be generally recognized as safe (GRAS) within the meaning of the Federal Food, Drug, and Cosmetic Act.

In addition the Panel has concluded that the additional exposure to the major component of *Dunaliella bardawil* powder, i.e., β-carotene, does not present a safety concern. The expected consumption of the carotenoids in processed foods containing added powder at the proposed level of 0.071 g/day is 6 mg/day. 95% of the carotenoids would be in the form of β-carotene and the balance would consist of certain minor carotenoids. The β-carotene in the Nikken product exists in nearly equal parts of all *trans*-isomer and the 9-*cis*-isomer. JECFA has concluded that the ADI for β-carotene from traditional sources is 5 mg/kg bw/day. (WHO 1975). Ample data exist to demonstrate that the isomeric forms of β-carotene do not differ toxicologically. Therefore, daily consumption of 0.071 g/day of *Dunaliella bardawil* powder containing 6 mg of carotenoids or 0.10 mg/kg bw/day carotenoids is well below JECFA's ADI of 5 mg/kg bw/day for β-carotene or the 37 mg/person/day calculated from all dietary sources that did not raise a safety concern for FDA (SCOGS, 1979).

This determination is predicated on Nikken's continued production of *Dunaliella bardawil* powder in conformance with GMP regulations.

3. Common Knowledge Elements of GRAS Determination

The first common knowledge element for a GRAS determination is that data and information relied upon to establish safety must be generally available; this is most commonly established by utilizing published, peer-reviewed scientific journals for the safety assessment. The majority of studies reviewed as part of this safety assessment have been published in the scientific literature. Limited use was made of unpublished studies, and these were deemed to be supportive and were not primary studies.

The second common knowledge element for a GRAS determination is the need for consensus among qualified scientists about the safety of the substance with its intended use.

Certainly, many competent researchers responsible for the data referred to in this report that have expertise in the fields of aquaculture, nutrition, toxicology and medicine view algae as a viable source of food and a viable source of certain nutrients, and they have been cited in this evaluation.

Considerable advances in thinking have occurred over the past few years regarding the safety evaluations of natural products in food. It is difficult to feed ingredients with nutritional value at high levels to laboratory animals because of the possibility of disturbing nutritional balance. Therefore, establishing large margins of safety from these studies is impractical. Seasoned scientific judgment must be applied in evaluating these studies. In addition, new concepts in safety evaluation (European Commission, 2003) such as "qualified presumption of safety," taking into account genetic background and compositional information of ingredients from novel organisms, are gaining increased scientific credibility and acceptance. The increasing comfort level with algal biomass use in food products is evidenced by FDA's acceptance of the GRAS Notice for spirulina.

The Panel recognizes that the safety of *Dunaliella bardawil* powder in human foods has been the subject of interest for many years. There is an additional high presumption of safety based on the fact that decades of research have not identified that any harmful metabolites are generated by this organism under any growth condition. A number of published clinical studies with *Dunaliella bardawil* powder are available, and no untoward events were seen in any of them (Morinobu et al., 1994; Tamai et al., 1996; Levy et. al., 1995: Ben-Amotz and Levy, 1996; Ben-Amotz et al., 1998). Therefore, this plant biomass which consists of normal common carbohydrate, protein and fat has a high presumption of safety. Only a handful of the published studies in animals where the algal biomass was administered by the oral route were designed as rigorous toxicology studies.

The Panel is cognizant of the scientific rigor and broad base of scientific expertise from various regulatory agencies around the world that resides within JECFA. However, JECFA has not updated its review since 1993, and it did not review the numerous studies which have been done during the last 17 years. The collection of independent studies in the composite viewed under emerging common sense criteria to evaluate sources of biomass for safety provides a firmer consensus within the scientific community that adequately offsets the outdated and now incomplete position on safety of *Dunaliella bardawil powder* contained in the cited JECFA report. The Panel believes that consensus exists

regarding the limited food intake levels identified in the Section IV Intended Dietary Uses and that such information supports a GRAS conclusion.

VII. CONCLUSIONS[10]

The Expert Panel unanimously agrees that Nikken's *Dunaliella bardawil* powder of appropriate food grade purity when consumed in the specified foods at a level not to exceed 1.2 mg/kg bw/day is generally recognized as safe. This designated human dietary exposure reflects a per person incremental β-carotene level of 0.12 mg/kg bw/day. Both of these designated amounts---for *Dunaliella bardawil* powder and its major nutrient component, β-carotene---are below appropriately determined ADIs, respectively.

The Panel offers the following conclusion:

> *Dunaliella bardawil* powder that is produced in accordance with FDA Good Manufacturing Practices requirements while meeting the purity specifications for *Dunaliella bardawil* powder, as set forth in Section III.D, Tables 2 and 3 of this notification, is generally recognized as safe when consumed at levels up to 0.071 gram per person per day or 1.2 mg/kg bw/day in the proposed foods and at the proposed food use levels.

This declaration is made in accordance with FDA's standard for food ingredient safety, i.e., reasonable certainty of no harm under the intended conditions of use.

In gathering data and making this decision the Expert Panel utilized the assistance of Dr. Jürgen Polle, Associate Professor, Department of Biology, Brooklyn College of the City University of New York, 2900 Bedford Ave, Brooklyn, NY. Professor Polle is a world-wide expert on *Dunaliella bardawil*.

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[10] The biographical information for Richard C. Kraska, Ph.D., DABT, Robert S. McQuate, Ph.D., and Ms. Robin Guy, MS, DABT can be found on GRAS Associates website---www.gras-associates.com. Dr. Jüergen Polle's curriculum vitae is at Attachment 12. Dr. Kraska served as Chair of the Panel.

(b) (6)

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(b) (6)

Robert S. McQuate, Ph.D. July 29, 2010

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ATTACHMENTS

1

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

Food and Drug Administration
5100 Paint Branch Parkway
College Park, Maryland 20740

AUG 1 5 2007

C. K. Gund, Ph.D., President
Phoenix Regulatory Associates, LTD.
21525 Ridgetop Circle, Suite 240
Sterling, Virginia 20166

Dear Ms. Gund:

This is to inform you that the notification which you submitted pursuant to 21 U.S.C. 350b(a)(2)(section 413(a)(2) of the Federal Food, Drug, and Cosmetic Act (the Act)) was filed by the Food and Drug Administration (FDA) on March 1, 2007. Additional information was received on April 9, 2007. Your notification concerned "Dunaliella Dried Powder" which you derive from *Dunaliella bardawil* Ben-Amotz & Avron and identify as a new dietary ingredient.

According to your notification, "A single serving of powder (1 mini teaspoon) is added to milk or a milk-containing product and stirred until the powder is dissolved. Usage is 1 to 3 times per day. One level mini teaspoon of powder is equal to approximately 100 mg or about 0.1mL. Nikken recommends that its product be taken daily over a person's lifetime. However, it does not recommend its product for children or for pregnant women. The label will carry the cautions that this product should be kept out of the reach of children and women who are pregnant or nursing should consult their physicians before taking this product."

Under 21 U.S.C. 350b(a), the manufacturer or distributor of a dietary supplement containing a new dietary ingredient that has not been present in the food supply as an article used for food in a form in which the food has not been chemically altered must submit to FDA, at least 75 days before the dietary ingredient is introduced or delivered for introduction into interstate commerce, information that is the basis on which the manufacturer or distributor has concluded that a dietary supplement containing such new dietary ingredient will reasonably be expected to be safe. FDA reviews this information to determine whether it provides an adequate basis for such a conclusion. Under 21U.S.C.350b(a)(2), there must be a history of use or other evidence of safety establishing that the new dietary ingredient, when used under the conditions recommended or suggested in the labeling of the dietary supplement, will reasonably be expected to be safe. If this requirement is not met, the dietary supplement is considered to be adulterated under 21U.S.C.342(f)(1)(B) because there is inadequate information to provide reasonable assurance that the new dietary ingredient does not present a significant or unreasonable risk of illness or injury.

Please note that acceptance of this notification for filing is a procedural matter, and thus, does not constitute a finding by FDA that the new dietary ingredient or supplement that contains the new dietary ingredient is safe or is not adulterated under 21 U.S.C. 342. FDA is not

precluded from taking action in the future against any dietary supplement containing your new dietary ingredient if it is found to be unsafe, adulterated or misbranded.

Your notification will be kept confidential for 90 days after the filing date March 1, 2007. After the 90-day date, the notification will be placed on public display at FDA's Docket Management Branch in docket number 95S-0316. Prior to that date, you may wish to identify in writing specifically what information you believe is proprietary, trade secret or otherwise confidential for FDA's consideration.

If you have any questions concerning this matter please contact me at (301) 436-1448.

Sincerely yours,

(b) (6)

Linda S. Pellicore, Ph.D.
Supervisory Team Leader, Senior Toxicologist
Division of Dietary Supplement Programs
Office of Nutritional Products, Labeling
and Dietary Supplements
Center for Food Safety and Applied Nutrition

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2

Product Description

Before submitting an order you will be asked to read and accept the terms and conditions of ATCC's [illegible] or, in certain cases, an MTA specified by the depositing institution.

Customers in Europe, Australia, China, Hong Kong, India, Japan, Korea, Macau, New Zealand, Singapore, and Taiwan, R.O.C. must contact a [illegible] for pricing information and to place an order for ATCC cultures and products.

Protozoa and Algae

ATCC® Number: 30861™ Order this item **Price:** $156.00

Organism: *Dunaliella bardawil* Amotz and Avron

Isolation: salt pond near Bardawil Lagoon, North Sinai, Israel, 1976

Depositors: A Ben-Amotz

Biosafety Level: 1 **Shipped:** test tube

Propagation: See Below

Permits/Forms: In addition to the MTA mentioned above, other ATCC and/or regulatory permits may be required for the transfer of this ATCC material. Anyone purchasing ATCC material is ultimately responsible for obtaining the permits. Please click here for information regarding the specific requirements for shipment to your location.

This material is cited in a U.S. and/or other Patent or Patent Application, and may not be used to infringe on the patent claims.

Related Products

Applications: produces glycerol, carotenes, and protein-rich material [2458]
produces carotene (carotenes; zeta-carotene) [2458]
produces glycerol (glycerin; glycerine) [2458]

Comments: Photosynthetic. Deposited as mixed culture. Bacteria present.
Source of beta-carotene in chicken feed [58073]
Chemical profile of selected species of microalgae [4662]
Stereoisomers of B-carotene and phytoene [4884] [50[illegible]]
Two-phase growth for B-carotene production [23[illegible]]

Propagation: ATCC Medium 1174: DA medium
Temperature: 20.0C to 25.0C

Cross Reference: GenBank: [illegible]: Dunaliella bardawil phytoene synthase mRNA, complete cds.

Classification: KINGDOM: Plantae

References:
2458: Avron M , Ben-Amotz A . Production of glycerol, carotenes and algae meal. US Patent 4,199,895 dated Apr 29 1980
4662: Ben-Amotz A , et al . Chemical profile of selected species of microalgae with emphasis on lipids. J. Phycol. 21: 72-81, 1985.
4884: Ben-Amotz A , Avron M . On the factors which determine massive Beta-carotene accumulation in the halotolerant alga Dunaliella bardawil. Plant Physiol. 72: 593-597, 1983.
4932: Ben-Amotz A . Accumulation of beta-carotene in halotolerant algae: purification and characterization of beta-carotene-rich globules form Dunaliella bardawil (Chlorophyceae). J. Phycol. 18: 529-537, 1982.
4933: Ben-Amotz A , et al . Massive accumulation of phytoene induced by norflurazon in Dunaliella bardawil (Chlorophyceae) prevents recovery from photoinhibition1. J. Phycol. 23: 176-181, 1987.
4934: Ben-Amotz A , Avron M . The wavelength dependence of massive carotene synthesis in

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Dunaliella bardawil (Chlorophyceae)1. J. Phycol. 25: 175-178, 1989.
5048: Ben-Amotz A , et al . Stereoisomers of -carotene and phytoene in the alga Dunaliella bardawil. Plant Physiol. 86: 1286-1291, 1988.
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58073: Ben-Amotz A , et al . Use of the beta-carotene rich alga Dunaliella bardawil as a source of retinol. Br. Poult. Sci. 27: 613-619, 1986. PubMed: [illegible]

Notices and Disclaimers

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All prices are listed in U.S. dollars and are subject to change without notice. A discount off the current list price will be applied to most cultures for nonprofit institutions in the United States and Canada. Cultures that are ordered as test tubes or flasks will carry an additional laboratory fee. Fees for permits, shipping, and handling may apply.

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30861 | ATCC Number Search | Clear Search |

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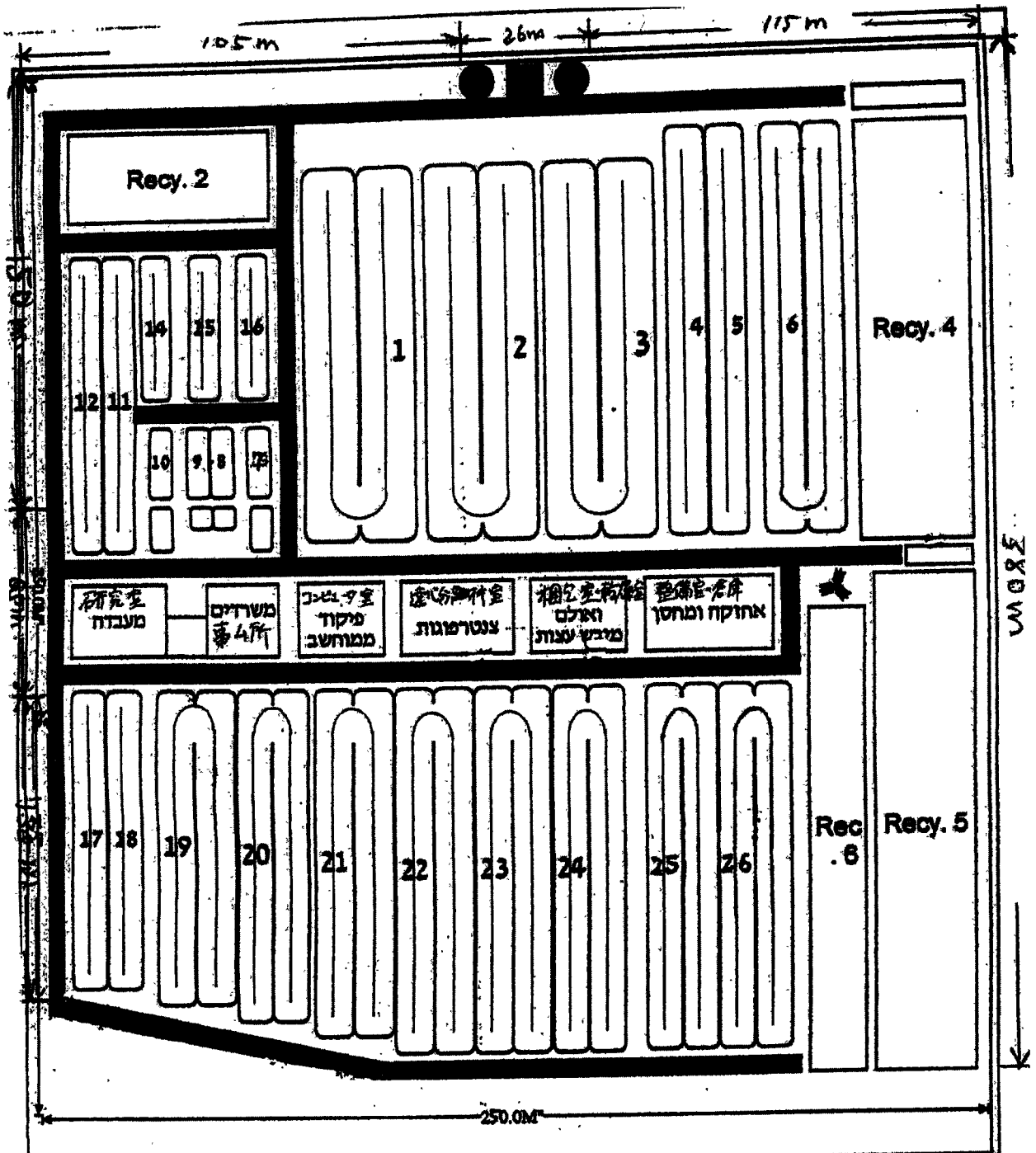

NBT
Nature Beta Technologies LTD.
P.O.Box: 626 Elat
ISRAEL, 88106
Telephone: 03-6331219
Fax: 03-6378107

Project Designer:	
Roman Goldin	
Project Contact:	
NBT LTD.	
Project #	WO#
#01	
Date:	Rev. Date:

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Attachment 4

NBT Monthly Report (6/10) to Marine Coastal Environment Division, Ministry of Environment, State of Israel

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	אצות דונליאלה בתעלה זבים
[illegible]	#	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
1/31/2010		48	8	2 5	2 3	15	5	None	0.05	0 5	0 5	0.5	1 8	0 05	0 05	90	90	90	None	None	
							Bactochen Analysis # 70/18941 (attached)					Photo attached									None
3/31/2010		54	8	0.5	2 8	5	0 3	none	0 05	0,5	0,65	<0 001	1 95	0.06	0 05	90	90	90	none	none	
							Bactochem Report # 61/107408 (attached)					Photo attached									none
4/30/2010	61/109013	55	8	2 6	1	10	0.5	none	0 05	0,5	0,51	<0 001	2 1	0 46	0.05	90	90	90	none	none	
								Attached photo													none
5/31/2010	61/110650	50	8	1	2	8	0 7	none	<0.05	<0 05	0 62	<0 001	2.1	0.46	0 05	9-	90	90	none	none	
									Attached photo												none
No. of samples		4	4	4	4	4	5	6	5	5	4	5	4	4	4	4	4	4	4	4	
Average		51 75	8	1 65	2.025	9 5	1.625	#DIV/0!	0.05	0 5	0 56	0.5	1.9875	0 2575	0 05	90	90	90	#DIV/0!	#DIV/0!	4
Minimum		48	8	0.5	1	5	0 3	0	0 05	0 5	0.5	0 5	1 8	0 05	0.05	90	90	90	0	0	#DIV/0!
Maximum		55	8	2 6	2 8	15	5	0	0 05	0.5	0 62	0 5	2.1	0.46	0.05	90	90	90	0	0	0
																					0

The last column to the right is Dunaliella algae discharge The columns (4 - 6) from the right are light Transmission at different wave lengths NBT did not provide a copy of the photograph but it as a monthly photo NBT takes of the discharged water

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אמינולאב בע"מ שירותי מעבדה אנליטיים לתעשיה, רפואה, חקלאות, מחקר ואיכות הסביבה
סניף ראשי: קרית רצמן, ת.ד. 4074, נס ציונה 70400 טל': 08-9409690, פקס: 08-9408474
סניף צפון: בנין ר.מ.א, חיפוריים 19, ת.ד. 1033, כרמיאל 20100 טל': 04-9586916, פקס: 04-9582184

10.02.2004
C 0633 0204

לכבוד
מר גרשון חיריפרסי
אנ.מי.טי
ת.ד 828 אילת 88106
טלפון: 08-6378770 08-6331219
פקס: 08-6378107
תגדון: תעודת בדיקה
מס' אמינולאב: 03631.04-C

תאריך קבלה: 02.02.04
תאור הדוגמאות: אבקת 011-004
נדגם ע"י: הלקוח
בדיקה: ארסן (As) ועופרת (Pb) ב- ICP-AES

תוצאות מעבדה ב- mg/kg:

מספר אמינולאב	03631
בדיקה	אבקת 011-004
ארסן (As)	<1
עופרת (Pb)	<2

נבדק ע"י: לילה איסקוב (b) (6)
אושר ע"י מנהל המעבדה: דרי צדוק שאבי (b) (6)

יש לפנות לעניינים המפורטים בפסקה זו רק בשלמות ואין לפרסם או לצטט, את כולם או חלקם, [illegible] המפורטים המופיעים בבדיקות אינו [illegible] מעבדה לרמה [illegible] לבדיקה.
אין לפרסם שום מאמר של אמינולאב בעיתון או בפרסום שלה, אלא אם כן [illegible] המוקדם בכתב.
הרשות לפרסומות [illegible] הבדיקות [illegible] המעבדה [illegible] אחוד לבדיקה עובדים.
דף 1 מתוך 1

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[redacted]

Aminolab Ltd. Analytical Laboratory Services

Main Branch: Weizmann Science Park, Ness-Ziona, Israel. Tel: (972)8-9303333, Fax: (972)8-9303300
North Branch: R.M.A. Building, P.O.B. 1033, Karmiel 20100 Israel. Tel: (972)4-9586916, Fax: (972)4-9582154

09/11/2009
Report No: P7690_R

ISRAC
ISO/IEC 17025
No 009

To:
Hirofumi Goto
N.B.T. Nature Beta Technologies Ltd.
P.O.Box 828
Eilat 88106
Tel: 08-6331219
Fax: 08-6378107

Re: Certificate of Analysis

Aminolab No: 51179.09-P **Sample reception:** 02/11/2009

Sample: Algae Powder #10168

Sampled by: The Customer

Analysis Method:

Non Fatty Food Products: FDA-PAM Vol 1, 3rd ed., sec. 302, by GC-MS EI Scan and/or SIM.

Pesticides determined: Organophosphorus, Organochlorine, Organonitrogen & Pyrethroids.
List of pesticides under accreditation was sent to the sponsor.
LOD: 0.01-0.05 ppm
LOQ: 0.02-0.20 ppm

Analyses Results:

Analysis	Units	Results	MRL	Remarks
Pesticide Residues by GC-MS	ppm	ND	-	1

MRL - Maximum Residue Limit
ND - Not Detected

Remarks:
(-)=No remarks
1 GC-MS amenable herbicides were not detected.

Quality Assurance:
The laboratory is accredited under ISO/IEC 17025 with standard operating procedures [redacted]
- Aminolab is accredited to perform this analysis under ISO/IEC 17025 by ISRAC.
- Aminolab has the recognition to perform this analysis by the Israeli Ministry of Health and the Ministry of Agriculture.

Data Analysis: Dr. Yoram Cohen- Lab. D. Manager Signature: (b) (6)
Approved by: Uri Golner - Lab. Manager Signature: (b) (6)

This certificate is valid only when it is presented in its complete format, and it is not permitted to extract part for inclusion in any other document. The data presented accurately expresses the results for the sample received only. It is not permitted to use Aminolab Ltds' name or its reputation in respect to the above - specified results, without Aminolab written consent.

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אמיולאב בע"מ עמוד תעבית אביבים לתעשיה, רפואה, חקלאות, מחקר ואיכות הסביבה
גיף ראשון קריית נתניה ת.ד. 4074, נס מדינה 70400 טל: 09-9203333, פקס: 09-9203399
גיף נמוך בנין ר.מ.ן, מיוחדים 19, ת.ד. 1083, פרדסיה 40100 טל: 09-8886016, פקס: 09-8882156

29/04/2008
C2924: דו"ח מס'

לכבוד:
Hirofumi Goto
מישטר בטח טכנולוגיות בע"מ
ת.ד. 828
אילת 88106
טל: 08-6331219
פקס: 08-6378107

הנדון: **תעודה לתוצאות בדיקה**

מס' אמיולאב: 18059.08-C
תאור הדוגמה: אבקה 0804085-0804089
נדגם ע"י: חלקוה
סוג הדיגום: לא ידוע

תאריך קבלה: 23/04/2008

תוצאות הבדיקה:

בדיקה	יחידות מידה	תוצאה	הערות
סריקת מתכות ב- ICP			
ארסן - As	mg/Kg	<2	1
עופרת - Pb	mg/Kg	<2	

Arsenic
Heavy metals

הערות לבדיקה:
(-) = אין הערות.
1. סריקת מתכות - תוצאה מלווה בסימן < - לא נמצאו עקבות בדוגמה בגבול הרגישות המצוין.

שיטות איכות:

בדיקה	שיטה / תקן	הערות
סריקת מתכות ב- ICP	SOP# CW-041	-

הערות / הערות:
למעבדה מערכת איכות מוסמכת לפי ISO/IEC 17025 והיא פועלת בהתאם לנהלי עבודה מסודרים.

(b) (6)

(b) (6)

נבדק ע"י: מרינה רוכמן

אושר ע"י: דר' צדוק שאבי - מנהל המחלקה

הערה:

יש להתייחס לתוצאות המופיעות במסמך זה כמלואם ואין לחתוך או לצטט, את כולם או חלקם, למסמכים אל
תוצאות המפורטים משקפים במדויק את תוצאות של הדוגמה שנמסרה לבדיקה, כפי שהתקבל במעבדה. אין לעשות שימוש בשמה של
המעבדה ו/או בתוצאות שלה לפרסומים ו/או לפרסומים עם התוצאות המופיעות. זה אלא בהסכמה לאישורה המוסמך בכתב.

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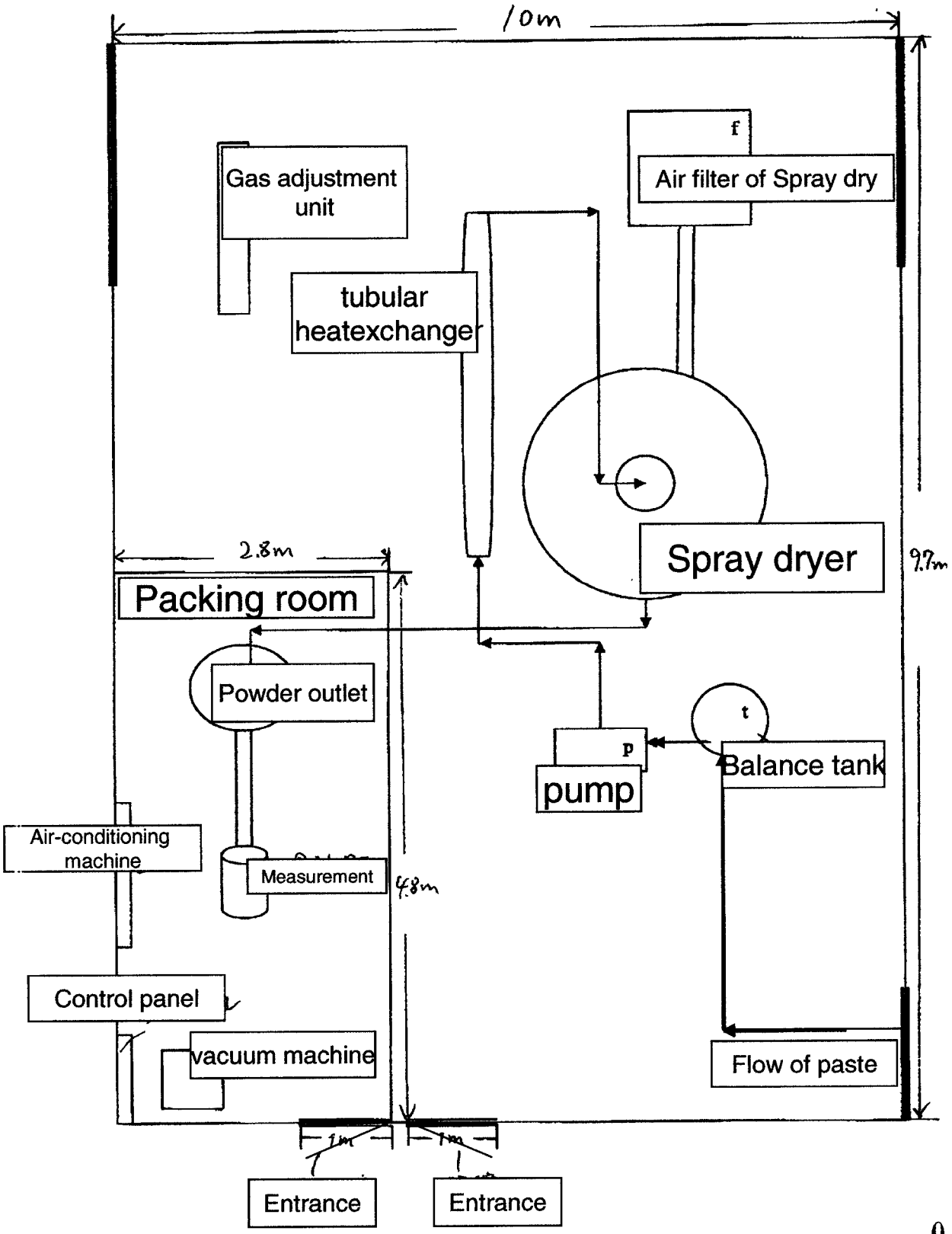
Dr y and Packing room



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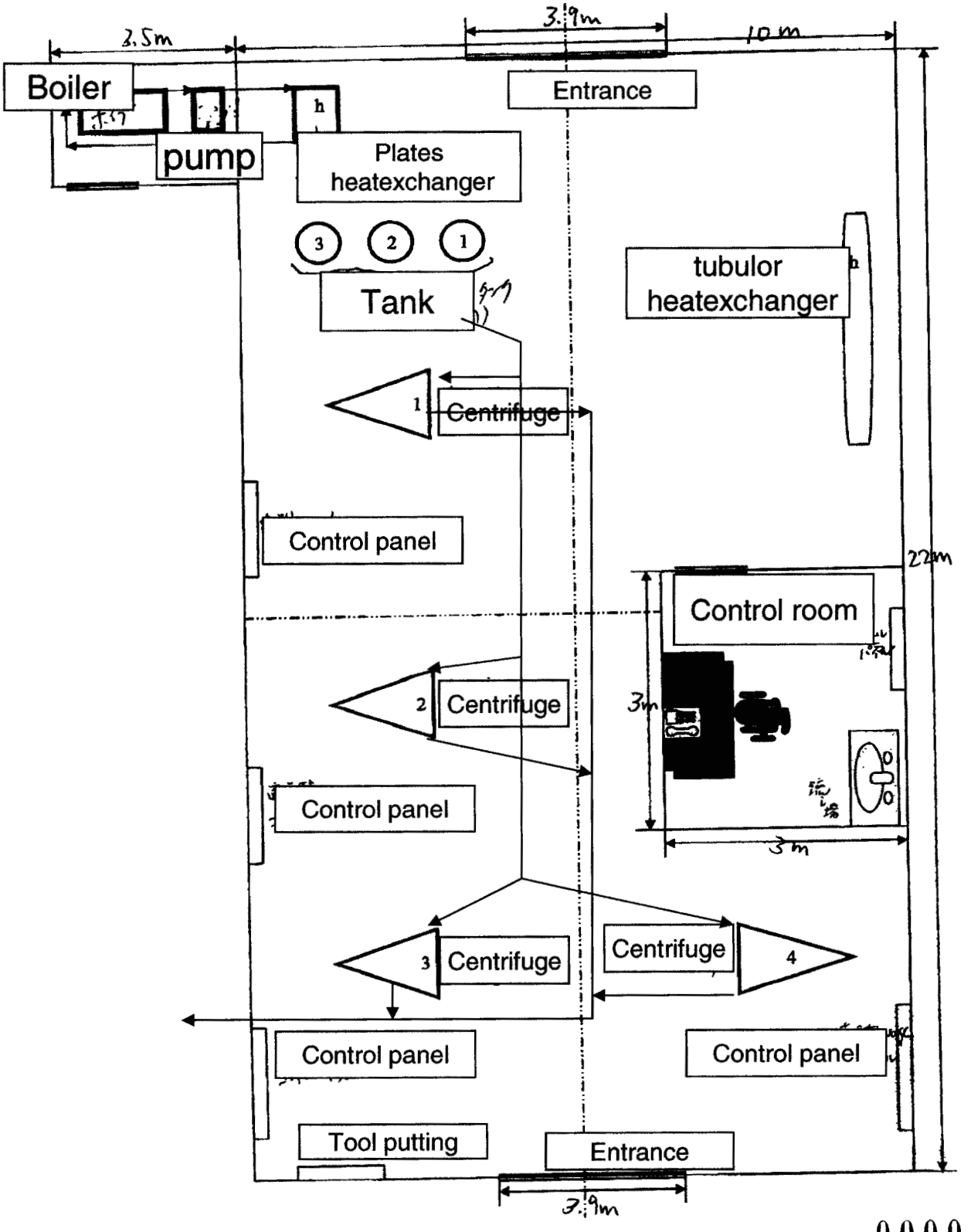
Centrifugal (separation) room



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Japan Food Research Laboratories

Authorized by the Japanese Government

52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062, Japan

No. 10050184001-03 1/1

June 17, 2010

CERTIFICATE OF ANALYSIS

Requested by: Nikken Sohonsha Corporation
1-32 Asahira, Fukuju-cho, Hashima-shi, Gifu 501-6255, Japan

Sample name: Dunaliella Lot# 0909 Manufacturing Date: Aug/12/2009

Received date: June 10, 2010

This is to certify that the following result(s) have been obtained from our analysis on the above mentioned sample(s) submitted by the client.

Test Result(s)

Test Item	Result	QL	N	M
Iodine	0.28 ppm	—		1
Lead	1.12 ppm	—		1
Cadmium	0.06 ppm	—		1

QL: Quantitation limit N: Notes M: Method

Method

1: Inductively coupled plasma mass spectrometry

(b) (6)

Noriko Imaizumi
Principal Investigator
Japan Food Research Laboratories

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Japan Food Research Laboratories
Authorized by the Japanese Government
52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062, Japan

No. 10050184001-04 1/1
June 17, 2010

CERTIFICATE OF ANALYSIS

Requested by: Nikken Sohonsha Corporation
1-32 Asahira, Fukuju-cho, Hashima-shi, Gifu 501-6255, Japan

Sample name: Dunaliella Lot# 0909 Manufacturing Date: Aug/12/2009

Received date: June 10, 2010

This is to certify that the following result(s) have been obtained from our analysis on the above mentioned sample(s) submitted by the client.

Test Result(s)

Test Item	Result	QL	N	M
Iodine	0.26 ppm	—		1
Lead	1.10 ppm	—		1
Cadmium	0.06 ppm	—		1

QL: Quantitation limit N: Notes M: Method

Method
1:Inductively coupled plasma mass spectrometry

JAPAN FOOD RESEARCH LABORATORIES

(b) (6)

Noriko Imaizumi
Principal Investigator
Japan Food Research Laboratories

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RCA0217-01

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Japan Food Research Laboratories
Authorized by the Japanese Government
52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0082, Japan

No. 10050184002-03 1/1
June 17, 2010

CERTIFICATE OF ANALYSIS

Requested by: Nikken Sohonsha Corporation
1-32 Asahira, Fukuju-cho, Hashima-shi, Gifu 501-6255, Japan

Sample name: Dunaliella Lot# 1001 Manufacturing Date: Jan/22/2010

Received date: June 10, 2010

This is to certify that the following result(s) have been obtained from our analysis on the above mentioned sample(s) submitted by the client.

Test Result(s)

Test Item	Result	QL	N	M
Iodine	0.28 ppm	—		1
Lead	1.11 ppm	—		1
Cadmium	0.06 ppm	—		1

QL: Quantitation limit N: Notes M: Method

Method
1: Inductively coupled plasma mass spectrometry

JAPAN FOOD RESEARCH LABORATORIES

(b) (6)

Noriko Imaizumi
Principal Investigator
Japan Food Research Laboratories

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Japan Food Research Laboratories
Authorized by the Japanese Government
52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062, Japan

No. 10050184002-04 1/1
June 17, 2010

CERTIFICATE OF ANALYSIS

Requested by: Nikken Sohonsha Corporation
1-32 Asahira, Fukuju-cho, Hashima-shi, Gifu 501-6255, Japan

Sample name: Dunaliella Lot# 1001 Manufacturing Date: Jan/22/2010

Received date: June 10, 2010

This is to certify that the following result(s) have been obtained from our analysis on the above-mentioned sample(s) submitted by the client.

Test Result(s)

Test Item	Result	QL	N	M
Iodine	0.26 ppm	—		1
Lead	1.12 ppm	—		1
Cadmium	0.06 ppm	—		1

QL: Quantitation limit N: Notes M: Method

Method
1:Inductively coupled plasma mass spectrometry

JAPAN FOOD RESEARCH LABORATORIES

(b) (6)

Noriko Imaizumi
Principal Investigator
Japan Food Research Laboratories

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Japan Food Research Laboratories

Authorized by the Japanese Government

52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062, Japan

No. 10050184003-03 1/1

June 17, 2010

CERTIFICATE OF ANALYSIS

Requested by: Nikken Sohonsha Corporation
1-32 Asahira, Fukuju-cho, Hashima-shi, Gifu 501-6255, Japan

Sample name: Dunaliella Lot# 1004 Manufacturing Date: Apr/6/2010

Received date: June 10, 2010

This is to certify that the following result(s) have been obtained from our analysis on the above mentioned sample(s) submitted by the client.

Test Result(s)

Test Item	Result	QL	N	M
Iodine	0.28 ppm	—		1
Lead	1.12 ppm	—		1
Cadmium	0.06 ppm	—		1

QL: Quantitation limit N: Notes M: Method

Method
1:Inductively coupled plasma mass spectrometry

JAPAN FOOD RESEARCH LABORATORIES

(b) (6)

Noriko Imaizumi
Principal Investigator
Japan Food Research Laboratories

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KCA0017-01

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Japan Food Research Laboratories
Authorized by the Japanese Government
52-1 Motoyoyogi-cho, Shibuya-ku, Tokyo 151-0062, Japan

No. 10050184003-04 1/1
June 17, 2010

CERTIFICATE OF ANALYSIS

Requested by: Nikken Sohonsha Corporation
1-32 Asahira, Fukuju-cho, Hashima-shi, Gifu 501-6255, Japan

Sample name: Dunaliella Lot# 1004 Manufacturing Date: Apr/6/2010

Received date: June 10, 2010

This is to certify that the following result(s) have been obtained from our analysis on the above-mentioned sample(s) submitted by the client.

Test Result(s)

Test Item	Result	QL	N	M
Iodine	0.25 ppm	—		1
Lead	1.09 ppm	—		1
Cadmium	0.06 ppm	—		1

QL: Quantitation limit N: Notes M: Method

Method
1: Inductively coupled plasma mass spectrometry

(b) (6)

Noriko Imaizumi
Principal Investigator
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אמינולאב בע"מ שרותי מעבדת אנליטיים לתעשיה, רפואה, חקלאות, מחקר ואיכות הסביבה
סניף ראשי: קרית ויצמן ת.ד. 4074, נס ציונה 70400 טל: 08-9303333, פקס: 08-9303300
סניף צפון: בנין ר.מ.א, תיוצרים 19, ת.ד. 1033, כרמיאל 20100 טל: 04-9586916, פקס: 04-9582154

11/03/2009
Report No: C1470T

To:
Hirofumi Goto
N.B.T. Nature Beta Technologies Ltd.
P.O.Box 828
Eilat 88106
Tel: 08-6331219
Fax: 08-6378107

CERTIFICATE OF ANALYSIS

Aminolab No.: **08650.09-C** **Sample reception:** 01/03/2009
Sample: Dnnaliella algae Medium
Sampled by: The Customer
Sampling type: Unknown

Analyses Results:

Analysis	Units	Results	Remarks
Mercury by Cold Vapor	mg/L	<0.0005	-
Metals scan by ICP			1
Arsenic - As	mg/L	<0.2	
Cadmium - Cd	mg/L	<0.1	
Lead - Pb	mg/L	<0.2	

Analyses Remarks:
(-)=No remarks
1. ICP scan - results including "<" = Below detection limits

Quality Assurance:

Analysis	Method / Standard	Remarks
Mercury by Cold Vapor	SOP# 20.WI.102	-
Metals scan by ICP	SOP# 20.S.111	-

Accreditations / Certifications:
The laboratory is accredited under ISO/IEC 17025 with standard operating procedures.

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Analyzed By: Marina Ruchman Signature: (b) (6)

Approved by: Dr. Zadok Shabi - Lab. Manager Signature:

This certificate is valid only when it is presented in its complete format, and it is not permitted to A[illegible] inclusion in any

BACTOCHEM

To:

N.B.T Ltd

P.O BOX 828,Eilat , Israel

Fax: 08-6378107

Report No. 61/89122

Sample	Sampling Date	Reporting Date
Dunaliella Algae Medium	19/04/09	20/04/09

The results are as follows:

Type of analysis	Unit	Results	MRL
Iodine	mg/liter	<0.1	-

We checked in the sample also iodine like iodide and we find 0.3 mg/liter.

Sincerely,
Bactochem Laboratories

(b) (6)

Dr. Eldad Elron
Environmental quality
Dept. Director
BACTOCHEM Ltd.

Bactochem Lab. Ltd • Department of Phytopathology • BACTOCHEM

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ATTACHMENT 8

Methods for Specifications and Stability Study of *Dunaliella Bardawil*

1. Moisture – Loss on Drying Test, Methods of Analysis in Health Science, *The Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pg 151 (2000)
2. Ash – Direct Incineration Method, Methods of Analysis in Health Science, *The Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pg 155 (2000)
3. Protein – Nitrogen Determination (Semimicro-Kjeldahl Method, Methods of Analysis in Health Science, *The Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pg 163 (2000)
4. Fat – Acid Hydrolysis Method, Methods of Analysis in Health Science, *The Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pg 192 (2000)
5. Carbohydrate – Subtraction Methods, Methods of Analysis in Health Science, *The Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pg 202 (2000)
6. Total Carotene – HPLC Methods, Methods of Analysis in Health Science, *The Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pg 4 (2000)
7. β-carotene – HPLC Methods, Methods of Analysis in Health Science, *The Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pg 4 (2000)
8. β-carotene inside Ratio of 9-*cis* in β-carotene – HPLC Methods, Methods of Analysis in Health Science, *The Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pg 4 (2000)
9. Sodium Chloride – Atomic Absorption Spectrophotometry, Methods of Analysis in Health Science, *The Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pg 161 (2000)
10. Heavy Metals (Pb) – Sulfuretted Sodium Methods, Methods of Analysis in Health Science, The *Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pg 609 (2000)
11. Arsenic (As) – DDTC Methods, Methods of Analysis in Health Science, *The Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pp 369-373 (2000)
12. Total Aerobic Mesophiles: Bacterial Count – Standard Agar Plate Method, Methods of Analysis in Health Science, *The Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pg 51 (2000)
13. *E-coli:* Colibacillus, BGLB Method, Methods of Analysis in Health Science, *The Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pg 151 (2000)
14. *Staphylococcus aureus* – Flat Applying Methods, Methods of Analysis in Health Science, *The Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pg 53.81 (2000), *AOAC Official Methods of Analysis*, pg 14 (1984)
15. Molds – Potato Dextrose Agar Methods, Methods of Analysis in Health Science, *The Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pp 100-103 (2000)
16. *Clostridium perfringens* – MPN Methods, Methods of Analysis in Health Science, *The Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pg 53.82-83 (2000), *AOAC Official Methods of Analysis*, pg 14 (1984)
17. *Salmonella* – Increased Breeding Methods, Methods of Analysis in Health Science, *The Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pg 88 (2000)
18. *Pseudomonas aeruginosa* – Flat Applying Methods, Methods of Analysis in Health Science, *The Pharmaceutical Society of Japan*, Kanehara & Co., Ltd., pg 53 (2000)

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STATEMENT OF ENGLISH TRANSLATION

Nihon Bioresearch Center Inc.
Hashima Laboratory
104, 6-chome, Nagura, Fukeguchi-cho,
Hashima-shi, Gifu Prefecture, Japan

Study title (in Japanese): Acute Oral Toxicity Study in Dunaliella Bardawil Spray Dried Powder in Mice

Date of original report: December 8, 1987

Test No.: 1

Study director: Fumihiko ARUGA

I, here by acknowledge that this English report is an exact translation of the above original Japanese final report on the study conducted in this institute.

Signed on June 2, 1990

by (b) (6)

Fumihiko ARUGA
Nihon Bioresearch Center Inc.
Hashima Laboratory

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CERTIFICATE OF TRANSLATED CONTENTS

Dated: 23th February 2007

We, the undersigned, hereby certify that the documents attached are the original manuscript text of the following as well as a matching translation of the relevant text faithfully rendered into English.

Text details:	試験計画書　試験番号：4087 Dunaliella Bardawil Spray Dried Powder のマウスにおける急性毒性試験 試験計画書　試験番号：5118 Dunaliella Bardawil Spray Dried Powder のラットにおける28日間経口投与による亜急性毒性試験 <Total pages: 28>

Translation details:	Protocol　Study No.4087 Oral administration acute toxicity study of Dunaliella Bardawil Spray Dried Powder in mice. Protocol　Study No.5118 28-day oral administration subacute toxicity study of Dunaliella Bardawil Spray Dried Powder in rats <Total pages: 28>

(b) (6)

Teruhisa Tsunoda
Director and General Manager
Tokyo Sales Division 2
HONYAKU CENTER INC.
2-4-1 Higashi Shinbashi, Minato-ku
Tokyo 105-0021, JAPAN

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株式会社日本バイオリサーチセンター

Study No.4087

STATEMENT

Study No. : 4087

Title: Acute Oral Toxicity Study on Dunaliella Bardawil Spray Dried Powder in Mice

This study was performed incompliance with the Good Laboratory Practice Standard for Safety Studies on Drugs (Notification No.313 of the Pharmaceutical Affairs Bureau, Japanese Ministry of Health and Welfare, March 31, 1982)

January 16, 2007

Hashima Laboratory, Nihon Bioresearch Inc.

(b) (6)

Study Director : _____

Fumihiko Aruga

000093

Quality Assurance Unit Statement

Study number: 4087

Title: Acute Oral Toxicity Study on Dunaliella Bardawil Spray Dried Powder in Mice

Quality assurance inspections of the study and review of the final report of the above referenced study were conducted according to the Good Laboratory Practice regulations and the Standard Operating Procedures of Nihon Bioresearch Center Inc. Findings from the inspection and final report review were reported to management and to the study director on the following dates.

To the best of my knowledge, there were no significant deviations from the Good Laboratory Practice regulations which affected the quality or integrity of the study. This report accurately describes the method of the study and the reported results accurately reflects the raw data of the study.

Inspection item	Date of inspection / Audits	Date of Findings Reported to the study Director and to Management
1. Study schedule and protocol	August 26, 1987	August 26, 1987
2. Receipt of animals	September 10, 1987	September 10, 1987
3. Test article, administration and animal breeding	September 18, 1987	September 18, 1987
4. Autopsy	October 2, 1987	October 2, 1987
5. Raw data, draft report	November 17-18, 1987	November 18, 1987
6. Final report	December 8, 1987	December 8, 1987

December 8, 1987

Nihon Bioresearch Center Inc.
Hashima Laboratory

Director of Quality Assurance Unit: (b) (6)
Kenji OKADA

000094

Acute Oral Toxicity Study on Dunaliella Bardawil Spray Dried Powder in Mice

SYNOPSIS

We trusted NIHON BIORESEARCH CENTER INC. with the investigation of acute oral toxicity study in mice about D. bardawil spray dried powder.

The test article, D. bardawil spray dried powder, was administered to three groups of mice, ten males and ten females, at dosage levels of 2.5 g/kg, 5.0 g/kg and 10 g/kg diluted with CMC-Na soln., once via oral intubation. CMC-Na soln. was used instead of test article as a control. Criteria evaluated for treatment effect during the subsequent 14-days observation period were mortality, general symptoms, body weights and gross necropsy examination.

The LD50 value was found to be greater than 10 g/kg for combined male and female mice. All mice survived to study termination. No significant findings were noted during the study period. No visible abnormalities were observed in mice at terminal sacrifice. In body weights, significant increases were observed at the group of 2.5 g/kg.

STUDY SUMMARY

STUDY TITLE: Acute Oral Toxicity Study in Mice
DATE INITIATED: 9/10/87 DATE TERMINATED: 10/2/87

000095

TEST FACILITY: NIHON BIORESEARCH CENTER INC., Gifu, Japan
OBJECTIVE: To evaluate the acute toxicity of D. bardawil spray dried powder in various doses by oral intubation study.

TEST SYSTEM:
Species: mice Strain: Crj:CD-1(ICR)
Source: Charles River Japan
The mouse was selected as the test system because it is an acceptable model for acute oral toxicity studies.
Body Weight Range: 20.9-23.3 g and 17.0-19.7 g at initiation, respectively.
Age at Start of Study: 5 wks old (young adult)
Diet: CRF-1, Oriental Yeast Inc., Japan
Environmental Conditions: Animal room with controlled temperature, humidity and light (12 hrs light and 12 hrs dark). Diet and water freely available; diet withheld approximately 18 - 20 hours prior to dosing.
Dosing Solutions: The test article was diluted with CMC-Na soln. at a dose volume of 40 ml/kg.
Dosage Groups: Three groups at dosage levels of 2.5 g/kg, 5.0 g/kg and 10 g/kg.
Administration: The test article was administered by oral gavage.

OBSERVATIONS:
Mortality and General symptoms: Observed 1/2, 2, 4 and 6 hrs after administration on the first day; twice daily thereafter for 14 days.
Body Weights: Immediately prior to test article administration, day 1, 3, 7, 10 and termination(day 14).

000096

Necropsy: Performed by qualified personnel.

RESULTS:

LD50 Value: The LD50 value was found to be greater than 10 g/kg for combined male and female mice.

Dose-Mortality Data: No mice died during the study period.

General symptoms: No significant obserbations were noted during the study period.

Body Weights: The significant increases of body weights were observed on day 10 in male mice of 2.5 g/kg.

Necropsy: No visible abnormalities were observed in mice at the terminal post-mortem examination.

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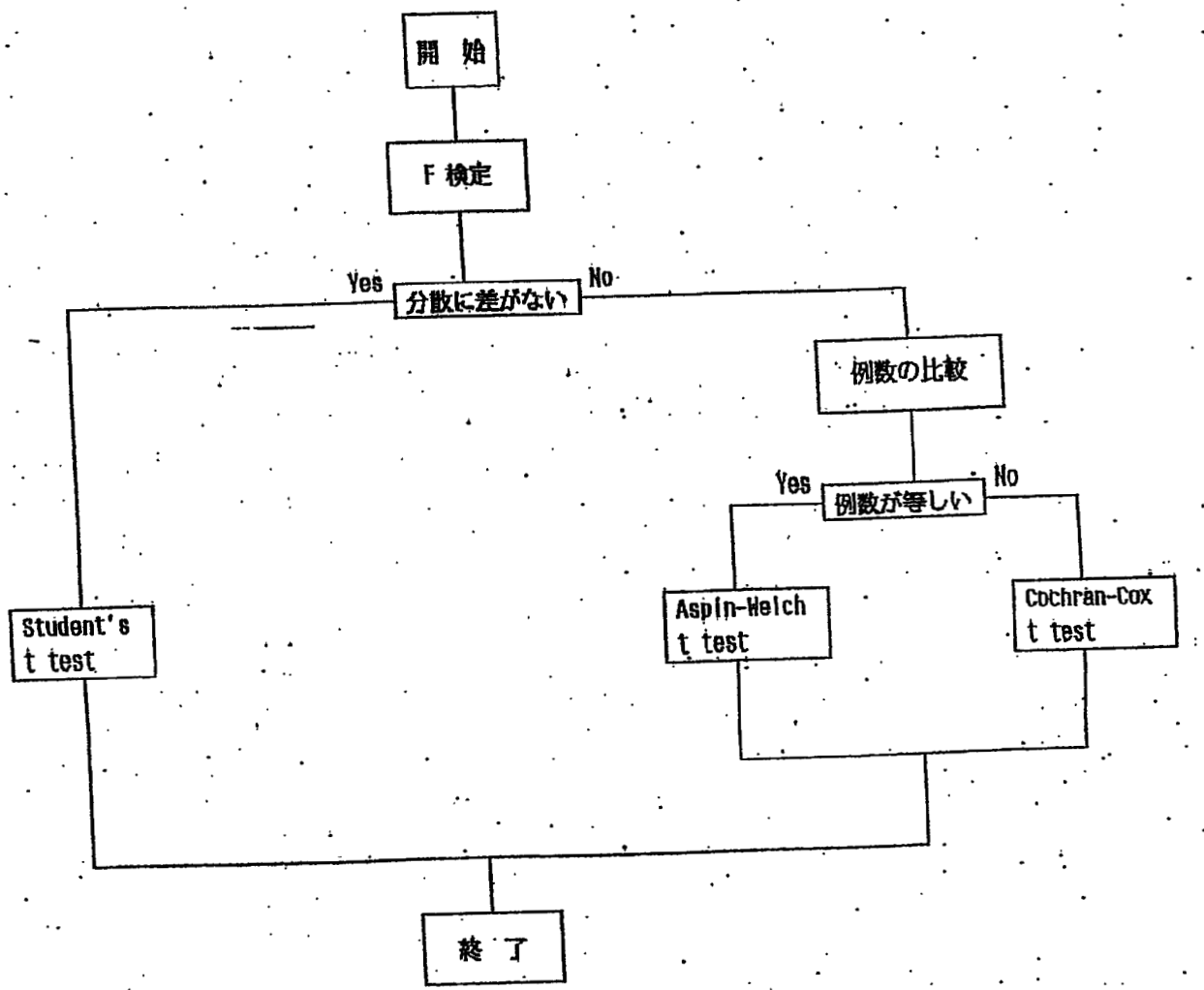
別表1　Dunaliella Bardawil Spray Dried powderのマウスにおける経口投与による急性毒性試験の統計手法のフローチャート



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別表2　Dunaliella Bardawil Spray Dried powderのマウスにおける経口投与による急性毒性試験のコンピュータの統計表示

(N) …… 例数

MEAN …… 平均

S. D. …… 標準偏差

VALUE …… 統計量

JUDGE …… 有意差マーク (*)、(**)

SIGN …… 対照群 (0mg/kg 群) に対して平均値が
大きい (+)、小さい (-)

METHOD …… 検定結果
TTH0 ： Student's t testで有意差なし

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表1　Dunaliella Bardawil Spray Dried powderのマウスにおける経口投与による急性毒性試験の経日死亡

性別	群	例数	投与後の時間 (h)				投与後の日数 (day)														総死亡数	LD_{50} (g/kg)
			0.5	2	4	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
雄	対照	10	0[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	>10
	DB 2.5 g/kg	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	DB 5 g/kg	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	DB 10 g/kg	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
雌	対照	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	>10
	DB 2.5 g/kg	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	DB 5 g/kg	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	DB 10 g/kg	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

a) 経時あるいは経日の死亡数。

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TEST NO. 4087

表2　Dunaliella Bardawil Spray Dried powderのマウスにおける経口投与による急性毒性試験の雄の体重推移

群	0	1	3	7	10	14 (日)
対 照						
MEAN	22.2	26.3	27.6	29.3	29.9	31.7
S.D.	0.69	0.90	0.78	1.25	1.12	1.39
(N)	10	10	10	10	10	10
DB 2.5G/KG						
JUDGE					*	
MEAN	22.2	26.3	28.1	29.7	31.1	32.6
S.D.	0.76	0.92	0.91	0.97	1.10	1.37
(N)	10	10	10	10	10	10
DB 5G/KG						
JUDGE						
MEAN	22.1	25.9	27.1	29.2	29.8	31.2
S.D.	0.68	0.82	0.97	1.37	1.25	1.59
(N)	10	10	10	10	10	10
DB 10G/KG						
JUDGE						
MEAN	22.2	25.9	27.5	29.4	30.1	31.5
S.D.	0.72	1.10	1.51	1.92	2.08	2.22
(N)	10	10	10	10	10	10

* : P< 0.05 で対照群に対して有意差あり

単位：g

TEST NO. 4087

表3： Dunaliella Bardawil Spray Dried powderのマウスにおける経口投与による急性毒性試験の雌の体重推移

群		0	1	3	7	10	14 (日)
対 照	MEAN	18.4	21.5	22.1	22.3	22.7	24.0
	S.D.	0.89	1.24	0.94	1.27	1.16	1.79
	(N)	10	10	10	10	10	10
DB 2.5G/KG	JUDGE MEAN	18.4	21.2	22.5	22.9	23.4	25.0
	S.D.	0.90	0.76	0.75	0.80	0.96	1.50
	(N)	10	10	10	10	10	10
DB 5G/KG	JUDGE MEAN	18.4	21.6	22.4	22.8	22.8	24.6
	S.D.	0.81	1.06	1.46	1.52	1.43	1.81
	(N)	10	10	10	10	10	10
DB 10G/KG	JUDGE MEAN	18.4	21.1	22.2	22.5	22.9	23.7
	S.D.	0.81	1.27	1.09	1.11	1.10	0.99
	(N)	10	10	10	10	10	10

単位：g

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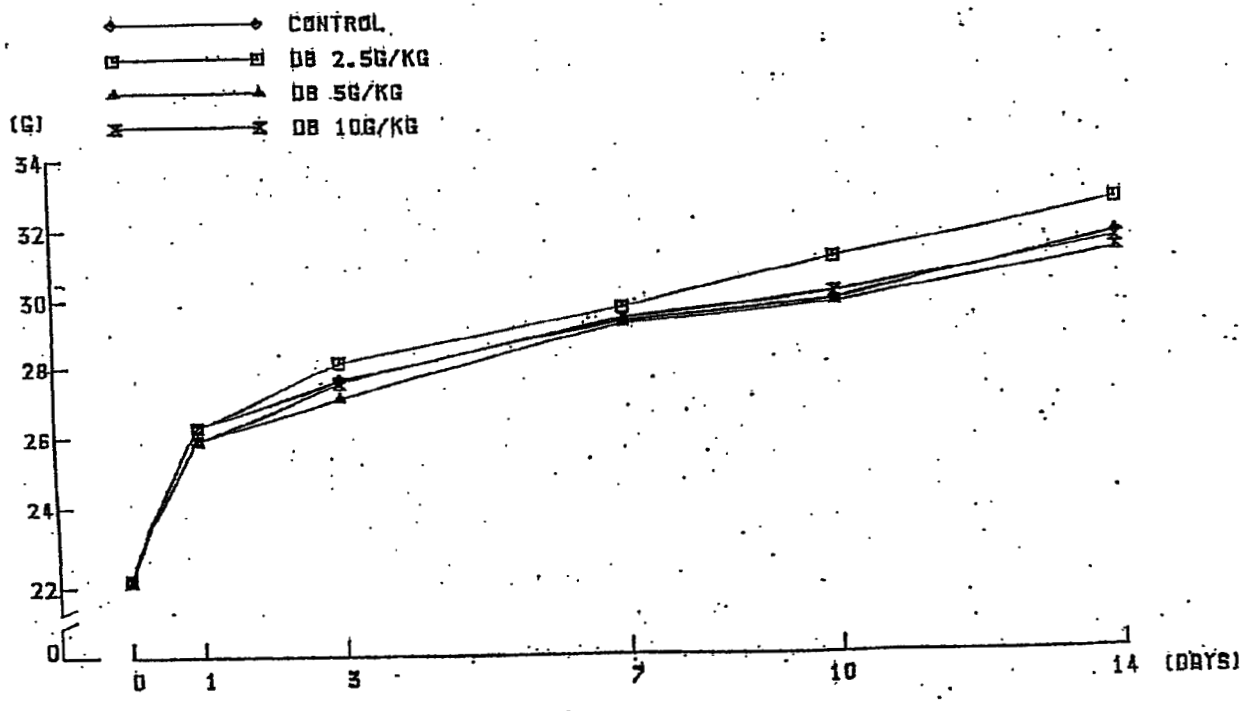

図1　Dunaliella Bardawil Spray Dried powderのマウスにおける経口投与による急性毒性試験の雄の体重推移

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000103

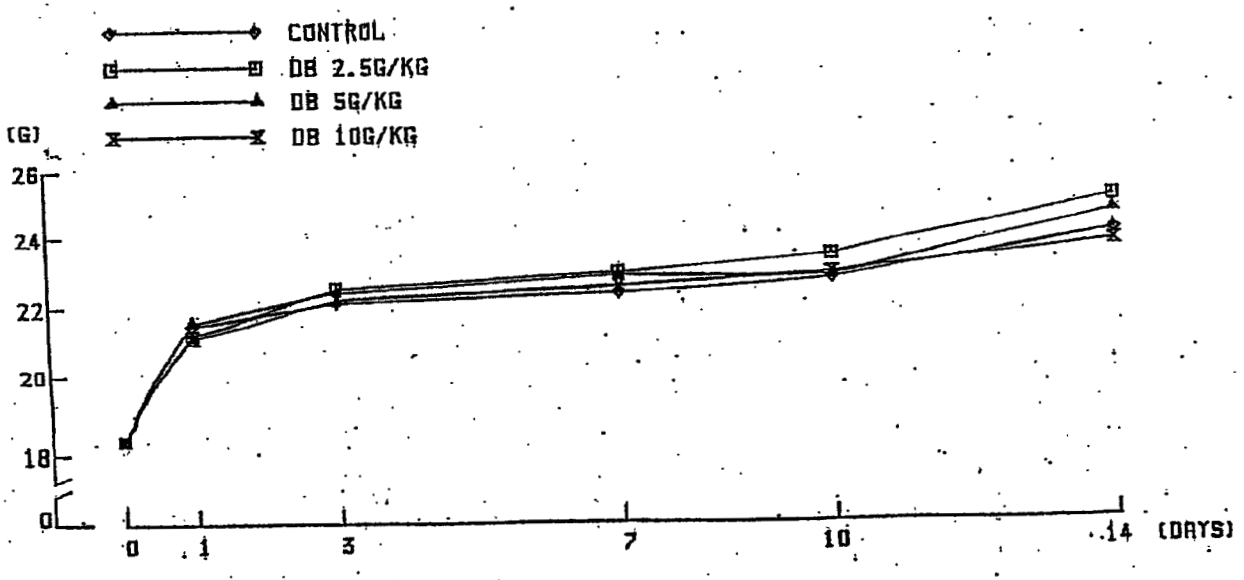

図2　Dunaliella Bardawil Spray Dried powderのマウスにおける経口投与による急性毒性試験の雌の体重推移

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TEST NO. 4087 APPENDIX: 2-1

(GROUP : CONTROL)

BODY WEIGHT OF MALE MICE

NO.	0	1	3	7	10	14 (DAYS)
001	21.3	26.4	26.7	27.7	28.8	30.2
002	22.1	26.1	27.3	29.3	29.5	31.3
003	21.4	25.1	27.8	29.5	30.3	32.0
004	22.1	26.1	28.2	30.0	30.8	32.4
005	23.2	27.1	27.8	30.0	31.0	32.3
006	22.8	27.1	29.0	31.1	31.2	33.8
007	23.0	27.5	28.0	30.0	30.3	32.6
008	21.7	25.0	26.2	26.8	28.0	29.6
009	22.6	25.7	27.4	28.8	28.4	29.9
010	21.6	27.3	27.8	29.9	30.2	32.8
(N)	10	10	10	10	10	10
MEAN	22.2	26.3	27.6	29.3	29.9	31.7
S.D.	0.69	0.90	0.78	1.25	1.12	1.39

UNIT : G

000105

TEST NO. 4087 APPENDIX: 2-2

(GROUP : DB 2.56/KG)

BODY WEIGHT OF MALE MICE

NO.	0	1	3	7	10	14 (DAYS)
101	20.9	25.4	27.3	29.2	30.3	31.9
102	22.7	25.5	27.5	28.2	29.8	30.9
103	21.5	26.0	27.6	29.7	31.4	32.5
104	22.5	27.2	28.6	30.7	32.3	33.6
105	21.9	25.6	26.7	29.6	30.4	32.0
106	22.3	26.3	28.0	29.0	30.1	31.9
107	21.8	26.3	27.9	28.9	30.1	30.9
108	21.7	25.6	28.3	30.7	32.0	33.5
109	23.2	27.3	29.4	29.7	31.8	34.7
110	23.3	28.1	29.6	31.4	32.9	34.5
(N)	10	10	10	10	10	10
MEAN	22.2	26.3	28.1	29.7	31.1	32.6
S.D.	0.76	0.92	0.91	0.97	1.10	1.37
VALUE	0.000	0.025	1.237	0.797	2.536	1.537
JUDGE					*	
SIGN			+	+	+	+
METHOD	TTH0	TTH0	TTH0	TTH0	TTH1	TTH0

* : SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP (P<0.05)

UNIT : G

TEST NO. 4087 APPENDIX: 2 - 3

(GROUP : DB 5G/KG)

BODY WEIGHT OF MALE MICE

NO.	0	1	3	7	10	14 (DAYS)
201	21.4	25.2	26.2	27.8	28.4	30.2
202	23.3	27.6	28.4	30.8	31.2	33.5
203	21.1	25.3	25.4	26.6	28.4	29.2
204	22.0	24.8	26.4	28.3	28.2	29.0
205	22.4	26.3	28.9	30.2	29.5	30.6
206	22.3	25.7	26.7	28.7	29.3	30.4
207	21.8	25.5	26.9	29.2	29.7	32.1
208	22.6	26.5	27.3	29.7	31.2	32.3
209	22.8	26.4	28.2	31.0	31.6	33.3
210	21.6	25.5	27.7	29.5	30.2	31.7
(N)	10	10	10	10	10	10
MEAN	22.1	25.9	27.1	29.2	29.8	31.2
S.D.	0.68	0.82	0.97	1.37	1.25	1.59
VALUE	0.164	1.194	1.268	0.221	0.150	0.688
JUDGE	-	-	-	-	-	-
SIGN						
METHOD	TTHO	TTHO	TTHO	TTHO	TTHO	TTHO

UNIT : G

TEST NO. 4087 APPENDIX: 2-4

(GROUP : DB 10G/KG)

BODY WEIGHT OF MALE MICE

NO.	0	1	3	7	10	14 (DAYS)
301	22.7	26.4	28.0	29.2	30.0	31.6
302	21.7	24.6	25.8	27.4	27.6	29.0
303	23.3	27.3	29.0	29.8	30.7	32.2
304	22.1	25.2	26.9	28.0	28.4	29.6
305	21.0	24.8	26.5	29.0	29.1	30.0
306	21.4	25.1	26.9	29.8	30.5	32.6
307	22.5	26.7	28.9	31.5	32.9	34.1
308	22.4	27.3	28.1	30.4	32.0	33.6
309	22.9	26.9	30.0	32.9	32.9	34.3
310	21.7	24.9	25.3	26.4	27.1	28.1
(N)	10	10	10	10	10	10
MEAN	22.2	25.9	27.5	29.4	30.1	31.5
S.D.	0.72	1.10	1.51	1.92	2.08	2.22
VALUE	0.032	0.936	0.149	0.179	0.361	0.217
JUDGE SIGN		-	-	+	+	-
METHOD	TTHO	TTHO	TTHO	TTHO	TTHO	TTHO

UNIT : G

000108

TEST NO. 4087 APPENDIX: 3 - 1

(GROUP : CONTROL)

BODY WEIGHT OF FEMALE MICE

NO.	0	1	3	7	10	14 (DAYS)
051	18.3	21.6	21.7	21.5	22.6	25.1
052	18.5	21.0	22.2	22.0	21.6	22.7
053	19.1	22.0	21.8	22.5	22.9	24.1
054	19.4	22.1	22.6	24.3	23.0	24.2
055	17.0	21.4	21.4	21.4	21.5	22.4
056	17.2	21.0	21.2	20.5	22.2	22.9
057	19.7	24.4	24.4	24.5	25.3	27.3
058	18.8	20.9	22.4	22.9	23.9	26.6
059	17.9	19.9	21.5	22.0	21.8	22.4
060	18.2	20.3	21.4	21.6	22.5	22.6
(N)	10	10	10	10	10	10
MEAN	18.4	21.5	22.1	22.3	22.7	24.0
S.D.	0.89	1.24	0.94	1.27	1.16	1.79

UNIT : G

TEST NO. 4087 APPENDIX: 3 - 2

(GROUP : DB 2.56/KG)

BODY WEIGHT OF FEMALE MICE

NO.	0	1	3	7	10	14 (DAYS)
151	18.5	22.1	21.9	22.4	23.9	26.8
152	19.7	22.2	23.5	24.0	24.1	24.5
153	18.3	20.0	21.5	21.8	23.5	25.8
154	19.3	21.1	23.8	23.9	23.7	23.7
155	17.2	21.2	22.3	22.7	23.4	25.8
156	19.3	21.9	22.6	22.6	22.8	23.8
157	18.7	21.0	22.9	24.1	24.8	26.1
158	18.0	21.2	22.3	22.7	22.9	23.8
159	17.0	20.0	21.7	22.2	21.2	22.6
160	17.9	21.2	22.9	23.0	23.5	27.0
(N)	10	10	10	10	10	10
MEAN	18.4	21.2	22.5	22.9	23.4	25.0
S.D.	0.90	0.76	0.75	0.80	0.96	1.50
VALUE	0.050	0.586	1.258	1.304	1.367	1.299
JUDGE SIGN		-	+	+	+	+
METHOD	TTHO	TTHO	TTHO	TTHO	TTHO	TTHO

UNIT : G

TEST NO. 4087 APPENDIX: 3 - 3

(GROUP 5 DB 5G/KG)

BODY WEIGHT OF FEMALE MICE

NO.	0	1	3	7	10	14 (DAYS)
251	18.6	21.6	22.1	23.9	24.6	26.7
252	19.5	21.7	22.3	23.9	23.2	24.0
253	18.3	21.0	21.1	22.5	21.9	22.0
254	17.1	20.1	20.9	21.0	20.4	23.6
255	18.9	22.4	23.7	24.1	24.1	25.0
256	18.1	22.3	22.5	22.3	22.2	24.7
257	19.4	23.5	25.1	24.8	24.3	26.9
258	19.1	22.2	24.0	23.9	23.7	26.5
259	17.5	21.0	21.8	21.5	22.1	24.8
260	17.8	20.2	20.6	20.3	21.1	21.8
(N)	10	10	10	10	10	10
MEAN	18.4	21.6	22.4	22.8	22.8	24.6
S.D.	0.81	1.06	1.46	1.52	1.43	1.81
VALUE	0.053	0.272	0.637	0.797	0.052	0.708
JUDGE SIGN		+	+	+	+	+
METHOD	TTHO	TTHO	TTHO	TTHO	TTHO	TTHO

UNIT : G

TEST NO. 4087 APPENDIX 3-4

(GROUP : D8 10G/KG)

BODY WEIGHT OF FEMALE MICE

NO.	0	1	3	7	10	14 (DAYS)
351	19.5	22.5	23.7	24.6	23.7	24.4
352	18.4	22.5	23.9	23.5	24.6	25.4
353	18.4	20.3	21.6	21.7	23.4	24.4
354	17.0	20.0	21.3	21.7	21.5	21.7
355	18.0	19.4	20.8	21.1	21.1	23.6
356	17.8	20.5	21.7	22.6	22.3	23.1
357	18.8	22.2	22.2	22.6	23.4	23.2
358	19.5	21.6	22.5	23.0	23.2	23.8
359	19.0	22.6	23.0	23.2	23.6	24.1
360	17.7	19.8	21.0	21.2	22.1	23.4
(N)	10	10	10	10	10	10
MEAN	18.4	21.1	22.2	22.5	22.9	23.7
S.D.	0.81	1.27	1.09	1.11	1.10	0.99
VALUE	0.000	0.571	0.241	0.374	0.317	0.495
JUDGE SIGN		-	+	+	+	-
METHOD	TTHO	TTHO	TTHO	TTHO	TTHO	TTHO

UNIT : G

SOP/TSB/07/RS-02

(DB) 使用記録用紙 (No. 1)

試験番号： 4087
LOT No.： NK－1001
入手年月日： 昭和 62年 9月 日
使用期限： 昭和 ~~年 月 日~~

正味量： 20g
風袋込み量： 157.65g
保管条件： 冷凍
保管形態： 密栓ガラス瓶
保管庫 No.： 冷凍庫 No.6

試験責任者 有賀文子 (印)

使用記録

年月日	採取量（使用量）（g）	風袋込み残量（使用後）（g）	使用者	調製指示書 No.
62.9.18	17.5	140.14	加藤	1
	仕解 17.5g	残量 140.14g		
		0.25.00g 確認	62.9.18	加藤

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CERTIFICATE OF TRANSLATED CONTENTS

Dated: 23th February 2007

We, the undersigned, hereby certify that the documents attached are the original manuscript text of the following as well as a matching translation of the relevant text faithfully rendered into English.

Text details:	試験計画書　試験番号：4087 Dunaliella Bardawil Spray Dried Powder のマウスにおける急性毒性試験 試験計画書　試験番号：5118 Dunaliella Bardawil Spray Dried Powder のラットにおける28日間経口投与による亜急性毒性試験 <Total pages: 28>

Translation details:	Protocol　Study No.4087 Oral administration acute toxicity study of Dunaliella Bardawil Spray Dried Powder in mice. Protocol　Study No.5118 28-day oral administration subacute toxicity study of Dunaliella Bardawil Spray Dried Powder in rats <Total pages: 28>

(b) (6)

Teruhisa Tsunoda
Director and General Manager
Tokyo Sales Division 2
HONYAKU-CENTER INC.
2-4-1 Higashi Shinbashi, Minato-ku
Tokyo 105-0021, JAPAN

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Study No. 4087

Protocol

Test article name: DB (abbreviation)

Title

Oral administration acute toxicity study of Dunaliella Bardawil Spray Dried Powder in mice

Sponsor

Nikken Seiyaku Kogyo Co.
4-41 Hirakata, Fukuju-cho, Hashima-shi, Gifu-prefecture

Testing facility

Hashima Institute, Nihon Bioresearch Center Co. (Facility Management: Kenichi Nakagawa)
6-104 Majima, Fukuju-cho, Hashima-shi, Gifu-prefecture

Study objective

According to Yakushin No. 118 "Guideline for toxicity studies of drugs" (dated February 15, 1984), acute toxicity of Dunaliella Bardawil Spray Dried Powder in mice is examined by oral administration.

Participating persons

Study Director: Fumihiko Ariga
Person responsible for the study: Kenji Uto

Quality assurance unit

Responsible person: Kenji Okada

Date of start of study (Date of adding signature and seal by Facility Management): August 25, 1987

Study period

Date of animal delivery: September 10, 1987
Date of the final necropsy: October 2, 1987

Date of submission of report (draft): November 30, 1987
Planned date of submission of final report: December 25, 1987

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Signature and seal

Study Director

Name: Fumihiko Ariga Seal August 25, 1987

Address: No. 302, Nishikawadaira Building, 8-40 Nishikawadaira, Gifu-shi

Affiliation: Hashima Institute, Nihon Bioresearch Center Co.

Approval of protocol

Sponsor: Teruo Shiraishi Seal August 25, 1987

Representative Director, Nikken Seiyaku Kogyo Co.,
4-41 Hirakata, Fukuju-cho, Hashima-shi, Gifu-prefecture

Facility Management: Kenichi Nakagawa Seal August 25, 1987
(Hashima Institute, Nihon Bioresearch Center Co.)

Confirmation

Quality assurance unit

Responsible person: Kenji Okada Seal August 25, 1987
(Hashima Institute, Nihon Bioresearch Center Co.)

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1. Test article

(1) Test article

Test article name: Dunaliella Bardawil Spray Dried Powder (abbreviation: DB)

Lot No. NK-1001

Description: It occurs as reddish brown powder and is insoluble in water.

Stability: It is stable in 0.5% CMC-Na aqueous solution up to 5 hours after preparation.

Date of reception: September 1, 1987

Amount received: 20 g

Storage conditions: After air is replaced by nitrogen, it is stored under freezing in the dark (free-oxygen absorber is used.)

Storage place: Test article storage cabinet (freezer) of Testing Facility

Supplier: Nikken Seiyaku Kogyo Co.

Vehicle: 0.5% carboxymethyl cellulose sodium aqueous solution (CMC-Na, Lot No. 1C4034, Junsei Chemical Co., Ltd.)

(2) Record

Date of receipt of the test article, Lot No., amount received, and amount of use at each time of use are recorded in designated forms.

2. Test animals

(1) Animal species and strain, and reason for the choice

Mouse (SPF) Crj: CD-1 (ICR)

The mouse is an animal species generally used in toxicity studies, and maintenance of the strain is clear and many data have accumulated.

(2) Age at the time of delivery

Mice are received at the age of 4 weeks.

(3) Date of delivery and number of animals delivered

September 10, 1987 52 males, 52 females

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(4) Body weight range at the time of delivery
Males: 15.1-21.4 g, Females: 17.0-21.4 g

(5) Supplier
Charles River Japan, Co.

(6) Quarantine and preliminary rearing
For delivered mice, 1-week preliminary rearing period is set. During the period, body weight is measured twice or more, and external appearance and clinical signs are observed. This is the period for quarantine and acclimation.

(7) Method of animal allocation to groups
Animals that showed no abnormalities in body weight gain, appearance or clinical signs during the preliminary rearing period are selected at the age of about 5 weeks, stratified by the body weight, and then, randomly allocated to groups so as to make the mean body weight and variation among the groups almost the same. Prior to administration, the animals are fasted from 4-5 p.m. the day before administration, and animal allocation to groups is performed on the day of administration. After allocation, the remaining animals are killed under anesthesia on the day of administration.

(8) Individual identification
Animals are identified by marking at the tail with oil-based ink during the preliminary rearing period, and by staining the fur with dyes after animal allocation. Cages are identified by putting a label carrying Study No., date of animal delivery and acclimation animal No. on each cage during the preliminary rearing period, and putting a label carrying Study No., test article name, dose and animal No. on each cage after animal allocation to groups.

(9) Conditions for animal care
The animals are reared in an animal room (Room 3, Building C) under the conditions of 20 -24℃, 40-70% humidity, 12-hour light and dark cycles (time of lighting with fluorescent lamp: 6 a.m. – 6 p.m.), and 12 air-changes/hour (using filter-sterilized fresh air).
During the preliminary rearing period and after animal allocation to groups, the animals are housed in plastic cages (175 x 245 x 125 mm) with bedding (chip, Chiba Animal Shizai, Co.). Up to 5 animals are put per cage. The plastic cages and water supply bottles are changed twice or more a week.

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(10) Food

Pellet food (CRF-1, Oriental Yeast Co.) within 3 months after reception is supplied freely. Contaminants of the food are analyzed by a third party organization, and the results of the lots used during this study are obtained from the manufacturer.

(11) Drinking water

City water is supplied freely using polycarbonate water supply bottles.

Quality of city water is examined by a third party organization almost every 3 months.

(12) Bedding

Contaminants, etc. of bedding are analyzed by a third party organization, and the results are obtained.

3. Method of preparation of test article fluid for administration

The test article is suspended in 0.5% CMC-Na aqueous solution at designated concentrations. Preparation is performed shortly before use, and after use, the remaining suspension is discarded.

4. Administration method and dose groups

(1) Route of administration and reason for the choice

Since DB is a food, its acute toxicity in mice is examined by oral administration in this study.

(2) Administration method

The test article is administered by gavage using plastic disposable syringe with metallic stomach tube.

(3) Dose volume, number of dosing and time of dosing

Based on the body weight measured shortly before administration, 40 ml/kg of test article suspension is administered once (from 8 a.m. to 12 noon, as a rule).

Administration is performed under deprivation of food and water. Food and water are supplied after completion of observation on the day of administration (at about 6 hours after dosing).

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(4) Dose groups, number of animals and Animal Nos.

The test groups are as follows:

Group	Test group	Dose	Color of label	Males (Animal Nos.)	Females (Animal Nos.)
Group 1	Control (vehicle)	-	White	10(001-010)	10(051-060)
Group 2	DB	2.5g/kg	Yellow	10(101-110)	10(151-160)
Group 3	DB	5g/kg	Blue	10(201-210)	10(251-260)
Group 4	DB	10g/kg	Red	10(301-310)	10(351-360)

(5) Reason for dose setting

Since DB is a food, it seems to be highly safe. No effect of its administration to rats at 10 g/kg/day for 3 weeks was reported. In consideration of suspendibility of DB in 0.5% CMC-Na aqueous solution and the possible largest dose-volume, 10 g/kg is set at the high dose, and the middle and low doses are set at 5 g/kg and 2.5 g/kg in a geometric series with a factor of 1/2.

5. Items of observation and examination

(1) Observation period

For 14 days after administration

(2) General clinical signs

Each animal is observed for 6 hours after dosing on the day of administration. From the next day, general clinical signs and the state of survival are observed twice daily in the observation period (only once on the day of necropsy).

(3) Body weight measurement

Each animal is weighed on the day of administration (shortly before dosing), and on days 1, 3, 7, 10 and 14 after administration at a constant time in the morning (8 a.m.-11 a.m., as a rule).

(4) Necropsy

Dead animals are necropsied as early as possible after being found. After the end of observation period, all the surviving animals are killed by exsanguination under ether anesthetization and necropsied. Findings are recorded. Organs and tissues that showed abnormalities are fixed in a 10% formalin solution and stored.

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6. Statistical assessment

(1) When calculation of LD_{50} and the 95% confidence limit are possible from the mortality by the end of the observation period, these values are calculated by the Probit method.

(2) For the body weight, the mean and standard deviation are calculated in each group. For testing significant difference between the control group and the test article administration group, the homogeneity of variances is examined by the *F* test. When variances are homogenous, Student's *t* test is used, and when variances are not homogenous, Aspin-Welch t test or Cochran-Cox *t* test is applied. In every test, the significant level is set at less than 5%.

7. Amendment to protocol

When changes of important contents of the protocol become necessary after making the study contract, the sponsor and the testing facility have to consult. Based on their agreement, changes are made, and the protocol amendment form in which the part of change, details of change and reason for the change are described is prepared and preserved together with the protocol.

8. Preservation of documents and specimens

All the documents and specimens concerning this study are preserved in the archives and specimen storage room of Nihon Bioresearch Center Co. The period of preservation will be decided under a separate contract.

The list of preserved items is as follows:

(1) Protocol, protocol amendment form, contract, chart of main plan

(2) Records on the test article
   1) Record of receipt and return of the test article
   2) Record on the description and stability of the test article (obtained from the sponsor)
   3) Record of use of the test article
   4) Record of preparation of the test article

(3) Records of analysis and environmental control
   1) Record of environment in animal room
   2) Record of animal care
   3) Record of analysis of food contaminants
   4) Record of quality analysis of drinking water

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(4) Records on test animals

1) Record of animal delivery
2) Record of receipt and acclimation of animals
3) Record of animal allocation to groups
4) Record of transfer and treatment of animals
5) Cage labels

(5) Records on administration

1) Record of calculation of dose-volumes
2) Record of administration

(6) Records of observation and examination

1) Record of deaths in the course
2) Record of observation of general clinical signs
3) Record of body weight measurement
4) Record of necropsy findings
5) Record of calculation of LD_{50} (when calculation is possible)
6) Record of confirmation of organs (when organs are preserved)

(7) Specimens

1) Formalin-fixed specimens (when organs are preserved)

(8) Preserved documents concerning report

1) Figures and tables used in preparing report
2) Statistical data
3) Manuscript of report
4) Copy of the final report

9. Return of remaining test article

After conclusion of the study, the remaining test article is returned to the sponsor together with the record of use (copy).

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Protocol Amendment Form (No. 1)

Study No. 4087

Title: Oral administration acute toxicity study of Dunaliella Bardawil Spray Dried Powder in mice

Part of correction, details of correction, reason for correction

p. 3 1. Test article

(1) Test article

Stability: Since the stability in 0.5% CMC is unknown now, the stability will be confirmed by the sponsor. Therefore, the data will be added after acquisition.

↓

The test article is stable in 0.5% CMC-Na aqueous solution up to 5 hours after preparation.

Vehicle: 0.5% carboxymethyl cellulose sodium aqueous solution (CMC-Na, Lot No. 1C4034, Junsei Chemical Co., Ltd.)

↓

Junsei

The above underlined parts are changed as shown in the lower line.

Reason for the changes: Concerning the stability, the data was decided to add later. [redacted]

[redacted]

pp. 3 – 4 2. Test animals

(3) Date of delivery and number of animals delivered

September 10, 1987 50 males, 50 females

↓ ↓

52 52

Continued to the next page

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(4) Body weight range at the time of delivery

↓

the day after delivery

Males: 15-25 g, Females: 15-25 g

↓ ↓

15.1-21.4 g 17.0-21.4 g

The above underlined parts are changed as shown in the lower line.

Reason for the changes: Owing to convenience of the animal supplier, the aforesaid number of animals with the above body weight ranges was delivered, and actual values are entered.

Study Director: Fumihiko Ariga Seal
Date of correction: November 10, 1987

Approval
Facility Management: Kenichi Nakagawa Seal
November 10, 1987

Sponsor: Teruo Shiraishi Seal
November 11, 1987

000124

信頼性保証部門陳述書

試験番号：４０８７

表　　題：Dunaliella Bardawil Spray Dried Powderのマウスにおける経口投与による急性毒性試験

上記の試験に関して、昭和６２年８月２６日から昭和６２年１２月８日にかけて検閲を実施し、その結果は運営管理者および試験責任者に報告されている。　この検閲記録は、㈱日本バイオリサーチセンター羽島研究所ＱＡＵ室にすべて保管されている。　この最終報告書は、試験で得られた生データを正確に反映している事をここに保証する。

昭和62年12月8日

㈱日本バイオリサーチセンター　羽島研究所

信頼性保証部門責任者 [illegible]

000125

Dunaliella Bardawil Spray Dried Powderのマウスにおける経口投与による急性毒性試験

報　告　書

提出日　昭和６２年１２月　８日

株式会社日本バイオリサーチセンター
羽島研究所

N.B R－1

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信頼性保証部門陳述書

試験番号：４０８７

表　　題：Dunaliella Bardavil Spray Dried Powderのマウスにおける経口投与による急性毒性試験

上記の試験に関して、昭和６２年８月２６日から昭和６２年１２月８日にかけて検閲を実施し、その結果は運営管理者および試験責任者に報告されている。　この検閲記録は、㈱日本バイオリサーチセンター羽島研究所ＱＡＵ室にすべて保管されている。　この最終報告書は、試験で得られた生データを正確に反映している事をここに保証する。

昭和62年12月8日

㈱日本バイオリサーチセンター　羽島研究所

信頼性保証部門責任者 [illegible]

000127

試験概要（試験番号：4087）

表　　題	Dunaliella Bardawil Spray Dried Powderのマウスにおける経口投与による急性毒性試験
試験番号	４０８７
被験物質名	Dunaliella Bardawil Spray Dried Powder
試験系	マウス(SPF),Crj:CD-1(ICR)
試験委託者	日健製薬工業株式会社 岐阜県羽島市福寿町平方４－41
試験実施場所	岐阜県羽島市福寿町間島６－１０４ 株式会社日本バイオリサーチセンター羽島研究所
試験目的	薬審第 118号（昭和59年 2月15日付）「医薬品のための毒性試験法ガイドライン」に基づき、Dunaliella Bardawil Spray Dried Powderのマウスに対する経口投与による急性毒性について検討する。
試験実施期間	自　昭和６２年　９月１０日（動物入荷日） 至　昭和６２年１０月　２日（剖検日）
試験期間	昭和６２年　８月２５日　～　昭和６２年１２月　８日
資料保管場所	岐阜県羽島市福寿町間島６－１０４ 株式会社日本バイオリサーチセンター羽島研究所

試験従事者の署名・捺印（試験番号：4087）

表　　題	Dunaliella Bardawil Spray Dried Powderのマウスにおける経口投与による急性毒性試験
試験番号	4087

試験責任者	(b) (6) [redacted]	昭和62年12月8日
試験担当責任者		昭和62年12月8日
試験担当者		昭和62年12月7日
		昭和62年12月7日
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		昭和62年12月7日
		昭和62年12月7日
		昭和62年12月7日
		昭和62年12月7日

昭和62年12月8日

運営管理者 (b) (6) [redacted]

NBR－3

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試験従事者および業務分担（試験番号：4087）

試験責任者：（試験の統括、試験計画書の作成、報告書の校正）

有賀文彦

試験担当責任者：（試験実施の指示、検体の投与、一般状態観察、剖検、データのまとめおよびチェック、報告書の作成）

宇土研二

試験担当者：（検体の投与、一般状態観察、動物の一般飼育管理、剖検）

山下恭
藤村高志
岩田晃治
森勝弘
浅野峰治
高井靖夫
溝口祥史
杉山浩子
山口笑

（剖検）　小池恒雄

（検体調製）　加藤正巳

（統計処理、図・表の作成）　鈴木麗子
豊田絵理子

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緒　言

今回、食品としての使用が予定されている、Dunaliella Bardawil Spray Dried Powderの安全性に関する試験の一環として、薬審第 118号（昭和59年 2月15日付）「医薬品のための毒性試験法ガイドライン」に基づき、Dunaliella Bardawil Spray Dried Powderのマウスに対する経口投与による急性毒性試験を実施したので、その結果を報告する。

当試験の日程は下記の通りであった。

動物入荷日：昭和62年 9月10日
投　与　日：昭和62年 9月18日
剖　検　日：昭和62年10月 2日

被験物質

被験物質Dunaliella Bardawil Spray Dried Powder（以下、DBと略す）は、赤褐色の粉末で、水に溶けない[1]。 DB（ロット番号： NK-1001）は昭和62年 9月 1日に、日健製薬工業株式会社より提供（正味量：20g）されたものを、試験実施機関の被験物質保管庫（冷凍庫）に遮光下・密栓ガラス瓶で保管した。

被験物質の調製は用時とし、媒体として 0.5%カルボキシメチルセルロースナトリウム水溶液（CMC-Na；純正化学株式会社、ロット番号：1C4034）を用いて懸濁し、各濃度毎に調製した。 投与後の残余検体液は廃棄した。

なお、DBの 0.5%CMC-Na水溶液中での安定性に関する試験委託者からのデータでは調製後 5時間まで安定であることが報告[1]されているため、調製後 5時間以内に使用した。

試験方法

1．試験動物および飼育条件

動物は、4 週齢の ICR系雌雄マウス［Crj:CD-1(ICR)］を、昭和62年 9月10日に雄52匹と雌52匹を日本チャールス・リバー株式会社より購入し、試験に用いた。 入荷翌日の体

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重範囲は、雄は15.1～21.4 g、雌は17.0～21.4 gであった。

入荷したマウスは 8日間の予備飼育期間を設け、この間 3回の体重測定と連日の外見、一般状態の観察を行い、異常がみられない 5週齢の動物を試験に用いた。

飼育は、室温20～24℃、湿度40～70%、明暗各12時間（蛍光灯による照明時間：午前 6時～午後 6時）、換気回数12回／時間（フィルターにより除菌された新鮮空気）に設定された動物飼育室（C棟３号室）で、予備飼育期間中および群分け後とも高圧蒸気滅菌した床敷（チップ、千葉アニマル資材株式会社）を敷いたプラスチック製ケージ（175 ×245 ×125 mm）に 1ケージ当たり 5匹までの群飼いとした。

プラスチック製ケージおよび給水瓶の交換は 1週間に 2回以上行った。

飼料は、絶食時を除き固型飼料（CRF-1 、オリエンタル酵母工業株式会社）を給餌器に入れ、飲料水は、上水道水を給水瓶に入れ、自由に摂取させた。

なお、当試験は経口投与であるため、投与に先立ち投与前日の夕刻 4～ 5時頃から摂水下で絶食（投与前18～20時間）を行った。 また、投与日の給餌および給水は投与後 5時間の観察の後に行った。

２．群分け方法

群分けは、体重を層別後、無作為に各群の平均体重および分散がほぼ等しくなるように、投与当日に行った。 群分け後の残余動物は、投与日に麻酔死処分した。

投与時の体重は、雄は 20.9g～ 23.3g、雌は 17.0g～ 19.7gであった。

３．個体識別方法

個体識別は、予備飼育期間中では油性インクペンで尾に印をつけることにより、群分け後では色素による染毛法により行った。 更に、各ケージには予備飼育期間中は試験番号、入荷年月日、馴化動物番号を、群分け後は試験番号、被験物質名、投与量、動物番号を記入したラベルをそれぞれ装着した。

４．投与方法

DBは食品としての使用が予定されているため、当試験においては経口投与を選択した。

投与は、金属製経口ゾンデを装着したプラスチック製ディスポーザブル注射筒を用い、絶食下で、投与直前に測定した体重を基準として、投与液量 40ml/kgで 1回投与（投与時間：午前10～12時）した。

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5．投与量および群構成

DBをラットに10g/kg/dayを 3週間投与しても影響はみられず、安全性は高いとの報告もあることから、DBの 0.5%CMC-Na水溶液に対する懸濁能および動物への投与可能な容量を考え合わせて、本試験の最高用量は10g/kgとし、以下公比 2で減じた 5g/kgおよび 2.5g/kgを中用量、低用量とした。 また、対照群として、媒体の 0.5%CMC-Na水溶液のみを被験物質投与群と同一液量投与する 1群を設けた。 1群の動物数は雌雄各10匹とした。

6．観察、検査項目

(1) 観察期間

観察期間は投与後14日間とした。

(2) 一般状態

投与日は投与後 6時間まで（投与後30分までと投与後 2、 4および 6時間）、投与翌日からの観察期間中は毎日 2回（午前と午後に各 1回、但し剖検日は剖検前の 1回のみ）、一般状態および死亡の有無を観察した。

(3) 体重測定

投与日の投与直前および投与後 1、 3、 7、10、14日の午前中のほぼ一定時刻（午前 8時～11時の間）に測定した。

(4) 剖検

観察期間終了後の生存動物は全例エーテル麻酔下で開腹して放血致死させたのちに剖検し、所見を記録した。

7．統計学的処理

観察期間終了後の死亡率からLD_{50}値およびその95%信頼限界値をProbit法により算出する予定であったが、死亡発現がみられなかったため、LD_{50}値は算出できなかった。

体重は、各群で平均値および標準偏差を算出した。 対照群との有意差検定は、分散の同等性を F検定を用いて確認した結果、各投与群とも分散に差がなかったため、 Studentの t検定を用いて行った（別表 1、2）。

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8. 資料の保管

当試験の実施により得られたすべての資料は、報告書提出後 1年間は試験実施機関の資料保管室に保管する。 その後は試験委託者と協議の上、決定する。

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試験成績および考察

1．一般状態およびLD_{50}値

経日死亡およびLD_{50}値を表 1に示した。

雌雄いずれの投与群とも観察期間中に死亡発現はなく、一般状態の観察においても異常症状は認められなかった。

従って、LD_{50}値は雌雄とも10g/kg以上であると推測された。

なお、対照群においても観察期間中に死亡発現および異常症状は認められなかった。

2．体重

雄の体重推移を表 2（個別表 2-1～2-4 ）および図 1に、雌の体重推移を表 3（個別表 3-1～3-4 ）および図 2に示した。

雌雄いずれの投与群とも対照群とほぼ同様な推移を示し、2.5g/kg 群では雌雄とも投与後 3日以降の値は対照群を上回り、雄では投与後10日に対照群に比して有意な高値を示した。

3．剖検

観察期間終了後の生存例の剖検では、対照群およびいずれの投与群の雌雄とも著変は認められなかった。

以上の結果、マウスにDBの10g/kgを投与しても死亡発現、一般状態の変化および体重増加抑制ならびに剖検での著変は認められなかったことから、DBの安全性は高いものと考えられた。

なお、当試験の実施期間中に、データの信頼性に影響を及ぼしたと思われる環境要因は認められなかった。

文　献

1．日健製薬工業株式会社から提供された資料

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要　約

Dunaliella Bardawil Spray Dried Powderの2.5g/kg、5g/kgおよび10g/kgをICR系雌雄マウス［Crj:CD-1（ICR）］に経口投与する急性毒性試験を行った。

雌雄のいずれの投与群とも観察期間中に死亡発現は認められず、LD_{50}値は雌雄とも10g/kg以上であり、一般状態の変化および体重増加抑制ならびに剖検での著変も認められなかった。　よって、Dunaliella Bardawil Spray Dried Powderの安全性は高いものであった。

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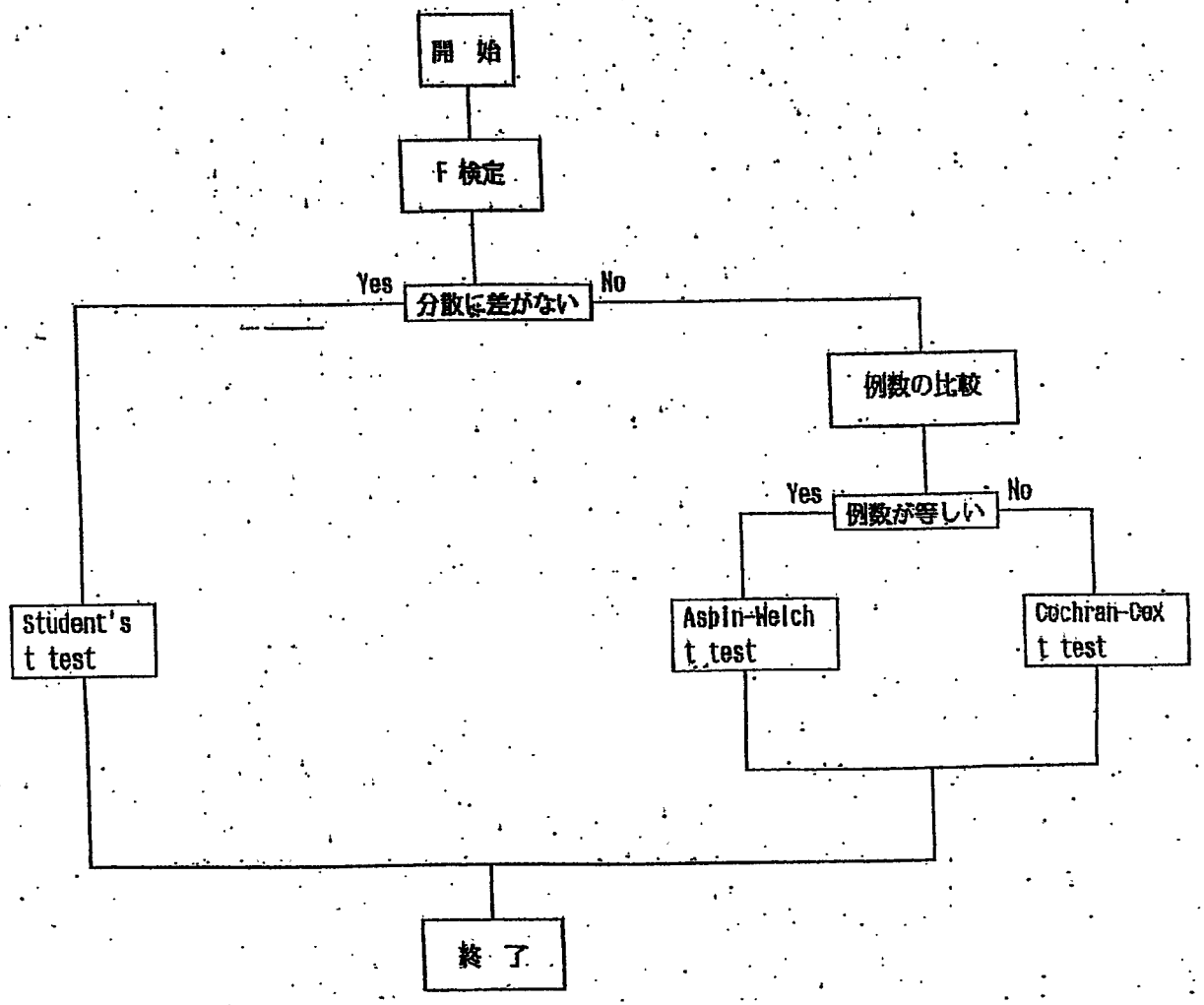
別表1　Dunaliella Bardawil Spray Dried powderのマウスにおける経口投与による急性毒性試験の統計手法のフローチャート



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別表2　Dunaliella Bardawil Spray Dried powderのマウスにおける経口投与による急性毒性試験のコンピュータの統計表示

(N.) …… 例数

MEAN …… 平均

S. D. …… 標準偏差

VALUE …… 統計量

JUDGE …… 有意差マーク (*)、(**)

SIGN …… 対照群 (0mg/kg 群) に対して平均値が
大きい (+)、小さい (-)

METHOD …… 検定結果
TTHO : Student's t testで有意差なし

表1　Dunaliella Bardawil Spray Dried powderのマウスにおける経口投与による急性毒性試験の経日死亡

性別	群	例数	投与後の時間 (h)				投与後の日数 (day)														総死亡数	LD_{50} (g/kg)
			0.5	2	4	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
雄	対照	10	0[a]	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	>10
	DB 2.5 g/kg	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	DB 5 g/kg	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	DB 10 g/kg	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
雌	対照	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	>10
	DB 2.5 g/kg	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	DB 5 g/kg	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	DB 10 g/kg	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

a) 経時あるいは経日の死亡数。

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TEST NO. 4087

表2　Duhaliella Bardawil Spray Dried powderのマウスにおける経口投与による急性毒性試験の雄の体重推移

群	0	1	3	7	10	14 (日)
対 照						
MEAN	22.2	26.3	27.6	29.3	29.9	31.7
S.D.	0.69	0.90	0.78	1.25	1.12	1.39
(N)	10	10	10	10	10	10
DB 2.5G/KG						
JUDGE					*	
MEAN	22.2	26.3	28.1	29.7	31.1	32.6
S.D.	0.76	0.92	0.91	0.97	1.10	1.37
(N)	10	10	10	10	10	10
DB 5G/KG						
JUDGE						
MEAN	22.1	25.9	27.1	29.2	29.8	31.2
S.D.	0.68	0.82	0.97	1.37	1.25	1.59
(N)	10	10	10	10	10	10
DB 10G/KG						
JUDGE						
MEAN	22.2	25.9	27.5	29.4	30.1	31.5
S.D.	0.72	1.10	1.51	1.92	2.08	2.22
(N)	10	10	10	10	10	10

* : P< 0.05 で対照群に対して有意差あり

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TEST NO. 4087

表3： Dunaliella Bardawil Spray Dried powderのマウスにおける経口投与による急性毒性試験の雌の体重推移

群		0	1	3	7	10	14 (日)
対照	MEAN	18.4	21.5	22.1	22.3	22.7	24.0
	S.D.	0.89	1.24	0.94	1.27	1.16	1.79
	(N)	10	10	10	10	10	10
DB 2.5G/KG	JUDGE						
	MEAN	18.4	21.2	22.5	22.9	23.4	25.0
	S.D.	0.90	0.76	0.75	0.80	0.96	1.50
	(N)	10	10	10	10	10	10
DB 5G/KG	JUDGE						
	MEAN	18.4	21.6	22.4	22.8	22.8	24.6
	S.D.	0.81	1.06	1.46	1.52	1.43	1.81
	(N)	10	10	10	10	10	10
DB 10G/KG	JUDGE						
	MEAN	18.4	21.1	22.2	22.5	22.9	23.7
	S.D.	0.81	1.27	1.09	1.11	1.10	0.99
	(N)	10	10	10	10	10	10

単位：g

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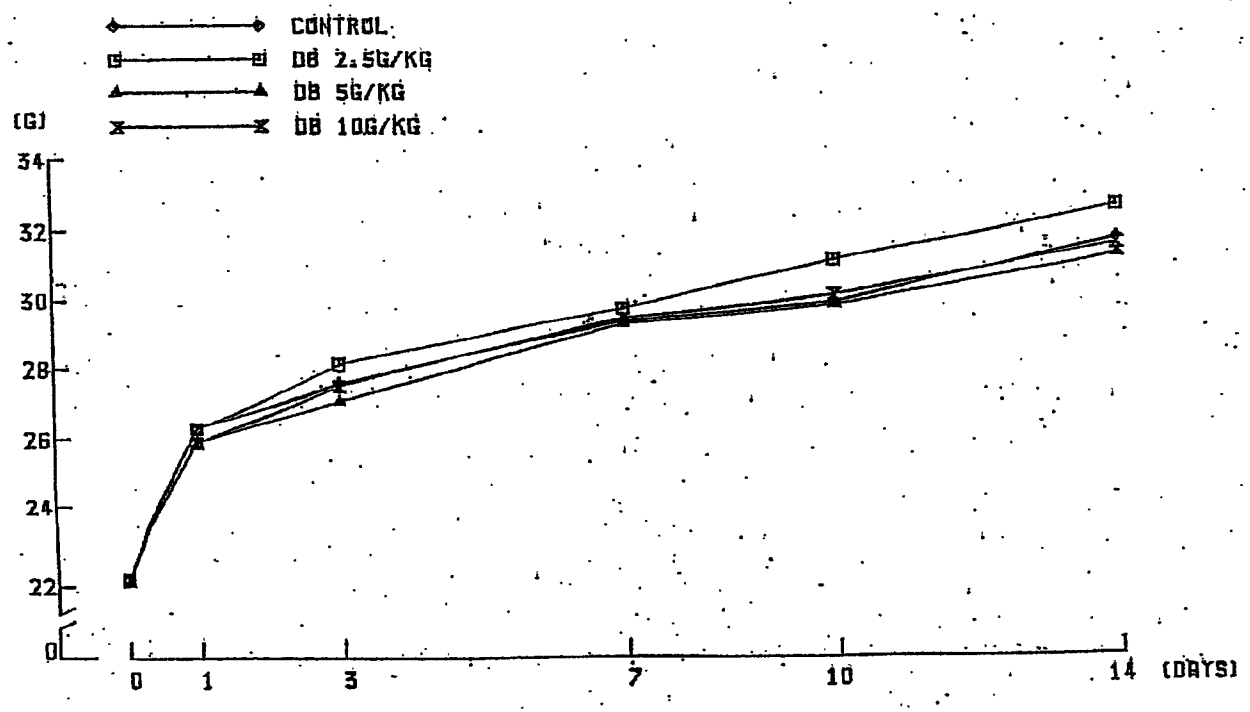

図1　Dunaliella Bardawil Spray Dried powderのマウスにおける経口投与による急性毒性試験の雄の体重推移

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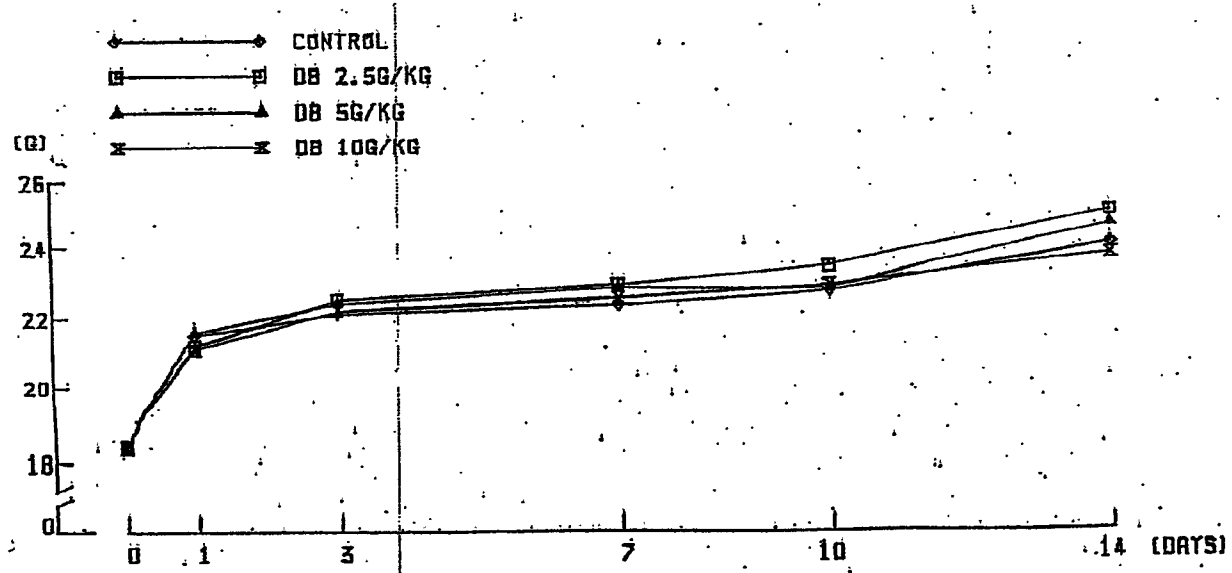

図2　Dunaliella Bardawil Spray Dried powderのマウスにおける経口投与による急性毒性試験の雌の体重推移

TEST NO. 4087 APPENDIX: 2-1

(GROUP : CONTROL)

BODY WEIGHT OF MALE MICE

NO.	0	1	3	7	10	14 (DAYS)
001	21.3	26.4	26.7	27.7	28.8	30.2
002	22.1	26.1	27.3	29.3	29.5	31.3
003	21.4	25.1	27.8	29.5	30.3	32.0
004	22.1	26.1	28.2	30.0	30.8	32.4
005	23.2	27.1	27.8	30.0	31.0	32.3
006	22.8	27.1	29.0	31.1	31.2	33.8
007	23.0	27.5	28.0	30.0	30.3	32.6
008	21.7	25.0	26.2	26.8	28.0	29.6
009	22.6	25.7	27.4	28.8	28.4	29.9
010	21.8	27.3	27.8	29.9	30.2	32.8
(N)	10	10	10	10	10	10
MEAN	22.2	26.3	27.6	29.3	29.9	31.7
S.D.	0.69	0.90	0.78	1.25	1.12	1.39

UNIT : G

TEST NO. 4087 APPENDIX 2-2

(GROUP : DB 2.5G/KG)

BODY WEIGHT OF MALE MICE

NO.	0	1	3	7	10	14 (DAYS)
101	20.9	25.4	27.3	29.2	30.3	31.9
102	22.7	25.5	27.5	28.2	29.8	30.9
103	21.5	26.0	27.6	29.7	31.4	32.5
104	22.5	27.2	28.6	30.7	32.3	33.6
105	21.9	25.6	26.7	29.6	30.4	32.0
106	22.3	26.3	28.0	29.0	30.1	31.9
107	21.8	26.3	27.9	28.9	30.1	30.9
108	21.7	25.6	28.3	30.7	32.0	33.5
109	23.2	27.3	29.4	29.7	31.8	34.7
110	23.3	28.1	29.6	31.4	32.9	34.5
(N)	10	10	10	10	10	10
MEAN	22.2	26.3	28.1	29.7	31.1	32.6
S.D.	0.76	0.92	0.91	0.97	1.10	1.37
VALUE	0.000	0.025	1.237	0.797	2.536	1.537
JUDGE					*	
SIGN			+	+	+	+
METHOD	TTH0	TTH0	TTH0	TTH0	TTH1	TTH0

* : SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP (P<0.05)

UNIT : G

Amendment

Study Director Tadakazu Furuhashi Seal September 25, 1989

Approval

Facility Management Kenichi Nakagawa Seal September 25, 1989

Study sponsor Hiroyuki Takenaka Seal September 28, 1989

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Protocol Amendment Form (No. 4)

Study number: 5118

Title: 28-day oral administration subacute toxicity study of Dunaliella Bardawil Spray Dried Powder in rats

Amendments and their reasons

p. 1 Study sponsor

Nikken Sohonsha Corporation

4-41 Hirakata, Fukuju-cho, Hashima -shi, Gifu-prefecture

↓

1-32 Asahira, Fukuju-cho, Hashima-shi, Gifu-prefecture

The underlined section is amended to as above.

Reasons: The address of the study sponsor has been changed.

Amendment

Study Director Tadakazu Furuhashi Seal October 17, 1989

Approval

Facility Management Kenichi Nakagawa Seal October 17, 1989

Study Sponsor Hiroyuki Takenaka Seal October 19, 1989

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TEST NO. 4087 APPENDIX: 2-3

(GROUP : DB 5G/KG)

BODY WEIGHT OF MALE MICE

NO.	0	1	3	7	10	14 (DAYS)
201	21.4	25.2	26.2	27.8	28.4	30.2
202	23.3	27.6	28.4	30.8	31.2	33.5
203	21.1	25.3	25.4	26.6	28.4	29.2
204	22.0	24.8	26.4	28.3	28.2	29.0
205	22.4	26.3	28.0	30.2	29.5	30.6
206	22.3	25.7	26.7	28.7	29.3	30.4
207	21.8	25.5	26.9	29.2	29.7	32.1
208	22.6	26.5	27.3	29.7	31.2	32.3
209	22.8	26.4	28.2	31.0	31.6	33.3
210	21.6	25.5	27.7	29.5	30.2	31.7
(N)	10	10	10	10	10	10
MEAN	22.1	25.9	27.1	29.2	29.8	31.2
S.D.	0.68	0.82	0.97	1.37	1.25	1.59
VALUE	0.164	1.194	1.268	0.221	0.150	0.688
JUDGE SIGN	-	-	-	-	-	-
METHOD	TTHO	TTHO	TTHO	TTHO	TTHO	TTHO

UNIT : G

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TEST NO. 4087 APPENDIX: 2-4

(GROUP : DB 10G/KG)

BODY WEIGHT OF MALE MICE

NO.	0	1	3	7	10	14 (DAYS)
301	22.7	26.4	28.0	29.2	30.0	31.6
302	21.7	24.6	25.8	27.4	27.6	29.0
303	23.3	27.3	29.0	29.8	30.7	32.2
304	22.1	25.2	26.9	28.0	28.4	29.6
305	21.0	24.8	26.5	29.0	29.1	30.0
306	21.4	25.1	26.9	29.8	30.5	32.6
307	22.5	26.7	28.9	31.5	32.9	34.1
308	22.4	27.3	28.1	30.4	32.0	33.6
309	22.9	26.9	30.0	32.8	32.9	34.3
310	21.7	24.9	25.3	26.4	27.1	28.1
(N)	10	10	10	10	10	10
MEAN	22.2	25.9	27.5	29.4	30.1	31.5
S.D.	0.72	1.10	1.51	1.92	2.08	2.22
VALUE	0.032	0.936	0.149	0.179	0.361	0.217
JUDGE SIGN		-	-	+	+	-
METHOD	TTHO	TTHO	TTHO	TTHO	TTHO	TTHO

UNIT : G

000150

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TEST NO. 4087 APPENDIX: 3-1

(GROUP : CONTROL)

BODY WEIGHT OF FEMALE MICE

NO.	0	1	3	7	10	14 (DAYS)
051	18.3	21.6	21.7	21.5	22.6	25.1
052	18.5	21.0	22.2	22.0	21.6	22.7
053	19.1	22.0	21.8	22.5	22.9	24.1
054	19.4	22.1	22.6	24.3	23.0	24.2
055	17.0	21.4	21.4	21.4	21.5	22.4
056	17.2	21.0	21.2	20.5	22.2	22.9
057	19.7	24.4	24.4	24.5	25.3	27.3
058	18.8	20.9	22.4	22.9	23.9	26.6
059	17.9	19.9	21.5	22.0	21.8	22.4
060	18.2	20.3	21.4	21.6	22.5	22.6
(N)	10	10	10	10	10	10
MEAN	18.4	21.5	22.1	22.3	22.7	24.0
S.D.	0.89	1.24	0.94	1.27	1.16	1.79

UNIT : G

000151

TEST NO. 4087 APPENDIX: 3 - 2

(GROUP : DB 2.5G/KG)

BODY WEIGHT OF FEMALE MICE

NO.	0	1	3	7	10	14 (DAYS)
151	18.5	22.1	21.9	22.4	23.9	26.8
152	19.7	22.2	23.5	24.0	24.1	24.5
153	18.3	20.0	21.5	21.8	23.5	25.8
154	19.3	21.1	23.8	23.9	23.7	23.7
155	17.2	21.2	22.3	22.7	23.4	25.8
156	19.3	21.9	22.6	22.6	22.8	23.8
157	18.7	21.0	22.9	24.1	24.8	26.1
158	18.0	21.2	22.3	22.7	22.9	23.8
159	17.0	20.0	21.7	22.2	21.2	22.6
160	17.9	21.2	22.9	23.0	23.5	27.0
(N)	10	10	10	10	10	10
MEAN	18.4	21.2	22.5	22.9	23.4	25.0
S.D.	0.90	0.76	0.75	0.80	0.96	1.50
VALUE	0.050	0.586	1.258	1.304	1.367	1.299
JUDGE SIGN		-	+	+	+	+
METHOD	TTHO	TTHO	TTHO	TTHO	TTHO	TTHO

UNIT : G

000152

000152

TEST NO. 4087 APPENDIX: 3-3

(GROUP 3 DB 5G/KG)

BODY WEIGHT OF FEMALE MICE

NO.	0	1	3	7	10	14 (DAYS)
251	18.6	21.6	22.1	23.9	24.6	26.7
252	19.5	21.7	22.3	23.9	23.2	24.0
253	18.3	21.0	21.1	22.5	21.9	22.0
254	17.1	20.1	20.9	21.0	20.4	23.6
255	18.9	22.4	23.7	24.1	24.1	25.0
256	18.1	22.3	22.5	22.3	22.2	24.7
257	19.4	23.5	25.1	24.8	24.3	26.9
258	19.1	22.2	24.0	23.9	23.7	26.5
259	17.5	21.0	21.8	21.5	22.1	24.8
260	17.8	20.2	20.6	20.3	21.1	21.8
(N)	10	10	10	10	10	10
MEAN	18.4	21.6	22.4	22.8	22.8	24.6
S.D.	0.81	1.06	1.46	1.52	1.43	1.81
VALUE	0.053	0.272	0.637	0.797	0.052	0.708
JUDGE						
SIGN		+	+	+	+	+
METHOD	TTHO	TTHO	TTHO	TTHO	TTHO	TTHO

UNIT : G

TEST NO. 4087 APPENDIX: 3-4

(GROUP : DB 10G/KG)

BODY WEIGHT OF FEMALE MICE

NO.	0	1	3	7	10	14 (DAYS)
351	19.5	22.5	23.7	24.6	23.7	24.4
352	18.4	22.5	23.9	23.5	24.6	25.4
353	18.4	20.3	21.6	21.7	23.4	24.4
354	17.0	20.0	21.3	21.7	21.5	21.7
355	18.0	19.4	20.8	21.1	21.1	23.6
356	17.8	20.5	21.7	22.6	22.3	23.1
357	18.8	22.2	22.2	22.6	23.4	23.2
358	19.5	21.6	22.5	23.0	23.2	23.8
359	19.0	22.6	23.0	23.2	23.6	24.1
360	17.7	19.8	21.0	21.2	22.1	23.4
(N)	10	10	10	10	10	10
MEAN	18.4	21.1	22.2	22.5	22.9	23.7
S.D.	0.81	1.27	1.09	1.11	1.10	0.99
VALUE	0.000	0.571	0.241	0.374	0.317	0.495
JUDGE SIGN		-	+	+	+	-
METHOD	TTHO	TTHO	TTHO	TTHO	TTHO	TTHO

UNIT : G

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SOP/TSB/07/RS-02

(DB) 使用記録用紙 (No. 1)

試験番号： 4087
LOT No.： NK－1001
入手年月日： 昭和 62年 9 月 1 日
使用期限： 昭和 ~~年 月 日~~

正味量： 20g
風袋込み量： 157.65g
保管条件： 冷凍
保管形態： 密栓ガラス瓶
保管庫No.： 冷凍庫 No.6

試験責任者 有賀文子

使用記録

年月日	採取量（使用量）（g）	風袋込み残量（使用後）（g）	使用者	調製指示書No.
62.9.18	17.5	140.14	加藤	1
	作製 17.5g	残量 140.14g		
		0.2%0.01g 確認	62.9.18	加藤

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試験番号：4087

試験計画書

被験物質名：DB（略称）

表　題

Dunaliella Bardawil Spray Dried Powderのマウスにおける経口投与による急性毒性試験

試験委託者

日健製薬工業株式会社

岐阜県羽島市福寿町平方 4 -41

試験機関

株式会社日本バイオリサーチセンター羽島研究所（運営管理者　仲川憲一）

岐阜県羽島市福寿町間島 6 - 104

試験目的

薬審第 118号（昭和59年 2月15日付）「医薬品のための毒性試験法ガイドライン」に基づき、Dunaliella Bardawil Spray Dried Powderのマウスに対する経口投与による急性毒性について検討する。

試験関係者

試験責任者　　有賀文彦

試験担当責任者　宇土研二

信頼性保証部門

担当責任者　　岡田謙司

試験開始日（運営管理者の署名捺印日）　　昭和62年 8月25日

試験実施期間

動物入荷日　　昭和62年 9月10日

最終剖検予定日　　昭和62年10月 2日

報告書（案）提出日　　昭和62年11月30日

最終報告書提出予定日　　昭和62年12月25日

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株式会社日本バイオリサーチセンター

試験番号：4087

陳述書

試験番号：４０８７

試験表題：Dunaliella Bardwil Spray Dried Powder のマウスにおける経口投与による急性毒性試験

当試験は、薬発第318号厚生省薬務局長通知（昭和57年3月31日付）「医薬品の安全性試験の実施に関する基準について」に従って実施されたものである。

2007年 1月 16日

株式会社日本バイオリサーチセンター　羽島研究所

試験責任者　[illegible]

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試験番号：4087

署名捺印

試験責任者

氏　名：(b) (6) 昭和 62 年 8 月 25 日

住　所：(b) (6)

所属先：(b) (6)

試験計画書の承認

試験委託者 岐阜県羽島市福寿町平方4丁目41番地 日健製薬工業株式会社

(b) (6) 昭和 62 年 8 月 25 日

（日健製薬工業株式会社）

運営管理者 (b) (6) 昭和 62 年 8 月 25 日

（株式会社日本バイオリサーチセンター羽島研究所）

確　認

信頼性保証部門

担当責任者 (b) (6) 昭和 62 年 8 月 25 日

（株式会社日本バイオリサーチセンター羽島研究所）

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1．被験物質

（1）被験物質

被験物質名：Dunaliella Bardawil Spray Dried Powder（略称：DB）

ロット番号：NK-1001

性　　状：赤褐色の粉末で、水に溶けない。

安 定 性：~~0.5%CMC 中での安定性は現在不明であるが、試験委託者において確認される予定であるので、データ入手後追記する。~~ 0.5%CMC-Na水溶液中では調製後4時間まで安定。

入 手 日：昭和62年 9月 1日

入 手 量：20g

保管条件：窒素置換後、遮光下で冷凍保存（脱酸素剤使用）

保管場所：試験機関の被験物質保管庫（冷凍庫）

提 供 元：日健製薬工業株式会社

媒　　体：0.5%カルボキシメチルセルロースナトリウム水溶液（CMC-Na、~~純生化学株式会社~~ 純正化学株式会社、Lot No.1C4034）

（2）記録

被験物質の入手日、ロット番号、入手量、使用ごとの使用量を所定の用紙に記録する。

2．試験動物

（1）動物種、系統および選択理由

マウス（SPF）Crj:CD-1(ICR)

マウスは毒性試験に一般的に用いられている動物種で、その系統維持が明らかであり、集積データも揃っているため。

（2）入荷時の週齢

マウスは 4週齢で入荷する。

（3）入荷日、入荷匹数

昭和62年 9月10日 ~~雄50匹、雌50匹~~ 52匹 52匹

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（4）入荷時の体重範囲

~~雄15～25g、雌15～25g~~
15.1～21.4g　17.0～21.4g

（5）供給業者

日本チャールス・リバー株式会社

（6）検疫および予備飼育

入荷したマウスは、約 1週間の予備飼育期間を設け、この間 2回以上の体重測定と外見、一般状態の観察を行い、検疫馴化とする。

（7）群分け法

予備飼育期間中の体重増加、外見および一般状態に異常の見られない約 5週齢の動物を選び、体重を層別後、無作為に各群の平均体重および分散がほぼ等しくなるように行う。 投与に先立ち投与前日の夕刻 4～ 5時頃から絶食を行うため、投与当日に群分けする。 群分け後の残余動物は、投与日に麻酔死処分する。

（8）個体識別

予備飼育期間中は尾に油性インクペンで印をつけることにより、群分け後は色素による染毛法により識別する。 予備飼育期間中の各ケージには試験番号、入荷年月日および馴化動物番号を記入したラベルを、群分け後の各ケージには試験番号、被験物質名、投与量、動物番号を記入したラベルを装着する。

（9）動物飼育環境

室温20～24℃、湿度40～70%、明暗各12時間（蛍光灯による照明時間：午前 6時～午後 6時）、換気回数12回／時間（フィルターにより除菌された新鮮空気）に設定された動物飼育室（C棟３号室）で飼育する。

飼育ケージは、予備飼育期間中および群分け後も床敷（チップ、千葉アニマル資材株式会社）を入れたプラスチック製ケージ（ 175× 245×125 mm）を用いて 1ケージ当たり 5匹までの群飼いとする。 プラスチック製ケージ、給水瓶交換は 1週間に 2回以上行う。

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（10）飼料

入荷後 3ヵ月以内の固型飼料（CRF-1 、オリエンタル酵母工業株式会社）を自由に摂取させる。 使用した飼料中の汚染物質の分析結果は、当該試験期間中に用いたロットについて第三者機関で実施したものを、製造業者から入手する。

（11）飲料水

上水道水をポリカーボネート製給水瓶を用いて自由に摂取させる。

上水道水の水質検査は、ほぼ 3ヵ月毎に第三者機関で実施する。

（12）床敷

床敷の汚染物質等の分析結果は第三者機関にて実施したものを入手する。

3．投与検体液の調製方法

被験物質は 0.5%CMC-Na水溶液に濃度毎に懸濁する。

また、調製は用時に行い、使用後の残液は廃棄する。

4．投与法および投与区分

（1）投与経路およびその選択理由

DBは食品であるため、マウスを用いた当試験では経口投与による急性毒性について検討する。

（2）投与方法

金属製経口ゾンデを装着したプラスチック製ディスポーザブル注射筒を用いて投与する。

（3）投与液量、投与回数、投与時刻

投与直前に測定した体重を基準として、40ml/kg を 1回投与（原則として午前8時～午前12時の間）する。

なお、投与は絶食・絶水下で行い、飼料および飲料水は投与日の観察終了後（投与約 6時間後）に与える。

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（4）投与区分、動物数および動物番号

試験群は下記の通りとする。

群	試験群	投与量	ラベルの色	雄（動物番号）	雌（動物番号）
第 1群	対照（溶媒）	—	白 色	10（001～010）	10（051～060）
第 2群	DB	2.5g/kg	黄 色	10（101～110）	10（151～160）
第 3群	DB	5 g/kg	青 色	10（201～210）	10（251～260）
第 4群	DB	10 g/kg	赤 色	10（301～310）	10（351～360）

（5）投与量設定の理由

DBは食品であり安全性は高いと考えられ、またラットに10g/kg/dayを 3週間投与しても影響がみられないという報告もあることから、 0.5%CMC-Na水溶液への懸濁能および動物に投与可能な容量とを考え合わせ10g/kgを高用量とし、以下公比 2により5g/kg および 2.5g/kgを中用量、低用量とする。

5．観察、検査項目

（1）観察期間

投与後14日間

（2）一般状態

投与日は投与後 6時間まで、投与翌日からの観察期間中は毎日 2回（但し剖検日は剖検前の 1回のみ）一般状態および死亡の有無を観察する。

（3）体重測定

投与日（投与直前）および投与後 1、 3、 7、10、14日の午前中のほぼ一定時刻（原則として午前 8～11時の間）に測定する。

（4）剖検

死亡動物については、発見後可及的速やかに、生存動物については観察期間終了時に全例についてエーテル麻酔下で放血死させたのちに剖検し、所見を記録する。 なお、異常の認められた臓器および組織は10%ホルマリン液で固定・保存する。

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6．統計学的処理

（1）観察期間終了後の死亡率からLD_{50}およびその95%信頼限界値が算出可能な場合は、Probit法により算出する。

（2）体重は各群で平均値および標準偏差を算出する。 対照群と被験物質投与群との有意差検定には分散の同等性をF 検定を用いて行い、分散が等しい場合はStudent のt 検定を、分散が等しくない場合はAspin-Welch のt 検定またはCochran-Cox のt 検定を用いて行う。 いずれの検定法においても危険率 5%未満を有意とする。

7．試験計画書の修正

試験受託契約の締結後に、試験計画書の重要な内容変更の必要性が生じた場合には、試験委託者と試験機関の両者間で協議して合意の上で変更し、変更箇所、変更内容、変更理由を記した試験計画書修正書を作成して試験計画書とともに保管する。

8．資料および標本の保管

この試験に関するすべての資料および標本は、株式会社日本バイオリサーチセンターの資料および標本保管室に保管する。 保管期間は、別途契約による。

以下にそのリストを示す。

（1）試験計画書、試験計画書修正書、契約書、主計画表

（2）被験物質に関する記録

1)被験物質の受領および返却記録

2)被験物質の性状および安定性に関する記録（試験委託者より入手したもの）

3)被験物質の使用記録

4)被験物質の調製記録

（3）分析、環境制御記録

1)飼育室内環境記録

2)飼育管理記録

3)飼料中の汚染物質分析記録

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4)飲料水の水質検査記録

(4) 試験動物に関する記録

1)動物入荷記録

2)動物受入れ、馴化記録

3)動物群分け記録

4)動物移動および処分記録

5)ケージラベル

(5) 投与に関する記録

1)投与液量算出記録

2)投与記録

(6) 観察、検査記録

1)経日死亡記録

2)一般状態観察記録

3)体重測定記録

4)剖検所見記録

5)LD_{50} 値算出記録（算出可能な場合）

6)臓器確認記録（臓器保存を行った場合）

(7) 標本

1)ホルマリン固定標本（臓器保存を行った場合）

(8) 報告書に関する保管資料

1)報告書作成に用いた図、表

2)統計処理データ

3)報告書の原稿

4)最終報告書の写し

9．残余被験物質の返却

残余の被験物質は、試験終了後に使用記録（写）を添えて試験委託者に返却する。

試験計画書修正書 (No.1)

試験番号：4087
表　　題：Dunaliella Bardawil Spray Dried Powderのマウスにおける経口投与による急性毒性試験

修正箇所、修正内容、修正理由

p.3　　1．被験物質

(1) 被験物質
安 定 性：0.5% CMC中での安定性は現在不明であるが、試験委託者において確認される予定であるので、データ入手後追記する。
↓
0.5%CMC-Na水溶液中では調製後 5時間まで安定。
媒　　体：0.5%カルボキシメチルセルロースナトリウム水溶液（CMC-Na、純生化学株式会社、Lot No.1C4034）
↓
純正化学株式会社

上記、アンダーラインの部分を下段の如く修正する。

修正理由：安定性についてはデータ入手後に追記することとなっていたため。
CMC-Na製造元の名称についてはタイプミス。

p.3～4　2．試験動物

(3) 入荷日、入荷匹数
昭和62年 9月10日　雄50匹、雌50匹
↓　↓
52匹　52匹

次頁へ続く

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(4) 入荷時の体重範囲
↓
入荷翌日
雄 15 ～ 25g、雌 15 ～ 25g
↓　　↓
15.1～21.4g　17.0～21.4g

上記アンダーラインの部分を下段の如く修正する。

修正理由：動物納入業者の都合により上記匹数および体重範囲の動物が入荷したため、実情に合わせる。

試験責任者：(b) (6) 修正日：昭和 62 年 11 月 10 日

承認
運営管理者：(b) (6) 昭和 62 年 11 月 10 日

試験委託者：(b) (6) 昭和 62 年 11 月 11 日

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GRAS Notice Review

GRAS Notification

***Dunaliella bardawil* Powder**
Food Usage Conditions for General Recognition of Safety

Volume 2 of 2

Submitted for:

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CERTIFICATE OF TRANSLATED CONTENTS

Dated: 8th February 2007

We, the undersigned, hereby certify that the documents attached are the original manuscript text of the following as well as a matching translation of the relevant text faithfully rendered into English.

Text details:	1. ヒトにおける β-カロテン長期間投与時の血中動態および効果に関する研究 2. 報告書：Dunaliella Bardawil Paste の変異原性試験（細菌を用いる復帰変異試験） 3. 最終報告書：Dunaliella Bardawil Spray Dried Powder のラットにおける 28 日間経口投与による亜急性毒性試験 <Total pages: 31>

Translation details:	1. Bioavailability of Synthesized and Naturally-occurring β-Carotene in Humans 2. Study Report: Mutagenecity Study of Dunaliella Bardawil Paste (Reversion test with bacteria) 3. Final Report: Subacute Toxicity Study of Dunaliella Bardawil Spray-Dried Powder by 28-Day Oral Administration in Rats <Total pages: 33>

(b) (6)

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Study No.5118

STATEMENT

Study No. : 5118

Title : Subacute Toxicity Study of Dunaliella Bardawil Spray-Dried Powder by 28-Day Oral Administration in Rats

This study was performed incompliance with the Good Laboratory Practice Standard for Safety Studies on Drugs (Notification No.313 of the Pharmaceutical Affairs Bureau, Japanese Ministry of Health and Welfare, March 31, 1982)

January 16, 2007

Hashima Laboratory, Nihon Bioresearch Inc.

(b) (6)

Study Director : [redacted]

Tadakazu Furuhashi

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Subacute Toxicity Study of Dunaliella Bardawil Spray-Dried Powder by 28-Day Oral Administration in Rats

Final Report

Submitted on October 31, 1989

Hashima Laboratory
Nihon Bioresearch Inc.

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Summary of the study (Study No.5118)

Title	Subacute toxicity study of Dunaliella Bardawil Spray-Dried Powder by 28-day oral administration in rats
Study No.	5118
Name of test substance	Dunaliella Bardawil Spray-Dried Powder
Test system	Sprague-Dawley Rat [SPF, Crj: CD (SD)]
Sponsor	NIKKEN SOHONSHA 1-32, Asahira, Fukuju-cho, Hashima-shi, Gifu-ken
Study site	Hashima Laboratory, Nihon Bioresearch Inc. 6-104, Majima, Fukuju-cho, Hashima-shi, Gifu-ken
Purpose of the study	To evaluate the safety of Dunaliella Bardawil Spray- Dried Powder
Study period	January 5, 1989 to October 31, 1989
Storage of data and samples	Hashima Laboratory, Nihon Bioresearch Inc. (All data and samples obtained for the implementation of the study shall be stored for five years in the data storeroom and sample storeroom in Hashima Laboratory, Nihon Bioresearch Inc. after submission of the final report. Subsequent storage shall be decided by discussion with the sponsor.)
Protocol deviation	There were no protocol deviations in the study.

Persons engaged in the study and job assignment (Study No.5118)

Study director:	Control of the study, preparation of the protocol, checking of the data, correction of the final report Tadakazu Furuhashi
Study managing director:	Supervision over implementation of the study, checking of the data, preparation of samples and of the final report Kenji Udo
Person in charge of study:	Administration and preparation of samples, observation of general signs, general animal care Takashi Fujimura, Koichi Aoyama, Kazuyoshi Naito, Chizuko Hori, Hiroko Sugiyama, Hiroshi Kobayashi
	Autopsy, organ weighing and histopathological examination Hitoshi Kimura, Tsuneo Koike, Kazuko Suganuma, Kenichi Yoshijima, Shoko Inoue, Yuki Mushiga, Megumi Hori, Rikiko Mori
	Laboratory test Fumihiko Ariga, Yukari Watanabe, Hitomi Washimi, Yukako Yamakida
	Fundus examination Hiroshi Wada
	Management and preparation of samples Masami Kato, Yasumi Goto
	Statistical processing, preparation of Tables/Figures Reiko Suzuki, Sachiko Hayashi

Study director:	Tadakazu Furuhashi	October 31, 1989
Study managing director	Kenji Udo	October 31, 1989
Facility manager	Kenichi Nakagawa	October 31, 1989

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Summary

0.5 or 2.5 g/kg of Dunaliella Bardawil Spray-Dried Powder was orally administered to male and female Sprague-Dawley Rats [Crj: CD(SD)] for 28 days to examine its toxicity. 10 mL/kg of 0.5%CMC-Na aqueous solution was orally administered to the control group.

The following results were obtained:

1. No deaths or abnormal general signs were recognized in male or female rats in any of the groups.
2. No effects of Dunaliella Bardawil Dried-Spray Powder were noted on weight, food intake or urinalysis.
3. Ophthalmological examination showed no abnormalities in male or female rats in any of the groups.
4. Hematological and blood chemistry examinations showed no effects of Dunaliella Bardawil Spray-Dried Powder.
5. Autopsy, organ weighing and histopathological examination showed no effects of Dunaliella Bardawil Spray-Dried Powder.
6. Based on the above, no signs of toxicity caused by Dunaliella Bardawil Spray-Dried Powder were observed in male or female rats. Therefore, Dunaliella Bardawil Spray-Dried Powder at 2.5 g/kg, the highest dose recommended for continuous administration, was confirmed to have no toxic effects.

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In the 21-hour urine, urinary specific gravity [S.G.] was measured using a refracting urometer (Uripet, Nippon Kogaku Kogyo CO., LTD.)

In the 24-hour urine, the weight of total urine volume [UV] obtained from three- and 21-hour urine was measured.

(5) Ophthalmological examination

After dilation of both eyes using a mydriatic drop (Mydrin P, SANTEN PHARMACEUTICAL CO., LTD.), funduscopy was performed using a fundus camera (RC-2 Model 621, Kowa Company, Ltd.), before initial administration and after completion of the dosing period.

(6) Hematological examination

On the day after the final administration, blood was collected by cannulation from the abdominal aorta under anesthesia with pentobarbital sodium (Tokyo Chemical Industry Co., Ltd.), and the following hematological parameters were examined. The remaining blood after testing was disposed on the testing day.

Prothrombin time [PT] was measured in plasma treated with 3.13% sodium citrate (Junsei Kagaku Co., Ltd.) by Quick's one-stage method, using FibroSystem (BBL, Div. of Becton, Dickinson & Co.)

Red blood cells [RBC]/white blood cells [WBC] counts, hemoglobin [HGB], hematocrit [HCT] and platelets [PLT] count were measured in blood collected in an EDTA-2K coating Sysmex cup, using an automated blood cell counter [Sysmex E-2000rn, Sysmex (former name was Toa Medical Electronics)].

For blood reticulocytes [RET] count, blood treated with EDTA-2K was stained supravitally (0.5% new methylene blue and 1.6% potassium oxalate mixture) and smeared on glass slides, followed by preparation of Giemsa-stained samples, to determine the percentage of RET in 1000 red cells under microscope.

For differential counts [L: lymphocytes, N: neutrophils, E:eosinophils, B: basophils, M: monocytes], blood was treated with EDTA-2K smeared on the glass slides, and stained with May-Grunwald-Giemsa, and 100 white cells were examined under microscope to obtain the differential count.

(6) Blood chemistry examination

Blood was left for 45-60 minutes at room temperature, then centrifuged for 15 minutes at 4°C and 3000 rpm (20PR-52, Hitachi Koki) to obtain serum.

The serum was stored at -80°C(MDF-390AT, SANYO DENKI CO., LTD.) until assayed. After the assays, the remaining serum was stored at -20°C (RS-70, HITACHI) and discarded after submission of the final report.

GOT and GPT were measured by Henry's method, alkaline phosphatase [ALP] by the pNPP method, creatinine phosphokinase [CPK] by Oliver's method, lactase dehydrogenase [LDH] by the Wroblewski-La Due method, total cholesterol [T-CHO] by the COD-DAOS method, triglycerides [TG] by the GPO-DAOS method, total protein by the Biuret method, blood urea nitrogen [BUN] by an enzymatic method, creatinine [CRE] by Jaffe's reaction, total bilirubin [T-BIL] by the azobilirubin method, glucose [GLU] by the glucose dehydrogenase method, Ca by the OCPC method and inorganic phosphorus [Pi] by the molybdenum blue method, using an autoanalyzer (AU 500, OLYMPUS).

5. Doses, group composition and reasons for dose setting

Doses and group composition are shown below.

Group	Test group	Dose	Label color	Male (Animal No.)	Female (Animal No.)
Group 1	Control (0.5% aqueous CMC-Na solution)	-	White	5 (011-015)	5 (061-065)
Group 2	Dunaliella Bardawil Spray Dried Powder	0.5 g/kg	Yellow	5 (111-115)	5 (161-165)
Group 3	Dunaliella Bardawil Spray Dried Powder	2.5 g/kg	Red	5 (211-215)	5 (261-265)

Doses for the study were decided together with the sponsor. The previous oral acute toxicity study at the dose of 10 g/40 mL/kg of DB[2)] showed no abnormal general signs, changes in weight or abnormal findings at the autopsy. However, it was difficult to administer the product continuously at this dose under nonfasting conditions. Therefore, for this study, 2.5 g/kg, was set as the high dose, and 0.5 g/kg as the low dose. 2.5 g/kg is the maximum dose for administration calculated based on 10 mL/kg, which is the dosing volume generally used for continuous administration. In addition, a control group administered the same volume of 0.5% aqueous CMC-Na solution as that used for the test substance was established.

6. Observation /test items

(1) General signs

The animals were checked twice a day (only once on the day of autopsy) for the presence of abnormal signs.

(2) Body weight

The animals were weighed twice a week.

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(3) Food intake

Food intake was measured once a week on two consecutive days, and daily food intake was calculated. Food intake was recorded on the day of measurement of residual feed.

(4) Urinalysis

Urine was collected using a urine collection cage, after completion of the dosing period under fasting conditions (including no water) for three hours (three-hour urine); and subsequently, under normal conditions of feeding and water supply for 21 hours (total 24-hour urine), for the following tests.

In the three-hour urine, color tone was evaluated by the naked eye. After dribbling of 200 μL of the obtained urine on the Ames Clinitek test paper (Miles-Sankyo), pH, occult blood, protein, glucose, ketone bodies, urobilinogen and bilirubin were tested, using Ames Urine Analyzer (CLINITEK SG, Miles-Sankyo). For sedimentation, epidermal cells, RBC and WBC, the urine was microscopically examined in 100 visual field at a 400-fold magnification, and for hemaglutination, blood casts and crystal the entire visual field at a 100-fold magnification was examined.

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Sodium [Na] and potassium [K] were measured by flame photometry, using a flame photometer (FLAME-30C, Nippon Bunko Medical).

Chloride [Cl] was measured by the coulometric titration method, using an autoanalyzer (Chloride meter C-200AP, JOKOH).

The protein fraction [albumin (ALB), and α1-, α2-, α3-, β-, γ-globulin] was measured by electrophoresis, using an automated electrophoresis unit (OLYMPUSAES600, OLYMPUS).

A/G(albumin/globulin) ratio and albumin [ALB] were calculated from total protein and the protein fraction.

(7) Autopsy

After blood was collected, the animals were sacrificed by exsanguination; an autopsy was conducted, organs and tissues were macroscopically observed, and findings were recorded. Representative cases of the sites where abnormalities were recognized were photographed, and fixed in 10% neutral buffered formalin.

(8) Organ weighing

The following organs (absolute weight) were weighed (paired organs were weighed in block). In addition, the ratio of the organ weight to the body weight before autopsy (relative weight) was calculated.

Heart, lungs (bronchi included), thymus, liver, spleen, kidneys, adrenal glands, testes for males and ovaries for females.

After being weighed, the organs were fixed in 10% neutral buffered formalin and stored.

(9) Histopathological examination

Liver, kidneys and adrenal glands were fixed in 10% neutral buffered formalin and paraffin-embedded, according to the common procedure, followed by preparation of hematoxylin-eosin staining for histopathological examination. As for the rest, the sites where abnormalities were recognized were also tested. In addition, representative cases were photographed.

7. Statistical methods

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For body weight, food intake, hematological examination, blood chemistry examination, organ weights (absolute weight and relative weight to the body weight), urine volume and urine specific gravity, the mean value and standard deviation were calculated for each group.

To test the significance of differences between each dose group and the control group, multiple comparison was used, considering less than 5% ($P<0.05$) of risk as a significant level. Practically, Bartlett's test for equality of variances was used, and when the value was homoscedastic, one-way analysis of variance was used, followed by intergroup comparison to the control by Dunnet's method if a significant difference was recognized (when the number of cases was the same). When the value was not homoscedastic, one-way rank-based ANOVA (Kruskal-Wallis test) was used, and if the value was significant, intergroup comparison with the control was used, employing Dunnet's test for rank-transformed data (when the number of cases was the same).

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Study schedule

The study schedule was as follows:

Date of arrival of animals:	February 9, 1989
Date of grouping:	February 21, 1989
Date of initial administration:	February 22, 1989
Date of completion of administration:	March 21, 1989
Date of autopsy:	March 22, 1989
Date of completion of sample observation:	June 30, 1989

No unexpected events that might have affected the reliability of the results were found during the implementation of the study.

Test results

1. Death and general signs during the study

No deaths or abnormal general signs were recognized either in males or females of any group, including the control group.

2. Body weight

Changes in body weight are shown in figure1 and Table 1 (Appendix 1-1 to 1-3) for males and figure 2 and Table 2 (Appendix 2-1 to 2-3) for females.

Changes in the body weight of males and females in each dosing group were similar to those of the control group, and showed no significant differences on any measuring day.

3. Food intake

Changes in food intake are shown in figure.3 and Table 3 (Appendix 3-1 to 3-3) for males and figure 4 and Table 4 (Appendix 4-1 to 4-3) for females.

In the 0.5 g/kg dose group, a significantly higher level of food consumption was found in males on Day 10 and in females on Day 17 compared with the control group. In the 2.5 g/kg dose group, changes in food intake both in males or females were similar to those of the control group, showing no significant differences on any measuring day.

4. Urinalysis

Among the results of urinalysis, urine volume and urine specific gravity are shown in Table 5 (Appendix 5-1 to 5-3) for males and Table 6 (Appendix 6-1 to 6-3) for females.

Both the urine volume and urine specific gravity of each of the male dose groups and the female 2.5 g/kg dose group were similar to those of the control groups, showing no

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significant differences. In the female 0.5 g/kg dose group, the level of gravity was similar to that of the control group, while urine volume was significantly higher than that of the control group.

As for other results of the urinalysis, color tone, pH, occult blood, protein, glucose, ketone bodies, urobilinogen and bilirubin are shown in Table 7 (Appendix: males; 7-1 to 7-3, females: 8-1 to 8-3), and sedimentation in Table 8 (Appendix: males; 9-1 to 9-3, females: 10-1 to 10-3).

The values of every parameter in males and females in each dose group were similar to those of the control group.

5. Ophthalmological examination

No abnormalities were recognized in the fundus of males and females in any group, including the control group.

6. Hematological examination

The results of the hematological examination are shown in Table 9-1 (Appendix 11-1 to 11-3) and Table 9-2 (Appendix 12-1 to 12-3) for males, and Table 10-1 (Appendix 13-1 to 13-3) and Table 10-2 (Appendix 14-1 to 14-3) for females.

White blood cells [WBC] count was significantly higher in males of the 0.5 g/kg dose group and females of the 2.5 g/g dose group, compared to that of the control groups. The values of other parameters in males and females in each group were similar to those of the control group, showing no significant differences.

7. Blood biochemistry examination

The results of blood biochemistry examination are shown in Table 11-1 (Appendix 15-1 to 15-3), Table 11-2 (Appendix 16-1 to 16-3), Table 11-3 (Appendix 17-1 to 17-3) and Table 11-4 (Appendix 18-1 to 18-3) for males; and in Table 12-1 (Appendix 19-1 to 19-3), Table 12-2 (Appendix 20-1 to 20-3), Table 12-3 (Appendix 21-1 to 21-3) and Table 12-4 (Appendix 22-1 to 22-3) for females.

The values of sodium [Na] and chloride [CL] were significantly lower in males of the 2.5 g/kg dose group than in those of the control group, but the difference was minimal. The values of other parameters were almost the same as those of the control in males and females of each dose group, showing no significant differences.

8. Autopsy

Table 13 shows the autopsy findings in males and females.

In the control group, thymus hemorrhage was found in one male and one female, and a unilateral white renal nodule was found in one female (No.065).

In the dose groups of DP, a yellowish-white nodule and partial thickening of the liver capsule and partial thickening of the spleen capsule were recognized in one female of the 2.5 g/kg dose group; thymus hemorrhage was found in one male of the 2.5 g/kg dose group; and splenic enlargement was noted in another male (N.215) of the 2.5 g/kg dose group.

9. Organ weights

The absolute and relative organ weights are shown in Table 14 (Appendix 23-1 to 23-3) and Table 15 (Appendix 24-1 to 24-3) for males; and in Table 16 (Appendix 25-1 to 25-3) and Table 17 (Appendix 26-1 to 26-3) for females.

The absolute weight of both males and females in each dose group was similar to that of the control group for any organ, showing no significant differences. On the other hand, the relative weight of the kidneys in one male of the 2.5/kg dose group was significantly higher than the control group.

10. Histopathological examination

The results of histopathological examination are shown in Table 18 (Appendix 27-1 to 27-3) for males and Table 19 (Appendix 28-1 to 28-3) for females.

In the control groups, infiltration of inflammatory cells was observed in the liver of one male and all females, and in the renal interstitium in one male and one female. Besides, thymus hemorrhage was found in one male and one female, but all of these findings were considered very mild or mild changes. Moreover, unilateral renal blastoma was observed in one female (No.065).

In the dose groups, infiltration of inflammatory cells was observed in the liver in one to three animals in each male or female dose group, a foreign-body granuloma was found in one female of the 0.5 g/kg dose group and thickening of the liver capsule and a granuloma were found in one male of the 2.5 g/kg dose group (No.264). Regarding the kidneys, infiltration of inflammatory cells in the interstium and a cyst were found in one male and one female, respectively, of the 2.5 g/kg dose group. Besides, a basophilic change of the tubular epithelium and a fibrotic focus were noted in one male and one female, respectively, of the 0.5 g/kg dose group. All these abnormalities were mild or very mild. As for the rest, thymus hemorrhage was recognized in one male of the 2.5 g/kg dose group, extramedullary hematopoiesis (No.215) and thickening of the spleen capsule (No.264) in one male and a female of the 2.5 g/kg dose group.

Conclusions

The toxicity of Dunaliella Bardawil Spray Dried Powder was examined by a 28-day oral administration in Sprague-Dawley Rats [Crj: CD(SD)], and the following results obtained.

Concerning general signs in males and females of each dose group, the changes in body weight were almost the same as those observed in the control groups. The transient increase in food consumption observed in males and females of the 0.5 g/kg dose group in the middle of the observation period, was not dose-dependent.

The significant differences observed between males and females in each dose group and the control group regarding some parameters of urinalysis, hematological examination and blood chemistry were not dose-dependent and not necessarily common to males and females. Thus, they were not considered not to be a consequence of the administration of DB.

The thymus hemorrhage found in one male of the 2.5 g/kg dose group at the autopsy, was also recognized in the control group; thus, it was not considered an effect of DB. Moreover, the splenic enlargement found in one male (N.215) and the yellowish-white

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hepatic nodule and thickening of the liver and spleen capsules seen one female (No.264) of the 2.5 g/kg dose group, were assumed to be accidental changes.

The absolute weight of every organ in males and females of each dose group was similar to the weight of that organ in the control group. On the other hand, a significant difference in the relative weight of the kidneys was found in one male of the 2.5 g/kg dose group, but since this change was not common to males and females and there were no differences in the absolute weight, this change was not considered to be caused by DB.

The histopathological examination showed thymus hemorrhage and infiltration of inflammatory cells in the hepatic and renal interstitium, which were also observed in the control groups. As for the rest, a foreign-body granuloma was recognized in the liver, a basophilic of tubular epithelium, cyst and fibrotic focus were found in the kidneys, but all these abnormal findings were recognized only in one case each and were not dose-dependent; thus, they were not considered to have been caused by DB. In addition, the autopsy showed thickening of the liver capsule and granuloma in one female with a yellowish-white nodule/thickening of the liver capsule and thickening of the spleen capsule (No.264), and extramedullar hematopoiesis in one male with splenic enlargement (N.215). All these changes were consistent with the results of the macroscopic observation at the autopsy.

The white nodule recognized at the autopsy in the kidney in one female of the control group (No.065) was a renal blastoma.

These results indicated that Dunaliella Bardawil Spray-Dried Powder did not induce toxicity in male nor in female rats. Therefore, the 2.5 g/kg dose level of Dunaliella Bardawil Spray-Dried Powder, which is considered to be almost the maximum dose for continuous administration under the conditions of the study, was assumed to be the no-effect level.

References

1. Data on features and safety of the test substance provided by NIKKEN SOHONSHA
2. Fumihiko Ariga *et al.*: Study No.4087 "Acute toxicity study by oral administration of Dunaliella Bardawil Spray-Dried Powder in rats", Nihon Bioresearch Center, Hashima Laboratory, August 25, 1987 to December 7, 1987

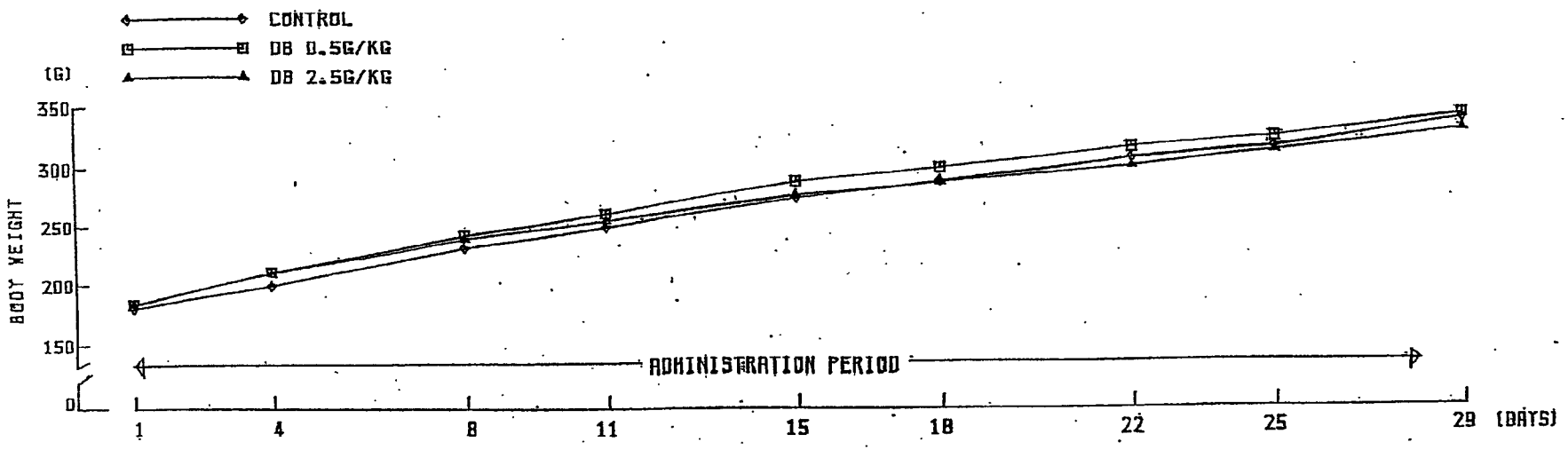

Fig. 1 Body weights of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

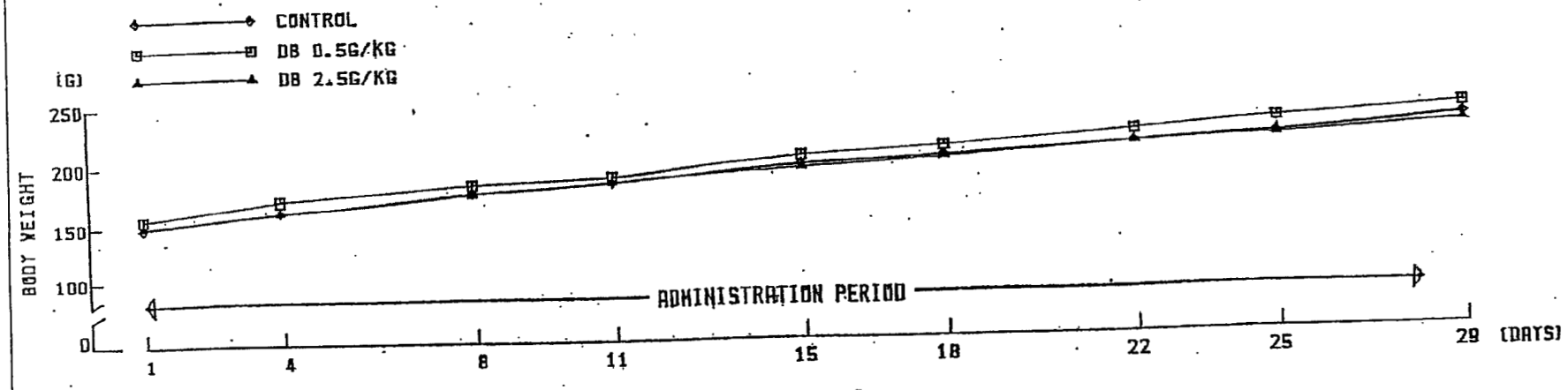

Fig. 2 Body weights of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

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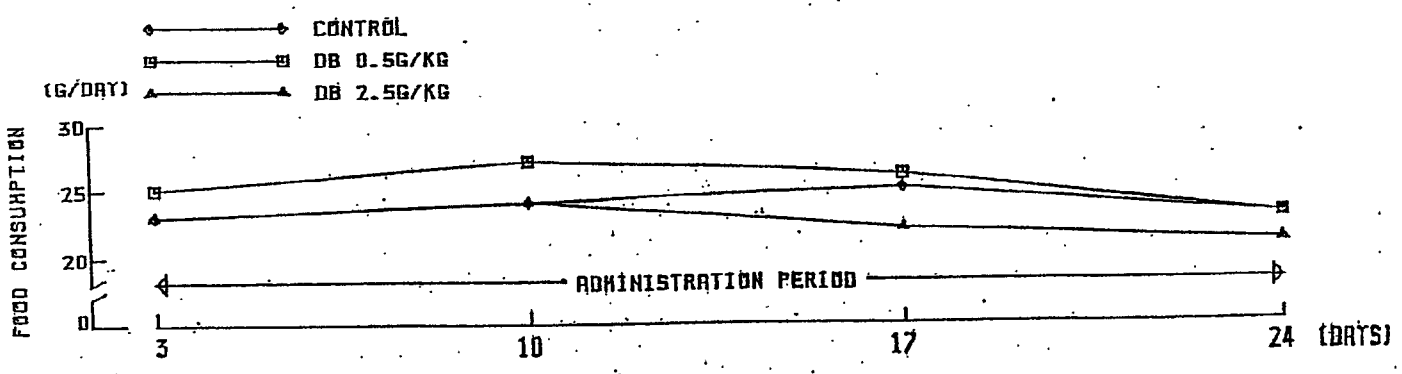

Fig. 3 Food consumptions by male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

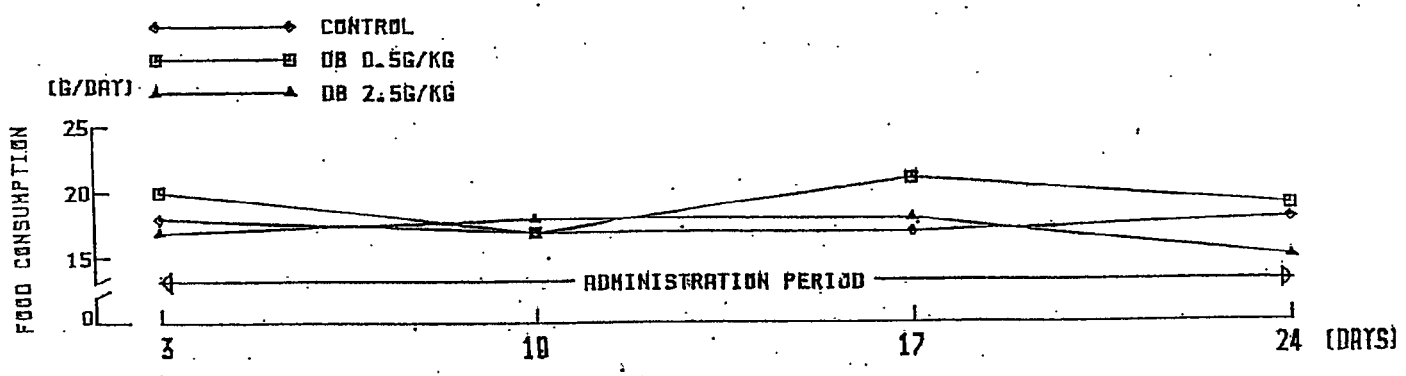

Fig. 4 Food consumptions by female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

Table 1 Body weights of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

GROUP	1	4	8	11	15	18	22	25	29 (DAYS)
CONTROL									
MEAN	180	199	230	248	273	287	305	314	337
S.D.	7.1	7.3	14.2	15.3	20.2	22.8	24.6	23.7	28.0
(N)	5	5	5	5	5	5	5	5	5
DB 0.5G/KG JUDGE									
MEAN	184	210	241	260	287	298	314	322	341
S.D.	6.2	8.2	8.8	7.9	5.1	7.0	8.7	8.5	12.5
(N)	5	5	5	5	5	5	5	5	5
DB 2.5G/KG JUDGE									
MEAN	184	209	238	254	276	286	299	310	328
S.D.	5.8	5.9	4.0	8.3	10.4	13.5	17.9	19.0	25.2
(N)	5	5	5	5	5	5	5	5	5

UNIT : G

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Table 2 Body weights of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

GROUP	1	4	8	11	15	18	22	25	29 (DAYS)
CONTROL									
MEAN	149	161	174	180	194	199	208	213	226
S.D.	3.7	8.8	9.8	11.7	12.0	12.8	12.5	16.4	18.0
(N)	5	5	5	5	5	5	5	5	5
DB 0.5G/KG JUDGE									
MEAN	157	170	180	186	201	207	218	226	236
S.D.	8.2	12.3	12.6	10.7	8.8	9.7	10.8	14.1	15.5
(N)	5	5	5	5	5	5	5	5	5
DB 2.5G/KG JUDGE									
MEAN	151	161	173	181	191	197	207	211	220
S.D.	7.6	11.3	11.2	16.1	17.0	15.3	16.7	16.3	18.5
(N)	5	5	5	5	5	5	5	5	5

UNIT : G

Table 3 Food consumptions by male rats treated orally with Duhallella Bardawil Spray Dried Powder for 28 days

GROUP	3	10	17	24 (DAYS)
CONTROL				
MEAN	23	24	25	23
S.D.	1.1	2.5	3.1	2.6
(N)	5	5	5	5
DB 0.5G/KG				
JUDGE		*		
MEAN	25	27	26	23
S.D.	2.3	1.7	2.2	1.6
(N)	5	5	5	5
DB 2.5G/KG				
JUDGE				
MEAN	23	24	22	21
S.D.	1.6	0.7	1.5	1.9
(N)	5	5	5	5

* : SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP ($P<0.05$)
UNIT : G/DAY

Table 4 Food consumptions by female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

GROUP	3	10	17	24 (DAYS)
CONTROL				
MEAN	18	17	17	18
S.D.	2.3	2.9	2.3	1.5
(N)	5	5	5	5
DB 0.5G/KG				
JUDGE			*	
MEAN	20	17	21	19
S.D.	2.6	1.5	1.6	2.2
(N)	5	5	5	5
DB 2.5G/KG				
JUDGE				
MEAN	17	18	18	15
S.D.	1.5	0.9	1.5	1.9
(N)	5	5	5	5

* : SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP ($P<0.05$)
UNIT : G/DAY

000192

Table 5 Urinalyses of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days --- (i)

GROUP		UV g	S.G.
CONTROL	MEAN	14.2	1.052
	S.D.	3.20	0.0099
	(N)	5	5
DB 0.5G/KG			
	JUDGE MEAN	14.6	1.052
	S.D.	5.36	0.0081
	(N)	5	5
DB 2.5G/KG			
	JUDGE MEAN	15.3	1.052
	S.D.	3.76	0.0108
	(N)	5	5

000193

Table 6 Urinalyses of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days -- (I)

GROUP		UV	S.G.
		0	
CONTROL			
	MEAN	7.3	1.061
	S.D.	1.62	0.0151
	(N)	5	5
DB 0.5G/KG			
	JUDGE	##	
	MEAN	12.4	1.054
	S.D.	2.43	0.0087
	(N)	5	5
DB 2.5G/KG			
	JUDGE		
	MEAN	10.4	1.050
	S.D.	2.80	0.0168
	(N)	5	5

: SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP (P<0.01)

Table 7 Urinalyses of male and female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (±)

Sex	Group	N[a]	Color	pH					Protein (mg/dl)					Glucose (g/dl)					Ketone body (mg/dl)					Bilirubin				Occult blood					Urobilinogen (E.U)				
				5	6	7	8	9	陰性	±trace	30	100	≧300	陰性	±trace	0.25	0.5	≧1	陰性	±trace	16	40	≧80	陰性	+	±	++	陰性	±trace	+	±	++	0.1	1	2	4	8
Male	Control	5	Light yellow			2	3			3	2			5					2	2	1			5				5					3	2			
	DB 0.5 g/kg	5	Light yellow				5			3	1	1		5						2	3			4	1			5					3	2			
	DB 2.5 g/kg	5	Light yellow			2	3		2	3				5						4	1			5				5					4	1			
Female	Control	5	Light yellow				4	1	1	1	2	1		5					2	3				2	3			5					2	3			
	DB 0.5 g/kg	5	Light Yellow			2	3		3	1	1			5					4	1				4	1			5					3	2			
	DB 2.5 g/kg	5	Light yellow			1	4		1	4				5					4	1				3	2			5					2	3			

a) Number of samples tested.
E.U ; Ehrlich Unit

000195

Table 8 Urinalyses of male and female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days··· (m)

Sex	Finding		Epithelial cells					Erythrocytes (RBC)					Leukocytes (WBC)					Blood coa.		Casts		Crys-tals	
	Group	N a)	0	20 ~ 21	100 ~ 101	200 ~ 201	V Coagulation	0	20 ~ 21	100 ~ 101	200 ~ 201	500 ~ 501 V	0	20 ~ 21	100 ~ 101	200 ~ 201	500 ~ 501 V	−	+	−	+	−	+
Male	Control	5	5					5					5					5		5		5	
	DB 0.5 g/kg	5	5					5					5					5		5		4	1
	DB 2.5 g/kg	5	5					5					5					5		5		4	1
Female	Control	5	5					5					4	1				5		5		5	
	DB 0.5 g/kg	5	5					5					5					5		5		4	1
	DB 2.5 g/kg	5	5					5					4	1				5		5		4	1

coa. : coagulation

a) Number of samples tested.

Table 9 - 1 Hematological findings of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days··· (i)

GROUP		RBC $10^4/mm^3$	HGB g/dl	HCT %	PLT $10^3/mm^3$	RET %	PT Sec.
CONTROL							
	MEAN	801	14.2	46.6	88.2	30	14.1
	S.D.	35.5	0.35	1.15	10.48	6.7	0.33
	(N)	5	5	5	5	5	5
DB 0.5G/KG							
	JUDGE						
	MEAN	811	14.4	46.7	95.5	26	14.0
	S.D.	36.4	0.49	1.49	16.31	4.7	0.16
	(N)	5	5	5	5	5	5
DB 2.5G/KG							
	JUDGE						
	MEAN	786	14.2	46.3	86.5	27	14.1
	S.D.	37.2	0.62	1.70	21.49	4.0	0.25
	(N)	5	5	5	5	5	5

000197

000197

Table 9 - 2 Hematological findings of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (II)

GROUP		RBC $10^3/mm^3$	L %	N %	E %	B %	M %
CONTROL	MEAN	55	87	12	0	0	0
	S.D.	8.1	5.0	5.5	0.9	0.0	0.9
	(N)	5	5	5	5	5	5
DB 0.5G/KG	JUDGE	*					
	MEAN	92	94	6	0	0	0
	S.D.	21.7	3.1	3.0	0.0	0.0	0.5
	(N)	5	5	5	5	5	5
DB 2.5G/KG	JUDGE						
	MEAN	81	93	7	0	0	0
	S.D.	27.2	3.8	3.8	0.9	0.0	0.4
	(N)	5	5	5	5	5	5

* : SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP ($P<0.05$)

000198

Table 10 - 1 Hematological findings of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days··· (I)

GROUP		RBC $10^4/mm^3$	HGB g/dl	HCT %	PLT $10^4/mm^3$	RET %	PT Sec.
CONTROL	MEAN	754	13.7	44.5	98.6	31	14.0
	S.D.	38.8	0.45	1.66	11.26	6.5	0.22
	(N)	5	5	5	5	5	5
DB 0.5G/KG	JUDGE MEAN	735	13.3	43.5	106.2	26	14.1
	S.D.	39.0	0.52	1.23	21.53	4.3	0.33
	(N)	5	5	5	5	5	5
DB 2.5G/KG	JUDGE MEAN	749	13.3	43.2	109.3	24	13.9
	S.D.	32.3	0.61	2.16	15.46	6.6	0.35
	(N)	5	5	5	5	5	5

Table 10 - 2 Hematological findings of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (I)

GROUP		RBC $10^4/\mu l$	L %	N %	E %	B %	M %
CONTROL	MEAN	42	88	11	0	0	1
	S.D.	10.9	5.7	5.9	0.0	0.0	1.1
	(N)	5	5	5	5	5	5
DB 0.5G/KG	JUDGE						
	MEAN	44	89	9	1	0	0
	S.D.	6.4	3.8	3.4	1.2	0.0	0.5
	(N)	5	5	5	5	5	5
DB 2.5G/KG	JUDGE	*					
	MEAN	59	89	9	0	0	1
	S.D.	9.0	4.4	4.7	0.5	0.0	0.0
	(N)	5	5	5	5	5	5

* : SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP (P<0.05)

Table 11 - 1 Blood biochemical analyses of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days··· (I)

GROUP		GOT IU/ℓ	GPT IU/ℓ	ALP IU/ℓ	LDH IU/ℓ	CPK IU/ℓ
CONTROL	MEAN	80.8	34.1	256.2	1361.7	389.2
	S.D.	22.50	9.11	32.86	542.50	140.74
	(N)	5	5	5	5	5
DB 0.5G/KG	JUDGE MEAN	74.6	34.0	295.0	1080.6	377.7
	S.D.	4.98	7.24	33.97	309.04	66.16
	(N)	5	5	5	5	5
DB 2.5G/KG	JUDGE MEAN	78.8	36.9	238.0	1126.5	349.4
	S.D.	12.72	4.22	25.54	674.88	139.81
	(N)	5	5	5	5	5

Table 11 - 2 Blood biochemical analyses of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (II)

GROUP		TP g/dl	ALB g/dl	A/G	T-BIL mg/dl	BUN mg/dl	CRE mg/dl	GLU mg/dl	T-CHO mg/dl	TG mg/dl
CONTROL	MEAN	5.4	3.24	1.49	0.05	17.9	0.42	153.9	52.2	92.2
	S.D.	0.21	0.154	0.202	0.007	0.85	0.022	7.93	4.64	28.49
	(N)	5	5	5	5	5	5	5	5	5
DB 0.5G/KG	JUDGE									
	MEAN	5.6	3.21	1.36	0.05	19.2	0.45	165.9	55.9	113.8
	S.D.	0.14	0.196	0.214	0.019	1.04	0.011	7.61	5.34	33.25
	(N)	5	5	5	5	5	5	5	5	5
DB 2.5G/KG	JUDGE									
	MEAN	5.4	2.96	1.21	0.05	18.9	0.43	159.7	51.9	121.0
	S.D.	0.25	0.186	0.087	0.004	1.25	0.015	8.91	6.62	42.55
	(N)	5	5	5	5	5	5	5	5	5

000202

Table 11 - 3 Blood biochemical analyses of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days··· (m)

GROUP		Na mEq/l	K mEq/l	Cl mEq/l	Ca mg/dl	Pi mg/dl
CONTROL	MEAN	142.3	4.43	107.3	10.2	7.6
	S.D.	0.57	0.239	1.12	0.19	0.82
	(N)	5	5	5	5	5
DB 0.5G/KG	JUDGE					
	MEAN	140.9	4.44	106.2	10.4	7.9
	S.D.	0.56	0.246	1.24	0.23	0.74
	(N)	5	5	5	5	5
DB 2.5G/KG	JUDGE	**		*		
	MEAN	139.9	4.72	105.1	10.2	7.2
	S.D.	1.44	0.087	1.13	0.19	0.69
	(N)	5	5	5	5	5

* : SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP (P<0.05)
** : SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP (P<0.01)

000203

Table 11 - 4 Blood biochemical analyses of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (II)

GROUP		alb %	α-1 %	α-2 %	α-3 %	β %	γ %
CONTROL	MEAN	59.7	16.3	5.5	6.0	10.8	1.7
	S.D.	3.23	2.99	0.82	0.39	0.73	0.33
	(N)	5	5	5	5	5	5
DB 0.5G/KG	JUDGE MEAN	57.4	17.0	6.1	5.9	11.4	2.1
	S.D.	3.72	3.46	0.43	0.30	0.66	0.38
	(N)	5	5	5	5	5	5
DB 2.5G/KG	JUDGE MEAN	54.9	20.1	6.3	6.4	10.4	1.9
	S.D.	1.73	1.26	0.52	0.76	0.80	0.57
	(N)	5	5	5	5	5	5

Table 12 - 1 Blood biochemical analyses of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (1)

GROUP		GOT IU/ℓ	GPT IU/ℓ	ALP IU/ℓ	LDH IU/ℓ	CPK IU/ℓ
CONTROL	MEAN	59.7	25.9	152.0	826.1	271.1
	S.D.	8.13	3.12	30.59	278.92	69.51
	(N)	5	5	5	5	5
DB 0.5G/KG	JUDGE MEAN	58.1	28.8	162.2	691.2	231.0
	S.D.	1.88	2.62	19.59	267.36	49.40
	(N)	5	5	5	5	5
DB 2.5G/KG	JUDGE MEAN	63.8	29.1	161.4	810.8	269.6
	S.D.	13.55	7.92	30.49	387.77	110.41
	(N)	5	5	5	5	5

Table 12 - 2 Blood biochemical analyses of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (1)

GROUP		TP g/dl	ALB g/dl	A/G	T-BIL mg/dl	BUN mg/dl	CRE mg/dl	GLU mg/dl	T-CHO mg/dl	TG mg/dl
CONTROL	MEAN	5.9	3.76	1.74	0.06	17.9	0.43	161.2	68.5	57.8
	S.D.	0.31	0.198	0.072	0.013	3.63	0.025	10.75	3.73	9.65
	(N)	5	5	5	5	5	5	5	5	5
DB 0.5G/KG	JUDGE									
	MEAN	6.1	3.74	1.61	0.05	18.8	0.43	166.7	69.6	66.8
	S.D.	0.33	0.346	0.240	0.008	2.84	0.041	11.94	10.94	31.06
	(N)	5	5	5	5	5	5	5	5	5
DB 2.5G/KG	JUDGE									
	MEAN	5.9	3.75	1.72	0.05	20.2	0.42	159.9	63.4	81.4
	S.D.	0.37	0.245	0.103	0.004	3.25	0.019	10.01	14.15	16.18
	(N)	5	5	5	5	5	5	5	5	5

000206

Table 12 - 3 Blood biochemical analyses of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (M)

GROUP		Na mEq/l	K mEq/l	Cl mEq/l	Ca mg/dl	Pi mg/dl
CONTROL	MEAN	140.5	3.98	107.8	10.5	7.5
	S.D.	1.93	0.142	1.60	0.36	0.68
	(N)	5	5	5	5	5
DB 0.5G/KG	JUDGE MEAN	139.7	4.12	106.5	10.6	7.1
	S.D.	1.34	0.135	1.94	0.43	0.52
	(N)	5	5	5	5	5
DB 2.5G/KG	JUDGE MEAN	139.2	4.32	106.9	10.8	6.7
	S.D.	2.27	0.287	1.08	0.39	0.80
	(N)	5	5	5	5	5

000207

Table 12 - 4 Blood biochemical analyses of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (Ⅳ)

GROUP		alb %	α-1 %	α-2 %	α-3 %	β %	γ %
CONTROL	MEAN	63.6	14.3	4.2	5.5	10.3	2.1
	S.D.	1.02	1.54	0.72	0.63	0.70	0.67
	(N)	5	5	5	5	5	5
DB 0.5G/KG	JUDGE MEAN	61.6	15.3	3.9	6.0	10.7	2.6
	S.D.	3.77	3.11	0.40	0.70	0.69	0.66
	(N)	5	5	5	5	5	5
DB 2.5G/KG	JUDGE MEAN	63.3	13.7	4.5	5.5	10.5	2.4
	S.D.	1.50	1.24	1.40	0.15	0.68	1.27
	(N)	5	5	5	5	5	5

802000
000208

Table 13 Necropsy findings of male and female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days.

Findings	Group	Male			Female		
		Control	DB		Control	DB	
			0.5 g/kg	2.5 g/kg		0.5 g/kg	2.5 g/kg
	Number of animals	5	5	5	5	5	5
Liver							
Yellowish white nodule		0	0	0	0	0	1 (264)
Thickening of capsule (partially)		0	0	0	0	0	1 (264)
Thymus							
Hemorrhage		1 (013)	0	1 (211)	1 (062)	0	0
Spleen							
Swelling		0	0	1 (215)	0	0	0
Thickening of capsule (partially)		0	0	0	0	0	1 (264)
Kidney							
White nodule (left)		0	0	0	1 (065)	0	0

( ) : Animal number

0002009

Table 14 Absolute organ weights of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

GROUP	BODY WEIGHT (G)	THYMUS (G)	LUNGS (G)	HEART (G)	LIVER (G)	SPLEEN (G)	KIDNEYS (G)	ADRENALS (MG)	TESTES (G)
CONTROL									
MEAN	337	0.45	1.11	1.05	12.59	0.66	2.31	46.6	2.97
S.D.	26.0	0.097	0.075	0.119	1.283	0.034	0.249	5.54	0.175
(N)	5	5	5	5	5	5	5	5	5
DB 0.5G/KG									
JUDGE									
MEAN	341	0.52	1.13	1.06	12.94	0.65	2.38	48.2	2.77
S.D.	12.5	0.088	0.079	0.039	1.041	0.038	0.070	3.11	0.145
(N)	5	5	5	5	5	5	5	5	5
DB 2.5G/KG									
JUDGE									
MEAN	328	0.38	1.13	1.07	12.07	0.80	2.46	48.8	2.87
S.D.	25.2	0.042	0.100	0.077	1.090	0.425	0.211	7.95	0.205
(N)	5	5	5	5	5	5	5	5	5

000210

Table 15 Relative organ weights of male rats treated orally with Dunaliella Bardawil Spray-Dried Powder for 28 days

GROUP	BODY WEIGHT (G)	ORGAN WEIGHT (G OR MG) PER 100G OF BODY WEIGHT							
		THYMUS G(%)	LUNGS G(%)	HEART G(%)	LIVER G(%)	SPLEEN G(%)	KIDNEYS G(%)	ADRENALS MG(%)	TESTES G(%)
CONTROL									
MEAN	337	0.13	0.33	0.31	3.73	0.20	0.68	13.8	0.88
S.D.	26.0	0.032	0.016	0.020	0.105	0.017	0.027	0.68	0.090
(N)	5	5	5	5	5	5	5	5	5
DB 0.5G/KG JUDGE									
MEAN	341	0.15	0.33	0.31	3.79	0.19	0.70	14.1	0.81
S.D.	12.5	0.023	0.022	0.017	0.194	0.007	0.026	0.44	0.026
(N)	5	5	5	5	5	5	5	5	5
DB 2.5G/KG JUDGE									
MEAN	328	0.12	0.34	0.33	3.68	0.24	0.75**	14.8	0.88
S.D.	25.2	0.018	0.027	0.022	0.219	0.114	0.030	1.62	0.069
(N)	5	5	5	5	5	5	5	5	5

** : SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP (P<0.01)

Table 16 Absolute organ weights of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

GROUP	BODY WEIGHT (G)	THYMUS (G)	LUNGS (G)	HEART (G)	LIVER (G)	SPLEEN (G)	KIDNEYS (G)	ADRENALS (MG)	OVARIES (MG)
CONTROL									
MEAN	226	0.52	0.97	0.86	8.89	0.49	1.80	59.8	88.2
S.D.	16.0	0.108	0.073	0.093	0.972	0.067	0.205	5.40	14.37
(N)	5	5	5	5	5	5	5	5	5
DB 0.5G/KG									
JUDGE MEAN	236	0.48	1.00	0.88	9.81	0.58	1.85	63.5	94.0
S.D.	15.5	0.046	0.042	0.097	1.328	0.128	0.123	4.53	20.49
(N)	5	5	5	5	5	5	5	5	5
DB 2.5G/KG									
JUDGE MEAN	220	0.51	0.94	0.82	9.06	0.52	1.75	59.7	88.8
S.D.	18.5	0.075	0.087	0.130	1.227	0.115	0.207	11.75	16.24
(N)	5	5	5	5	5	5	5	5	5

Table 17 Relative organ weights of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

GROUP	BODY WEIGHT (G)	ORGAN WEIGHT (G OR MG) PER 100G OF BODY WEIGHT							
		THYMUS G(%)	LUNGS G(%)	HEART G(%)	LIVER G(%)	SPLEEN G(%)	KIDNEYS G(%)	ADRENALS MG(%)	OVARIES MG(%)
CONTROL									
MEAN	226	0.23	0.43	0.38	3.93	0.22	0.79	26.5	39.0
S.D.	16.0	0.040	0.027	0.030	0.178	0.019	0.045	1.62	5.14
(N)	5	5	5	5	5	5	5	5	5
DB 0.5G/KG JUDGE									
MEAN	236	0.20	0.43	0.37	4.15	0.25	0.78	27.0	39.9
S.D.	15.5	0.013	0.029	0.034	0.341	0.052	0.011	0.22	7.90
(N)	5	5	5	5	5	5	5	5	5
DB 2.5G/KG JUDGE									
MEAN	220	0.23	0.43	0.37	4.10	0.24	0.80	27.0	40.3
S.D.	18.5	0.029	0.042	0.031	0.230	0.053	0.063	3.73	6.15
(N)	5	5	5	5	5	5	5	5	5

Table 18 Histopathological findings of male rats treated orally with Dunaliella Bardawil spray Dried Powder for 28 days

Findings	Group	control					DB 0.5g/kg					DB 2.5g/kg				
	Number of animals	5					5					5				
	Grade	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++
Liver																
Cellular infiltration		4	1	0	0	0	3	2	0	0	0	4	0	1	0	0
Kidney																
Cellular infiltration(interstitial)		4	1	0	0	0	5	0	0	0	0	4	1	0	0	0
Basophilic change of renal tubules		5	0	0	0	0	4	1	0	0	0	5	0	0	0	0
Cyst		5	0	0	0	0	5	0	0	0	0	4	0	1	0	0
Thymus*																
Hemorrhage		0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
Spleen*																
Extramedullary hematopoiesis		0	0	0	0	0	0	0	0	0	0	0	0	1	0	0

−: No remarkable chenges ±: Minimal +: Slight ++: Moderate +++: Marked

* : Abnomal findings were observed at necropsy

No remarkable changes in Adrenal gland

Table 19 Histopathological findings of female rats treated orally with Dunaliella Bardawil spray Dried Powder for 28 days

Findings / Group	control					DB 0.5g/kg					DB 2.5g/kg				
Number of animals	5					5					5				
Grade	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++
Liver															
Cellular infiltration	0	2	3	0	0	3	2	0	0	0	2	3	0	0	0
Foreign body granuloma	5	0	0	0	0	4	0	1	0	0	5	0	0	0	0
Thickening of capsule	5	0	0	0	0	5	0	0	0	0	4	0	1	0	0
Granuloma	5	0	0	0	0	5	0	0	0	0	4	0	1	0	0
Kidney															
Cellular infiltration(interstitial)	4	1	0	0	0	5	0	0	0	0	4	1	0	0	0
Cyst	5	0	0	0	0	5	0	0	0	0	4	0	1	0	0
Focal fibrosis	5	0	0	0	0	4	0	1	0	0	5	0	0	0	0
Nephroblastoma#	4	−	1	−	−	5	−	0	−	−	5	−	0	−	−
Thymus*															
Hemorrhage	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Spleen*															
Thickening of capsule	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0

−: No remarkable changes ±: Minimal +: Slight ++: Moderate +++: Marked
* : Abnomal findings were observed at necropsy
No remarkable changes in Adrenal gland
: This finding is indicated by only + or −

Protocol

Title

28-day oral administration subacute toxicity study of Dunaliella Bardawil Spray Dried Powder in rats

Sponsor

Nikken Sohonsha Corporation
1-32 Asahira, Fukuju-cho, Hashima-shi, Gifu-prefecture

Testing facility

Hashima Institute, Nihon Bioresearch Center Co.
6-104, Majima, Fukuju-cho, Hashima-shi, Gifu-prefecture

Study objective

As part of the safety evaluation of Dunaliella Bardawil Spray Dried Powder, the safety of 28-day oral administration of Dunaliella Bardawil Spray Dried Powder is investigated using rats.

Participating persons

Study Director:	Tadakazu Furuhashi
Person responsible for the study:	Kenji Uto

Schedule

Date of start of study (Date of adding signature and seal by Facility Management): January 5, 1989

Date of animal delivery:	February 9, 1989
Grouping:	February 21, 1989
Start of administration:	February 22, 1989
End of administration:	March 21, 1989
Animal autopsy:	March 22, 1989
End of sample observation:	July 31, 1989
Submission of a report (draft):	September 30, 1989
End of the study (submission of a final report):	October 30, 1989

Signature/sealing

Study Director

Tadakazu Furuhashi Seal January 5, 1989
Hashima Institute, Nihon Bioresearch Center Co.

Approval of protocol

Study sponsor

Hiroyuki Takenaka Seal January 17, 1989
Nikken Sohonsha Corporation

Facility Management

Kenichi Nakagawa Seal January 5, 1989
Hashima Institute, Nihon Bioresearch Center Co.

1. Test article

(1) Test article

Name: Dunaliella Bardawil Spray Dried Powder (DB)

Lot number: NSK-1207

Description: reddish brown water-insoluble powder

Stability: The bulk powder is stable for at least one year after nitrogen replacement when stored in a tightly closed container, as well as for at least two months when stored in a refrigerator protected from light or in a freezer. Also, the bulk powder is stable for up to five hours when diluted or dissolved at concentrations of 5% and 25% in a 0.5% carboxymethyl cellulose sodium aqueous solution and stored in a refrigerator (approximately 5°C) protected from light

Date of reception: February 2, 1989

Amount received: 416.0 g

Storage place: test article storeroom in the study facility

Storage conditions: stored in a freezer (approximately -20°C) protected from light

Supplier: Nikken Sohonsha Corporation

(2) Return of unused test article

After the completion of administration, the unused test article is returned to the study sponsor with a copy of the usage record.

2. Preparation of test solution

The test article is dissolved or diluted at the specific concentration in necessary amounts of 0.5% carboxymethyl cellulose sodium aqueous solution (CMC-Na: lot number; 109E5046, manufacturer; Kanto Kagaku Kogyo, distilled water for injection: lot number; 8F82N, manufacturer: Otsuka Pharmaceutical Co., Ltd.). The test solution should be used within five hours of preparation and the unused test solution is discarded.

3. Animals

(1) Species, strain and reasons for selection

1) Species/strain: rat (SPF), Crj: CD (SD)

2) Reasons for selection: Rats are widely used in toxicity studies. Rats used in the present study have a clear strain history, and data in rats have been accumulated.

(2) Age (week) upon arrival
Four weeks

(3) Arrival date, sex and quantity
21 males and 21 females on February 9, 1989

(4) Body weight range on the day following arrival
Males: 55-105 g
Females: 55-105 g

(5) Supplier
Charles River Laboratories Japan, Inc.

(6) Quarantine and acclimatization
For quarantine and acclimatization, rats are subjected to a two-week preliminary breeding period, during which their weights are measured at least twice and their appearance and general conditions inspected.

(7) Grouping
Rats, aged approximately six weeks, which do not show any abnormality during preliminary breeding period in weight changes, appearance or general conditions are divided into specific groups using a random sampling method after being weighed one day before the start of administration, so that average weights and their dispersions are comparable between the groups.
Unused rats are killed by anesthetics on the day of first administration.

(8) Individual identification method
Rats are identified by staining fur and putting marks on their tails by oil-based ink during the preliminary breeding period, as well as by staining fur and ear-punching after grouping. During the preliminary breeding period, labels bearing study number, arrival date and acclimatization animal number are attached to each cage, and after grouping, labels in different colors bearing study number, test article name, dose and animal number to each cage.

(9) Environmental conditions
Rats are fed in the animal room (Room 4, Building C) under the following conditions – room temperature: 20-24°C, humidity: 40-70%, light-dark cycle: 12:12 hours (fluorescent lighting

6:00-18:00), and ventilation: 12 times/hour (air sterilized by filtering).

For animal cages, during the preliminary breeding period, plastic cages (300 x 355 x 175 mm) with bedding (chip, Chiba Animal Shizai) are used. Up to five rats are housed in one cage. After grouping, rats are individually housed in five-compartment stainless steel cages (755 x 210 x 170 mm). The plastic cage, plate in the stainless steel cage and water bottle are changed at least twice a week. The stainless steel cage (with a feeder) is changed at least once every two weeks.

(10) Food

Rats have free access to the pallet food (CRF-1, Oriental Yeast Co., Ltd.), which was purchased in the last three months. For food contamination, the food used during a given period is analyzed on a lot basis by the Japan Food Research Laboratories, and results are provided by the food manufacturer.

(11) Drinking water

Rats have free access to tap water from polycarbonate water supply bottles. For the quality of water, the results of an analysis by the Gifu Prefectural Public Health Inspection Center are provided every three months.

(12) Bedding

For bedding material contamination, the results of an analysis conducted once a year by the Tokyo Kenbikyoin Foundation are provided.

4. Dosage/administration

(1) Administration route and reasons for selection

DB is food, so the sub-acute toxicity of its oral administration is investigated using rats.

(2) Administration method and volume of test solution

The test solution, the volume of which is calculated from the body weight measured on the day closest to the administration day and the specific volume of 10 ml/kg, is administered by gavage using a metal stomach tube.

(3) Dose frequency and administration period

The test solution is administered once a day every day for 28 days (basically between 8:00am and 12:00am).

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(4) Dose and group

Group	Study product	Dose	Label color	Male (animal number)	Female (animal number)
Group 1	Control(0.5% CMC-Na aqueous solution)	-	White	5(001-015)	5(061-065)
Group 2	DB	0.5g/kg	Yellow	5(111-115)	5(161-165)
Group 3	DB	2.5g/kg	Red	5(211-215)	5(261-265)

(5) Reasons for dose setting

The dose was determined at a discussion with the study sponsor referring to the results of previous acute toxicity study with DB in mice (fasting). No abnormalities were observed in general conditions, body weight changes or autopsy findings even when a dose of 10 g/40 ml/kg, thought to be the maximum usable dose, was administered to mice. However, continuous non-fasting administration under the conditions above is considered to be difficult. By converting the maximum dose above according to a common rate for solution volumes of 10 ml/kg, the dose in the present study is set at 2.5 g/kg for the high dose group and 0.5 g/kg for the low dose group. In the control group, the same volume of a 0.5% CMC-Na aqueous solution as the test solution is used.

5. Observation/examination items

(1) General conditions

General conditions and deaths are checked twice a day during the study period (once on the autopsy day). Autopsy is conducted for macroscopic investigation of organs and tissues of dead animals as soon as possible after death. As upon autopsy of living animals, histopathological examinations should be conducted, if possible. For animals likely to be close to death (dying animals), autopsy is conducted immediately after blood sampling, and as upon autopsy of living animals, hematological examinations, blood biochemical examinations, organ weight measurements and histopathological examinations should be conducted, if possible.

(2) Body weight measurement

Twice a week

(3) Food consumption measurement

Once a week (Daily consumption is calculated by dividing the consumption for two consecutive days by two.)

(4) Urinalysis

Before the completion of administration, rats are transferred to a urine sampling cage, and urine is sampled for three hours (three-hour urine) from all rats in a fasting/water intake state and then for 21 hours from all rats in a non-fasting/water intake state (21-hour urine; totaling 24 hours (24-hour urine)). The following examinations are conducted using the urine samples above.

Examination items

Urine volume:	weight of the 24-hour urine (24-hour urine)
Color:	appearance (three-hour urine)*
Specific gravity:	refractometer (21-hour urine, refractory specific gravimeter: Uripet (Nikon Corporation))*
pH, occult blood, protein, glucose, ketone body, urobilinogen, bilirubin:	(three-hour urine, urine test strip for Ames Clinitek: Miles (Sankyo))*
Urine sediment:	microscopic examination (three-hour urine)*

*: If no urine is taken in three hours after administration (between about 10:00 and 13:00), the relevant items are not investigated.

(5) Ophthalmic examination

Before the first administration and before the end of administration period, a mydriatic drug (Mydrin P, Santen Pharmaceutical Co., Ltd.) is administered for mydriasis and ophthalmography is conducted for both eyes using a fundus camera (RC-2 Model 621, Kowa Company, Ltd.).

(6) Hematological examination

On the day following the last administration, blood samples are taken from the abdominal aorta into Sysmex's EDTA-2K-coating cup under intraperitoneal anesthesia with pentobarbital sodium, and the hematological examinations below are conducted basically in all animals. (Unused blood samples are discarded.) For prothrombin time, plasma samples prepared with 3.13% sodium citrate are used.

1) Red blood cell count, white blood cell count, hemoglobin level, hematocrit level, platelet count: Sysmex E-2000 (Toa Medical Electronics Co., Ltd.)

2) Prothrombin time: FibroSystem (BBL, Division of Becton, Dickinson & Co.)

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3) Reticulocyte: visual counting method

4) White blood cell differential count: visual counting method

(7) Blood biochemical examination

On the day following the last administration, blood samples are taken from the abdominal aorta under intraperitoneal anesthesia with pentobarbital sodium, and serum samples obtained from the blood are used for following blood biochemical examinations in all animals. The serum samples are stored in a -80°C freezer until measurement. After measurement, they are stored in a -20°C freezer until submission of a final report. Subsequent storage is determined by discussions with the study sponsor.

GOT, GPT, ALP, CPK, LDH, total cholesterol, triglyceride, total protein, urea nitrogen, creatinine, total bilirubin, glucose, Ca, inorganic phosphorus:

AU500 (Olympus Corporation)

Na, K: FLAME-30C (flame photometer, JASCO Corporation))

Cl: chloride meter C-200AP (coulometric titration, JOKOH Co., Ltd.)

Protein fraction (including albumin levels), A/G ratio:

AES600 (electrophoresis, Olympus Corporation)

(8) Autopsy

On the day following the final administration, animals from which blood samples were taken for (6) and (7) above are exsanguinated. Organs and tissues are macroscopically investigated and findings are recorded. Typical photos of abnormal organs and tissues of the animals and the corresponding parts of animals in the control group are taken. They are fixed/stored in 10% neutral buffer formalin.

(9) Organ weight measurement

The weights of the organs below of all animals are measured during autopsy on the day following the final administration (for paired organs, the weight of a pair is measured).

The rates of organ weights and the body weight before autopsy (relative weights) are calculated. After measurement, the organs below are fixed/stored in 10% neutral buffer formalin: heart, lungs (including the bronchus), thymus gland, liver, spleen, kidneys, adrenal gland, testicles (male) and ovaries (female)

(10) Histopathological examination

The livers, kidneys and adrenal glands of all animals are fixed in 10% neutral buffer formalin. Following routine method, they are paraffin-embedded and HE-stained for histopathological examinations. Examinations are also conducted for abnormal sites found by autopsy. The photos of typical cases are taken.

6. Statistical method

For body weight, food consumption, hematological findings, blood biochemical findings, organ weight, relative weight, urine volume and urine specific gravity, means and standard deviations for each group are calculated.

Tests of significance between the DB and control groups are carried out using a multiple comparison method (ASSIT analysis; refer to attachments 1 and 2). Differences are judged as significant at risk rates of <5%.

7. Amendment of protocol

If the protocol needs to be amended after an outsourcing agreement is signed, it will be amended by discussions between the study sponsor and study facility. The protocol amendment form which indicates sections before and after amendment and reasons for amendment needs to be stored with the protocol.

8. Storage of materials and samples

Materials and samples related to the study are all stored in the material/sample storeroom of Nihon Bioresearch Inc. Storage periods are subject to separate agreements.

(1) Protocol, main schedule table, protocol amendment form, contract

(2) Test article record
   1) Receipt/return record
   2) Record on characteristics and stability (received from study sponsor)
   3) Usage record
   4) Storage record

(3) Record on environmental conditions and contamination analysis
   1) Record on animal room environments
   2) Breeding control record
   3) Record on food contamination analysis
   4) Drinking water quality test record

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5) Record on bedding contamination analysis

(4) Record on animals
1) Animal arrival record
2) Quarantine/acclimatization record
3) Animal grouping record
4) Record on transfer and disposal of animals
5) Cage label

(5) Administration-related record
1) Record on calculation of volume of the test article solution
2) Administration record

(6) Observation/examination record
1) Daily death record
2) General condition record
3) Body weight record
4) Food consumption record
5) Urinalysis record
6) Ophthalmic examination record
7) Hematological examination record
8) Blood biochemical examination record
9) Organ weight record, relative weight record
10) Autopsy record
11) Organ inspection record
12) Histopathological processing record
13) Histopathological examination record
14) Photography-related record

(7) Samples
1) Formalin-fixed samples and paraffin-embedded samples
2) Histopathological samples

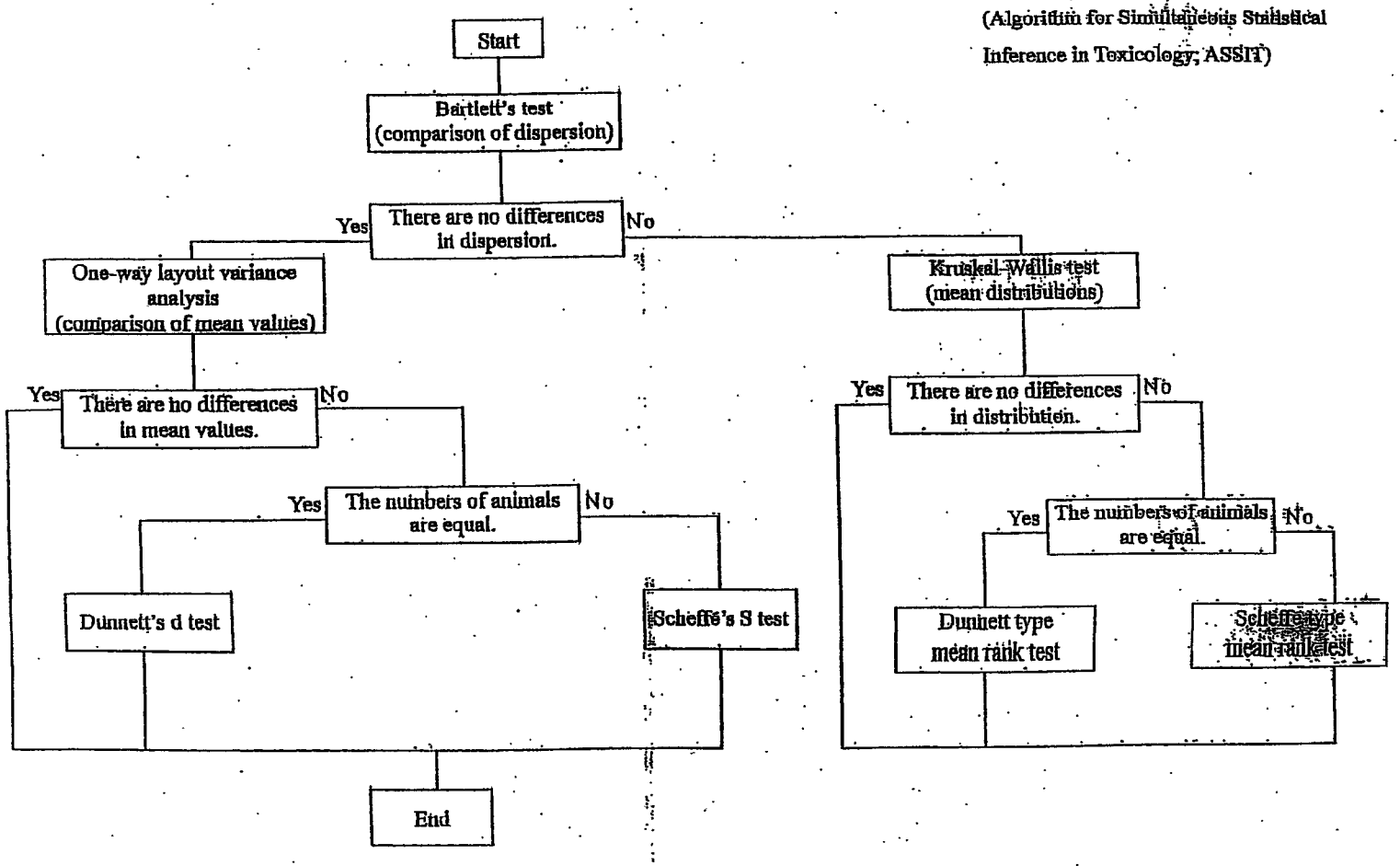
3) Blood smears
4) Serum samples (until the submission of the final report)
5) Photos and negatives

(8) Report-related materials
1) Figures and tables prepared for reports
2) Statistical data
3) Report (draft)
4) Copy of the final report

Attachment 1

ASSIT analysis

(Algorithm for Simultaneous Statistical Inference in Toxicology; ASSIT)



Attachment 2

Test results are indicated at the bottom part of Appendix by the abbreviations below.

ANH0: A one-way layout variance analysis shows the absence of significant differences.
ANH1: A one-way layout variance analysis shows the presence of significant differences.

KWH0: A Kruskal-Wallis test shows the absence of significant differences.
KWH1: A Kruskal-Wallis test shows the presence of significant differences.

DUH0: Dunnett's d test shows the absence of significant differences.
DUH1: Dunnett's d test shows the presence of significant differences.

SCH0: Scheffé's S test shows the absence of significant differences.
SCH1: Scheffé's S test shows the presence of significant differences.

DTH0: A Dunnett type mean rank test shows the absence of significant differences.
DTH1: A Dunnett type mean rank test shows the presence of significant differences.

STH0: A Scheffé type mean rank test shows the absence of significant differences.
STH1: A Scheffé type mean rank test shows the presence of significant differences.

000228

Protocol Amendment Form (No. 1)

Study number: 5118

Title: 28-day oral administration subacute toxicity study of Dunaliella Bardawil Spray Dried Powder in rats

Amendments and their reasons

p. 1 Study sponsor

Nikken Seiyaku Kogyo

↓

Nikken Sohonsha Corporation

p. 2 Approval of protocol

Study sponsor

Teruo Shiraishi Seal January 7, 1989

Nikken Seiyaku Kogyo

↓

Hiroyuki Takenaka Seal January 17, 1989

Nikken Sohonsha Corporation

The underlined sections are amended as above.

Reasons: Persons responsible for in the study sponsor have been changed.

Amendment

Study Director Tadakazu Furuhashi Seal January 17, 1989

Approval

Facility Management Kenichi Nakagawa Seal January 17, 1989

Study Sponsor Hiroyuki Takenaka Seal January 17, 1989

000229

Protocol Amendment Form (No. 2)

Study number: 5118

Title: 28-day oral administration subacute toxicity study of Dunaliella Bardawil Spray Dried Powder in rats

Amendments and their reasons

p. 3 1. Test article

(1) Test article

Lot number: mentioned after obtaining → NSK-1207

Date of obtaining MM DD, YYYY (mentioned after obtaining) → February 2, 1989

Quantity: mentioned after obtaining → 416.0 g

Storage conditions: stored in a refrigerator (approximately 5°C) protected from light → stored in a freezer (approximately -20°C) protected from light

The underlined sections are modified as above.

Reasons: It was planned to mention after obtaining of the test article. The storage conditions were modified as instructed by the study sponsor.

p. 4 (8) Individual identification method

Rats are identified by marking on their tails by oil-based ink during the preliminary breeding period.

Rats are identified by staining fur and putting marks on their tails by oil-based ink during the preliminary breeding period.

The underlined section is amended as above.

Reasons: This amendment is related to the revision of the standard operating procedures (SOP).

Amendment

Study Director Tadakazu Furuhashi Seal February 6, 1989

Approval

Facility Management Kenichi Nakagawa Seal February 6, 1989

Study Sponsor Hiroyuki Takenaka Seal February 8, 1989

000230

Protocol Amendment Form (No. 3)

Study number: 5118

Title: 28-day oral administration subacute toxicity study of Dunaliella Bardawil Spray Dried Powder in rats

Amendments and their reasons

p. 3 1. Test article

(1) Test article

Supplier: Nikken Seiyaku Kogyo → Nikken Sohonsha Corporation

The underlined section is amended as above.

Reasons: The company name has been changed.

p. 3 2. Preparation of test solution

The test article is dissolved or diluted at the specific concentration in necessary amounts of a 0.5% carboxymethyl cellulose sodium aqueous solution (CMC-Na: lot number: 109E5046, manufacturer: Kanto Kagaku Kogyo, distilled water for injection: lot number: 8F82N, manufacturer: Otsuka Pharmaceutical Co., Ltd.).

The underlined section is added.

Reasons: It was planned to mention after purchase.

p. 4 (3) Arrival date, sex and quantity

20 males and 20 females on February 9, 1989

21 males and 21 females on February 9, 1989

The underlined section is amended as above.

Reasons: Twenty-one each of male and female animals were delivered according to the animal supplier's convenience. For dates, era names are changed.

000231

Amendment

Study Director Tadakazu Furihashi Seal September 25, 1989

Approval

Facility Management Kenichi Nakagawa Seal September 25, 1989

Study sponsor Hiroyuki Takenaka Seal September 28, 1989

000232

Protocol Amendment Form (No. 4)

Study number: 5118

Title: 28-day oral administration subacute toxicity study of Dunaliella Bardawil Spray Dried Powder in rats

Amendments and their reasons

p. 1 Study sponsor

Nikken Sohonsha Corporation

4-41 Hirakata, Fukuju-cho, Hashima -shi, Gifu-prefecture

↓

1-32 Asahira, Fukuju-cho, Hashima-shi, Gifu-prefecture

The underlined section is amended to as above.

Reasons: The address of the study sponsor has been changed.

Amendment

Study Director Tadakazu Furuhashi Seal October 17, 1989

Approval

Facility Management Kenichi Nakagawa Seal October 17, 1989

Study Sponsor Hiroyuki Takenaka Seal October 19, 1989

000233

株式会社日本バイオリサーチセンター

試験番号：5118

陳 述 書

試験番号：5118

試験表題：Dunaliella Bardwil Spray Dried Powder のラットにおける 28 日間経口投与による亜急性毒性試験

当試験は、薬発第 313 号厚生省薬務局長通知（昭和 57 年 3 月 31 日付）「医薬品の安全性試験の実施に関する基準について」に従って実施されたものである。

2007 年 1 月 16 日

株式会社日本バイオリサーチセンター　羽島研究所

試験責任者 (b) (6)

000234

000234

Dunaliella Bardawil Spray Dried Powderのラットにおける28日間経口投与による亜急性毒性試験

最終報告書

提出日 平成 元年10月31日

株式会社日本バイオリサーチセンター
羽島研究所

NBR-1

000235

試験概要（試験番号：5118）

表　　題	Dunaliella Bardawil Spray Dried Powderのラットにおける28日間経口投与による亜急性毒性試験
試験番号	5118
被験物質名	Dunaliella Bardawil Spray Dried Powder
試験系	Sprague-Dawley [SPF 、Crj:CD(SD)] 系ラット
試験委託者	株式会社　日健総本社 岐阜県羽島市福寿町浅平１丁目３２番地
試験施設	株式会社日本バイオリサーチセンター　羽島研究所 岐阜県羽島市福寿町間島６丁目１０４番地
試験目的	Dunaliella Bardawil Spray Dried Powderの安全性に関する試験の一環として、Dunaliella Bardawil Spray Dried Powderをラットに28日間連続経口投与し、その安全性について検討した。
試験期間	昭和６４年　1月　5日　～　平成　元年１０月３１日
資料・標本の保管場所	株式会社日本バイオリサーチセンター　羽島研究所 （当試験の実施により得られたすべての資料および標本は、当試験の最終報告書提出後 5年間は株式会社日本バイオリサーチセンター　羽島研究所の資料保管室および標本保管室に保管する。　その後の保管は、試験委託者と協議のうえ決定する。）
試験計画書からの逸脱	当試験では、試験計画書からの逸脱は認められなかった。

000236

試験従事者および業務分担（試験番号：5118）

試験責任者：（試験の統括、試験計画書の作成、データのチェック、
最終報告書の校正）

古橋忠和

試験担当責任者：（試験実施の指示、データのチェック、検体の調製、
最終報告書の作成）

宇土研二

試験担当者：（検体の投与、検体調製、一般状態の観察、動物の一般飼育管理）

藤村高志　青山孝一　内藤一嘉
堀　千鶴子　杉山浩子　小林　広

（剖検、臓器重量測定および病理組織学的検査）

木村　均　小池恒雄　菅沼和子
吉島賢一　井上尚子　虫賀勇樹
堀　恵　森　力子

（臨床検査）

有賀文彦　渡邊ゆかり　鷲見ひとみ
山木田　有加子

（眼底検査）

和田　浩

（検体の管理・調製）

加藤正巳　後藤恭見

（統計処理、図・表の作成）

鈴木麗子　林　幸子

試験責任者 (b) (6) 平成元年10月31日

試験担当責任者 (b) (6) 平成元年10月31日

（最終報告書作成者）

運営管理者 (b) (6) 平成元年10月31日

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要　約

Dunaliella Bardawil Spray Dried Powderの 0.5および 2.5g/kgをSprague-Dawley [Crj:CD(SD)] 系ラットの雌雄に28日間経口投与してその毒性について検討し、以下の成績を得た。　対照群には、 0.5%CMC-Na水溶液 10ml/kgを経口投与した。

1．いずれの群も雌雄ともに、死亡発現はなく、一般状態の異常も認められなかった。

2．体重、摂餌量および尿検査では、Dunaliella Bardawil Spray Dried Powderの投与による影響は認められなかった。

3．眼科的検査では、いずれの群の雌雄とも異常は認められなかった。

4．血液学的検査および血液生化学的検査では、Dunaliella Bardawil Spray Dried Powderの投与による影響は認められなかった。

5．剖検、器官重量および病理組織学的検査では、Dunaliella Bardawil Spray Dried Powderの投与による影響は認められなかった。

6．以上の結果から、雌雄のラットに対してDunaliella Bardawil Spray Dried Powderによる毒性兆候を示唆する成績は得られなかった。　したがって、当試験条件下では連続投与可能なほぼ最大量と考えられたDunaliella Bardawil Spray Dried Powderの高用量の 2.5g/kgは、毒性学的無影響量と推定された。

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緒　言

Dunaliella Bardawil Spray Dried Powderの安全性に関する試験の一環として、Dunaliella Bardawil Spray Dried Powderのラットにおける28日間経口投与による亜急性毒性試験を実施したので、その結果を報告する。

被験物質

被験物質であるDunaliella Bardawil Spray Dried Powder（ロット番号：NSK-1207、略称：DB）は、赤褐色の粉末で、水に溶けない。 本物質は、窒素置換後、密封保存で 1年以上安定であり、冷蔵・遮光あるいは冷凍保存で最低 2カ月間安定とされている[1]。

Dunaliella Bardawil Spray Dried Powderは、平成元年 2月 2日に416.0gを株式会社日健総本社から提供された。 入手後は、試験施設の被験物質保管室の冷凍庫（約－20℃）内で気密容器に入れ、遮光条件下で保管した。

投与検体の調製は、Dunaliella Bardawil Spray Dried Powderに必要量の 0.5%カルボキシメチルセルロースナトリウム水溶液（CMC-Na：ロット番号；109E5048、製造元；関東化学工業株式会社、注射用蒸留水：ロット番号；8F82N 、製造元；株式会社大塚製薬工場）を加え、乳鉢を用いて懸濁し、所定濃度とした。 調製は毎日行い、投与後の残余検体は廃棄した。

なお、本被験物質は 0.5%カルボキシメチルセルロースナトリウム水溶液中では、冷蔵（約 5℃）・遮光保存で、 5および25%の濃度で調製後 5時間まで安定であることが、試験委託者側で確認されている[1]。

試験方法

1．試験動物および飼育条件

試験には、毒性試験に一般的に用いられている動物種でその系統維持が明らかであり、集積データも揃っているSprague-Dawley [Crj:CD(SD)] 系ラットの雌雄を使用した。

当試験用として、 4週齢の雌雄各21匹のラットを平成元年 2月 9日に日本チャールス・リバー株式会社から購入した。 動物の入荷翌日の体重範囲は、雄で77～ 87g、雌で68～81gであった。

入荷後の動物には、検疫および馴化のための予備飼育期間を12日間設け、この間に 3回の体重測定と毎日外見ならびに一般状態を観察した。

飼育は、室温20～24℃、湿度40～70% 、明暗各12時間（蛍光灯による照明：午前 6時～

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午後 6時）、換気回数12回／時（フィルターにより除菌した新鮮空気）に設定した動物飼育室（C棟4号室）で行った。

予備飼育期間中ではオートクレーブ処理した床敷（チップ、千葉アニマル資材株式会社）を入れたプラスチック製ケージ（W:300 ×D:355 ×H:175mm ）を用いて 1ケージ当たり 5匹までの群飼育とし、群分け後ではステンレス製五連ケージ（W:755 ×D:210 ×H:170mm ）を用いて個別に飼育した。

プラスチック製ケージおよびステンレス製五連ケージの受けtrayの交換は 1週間に 2回以上、ステンレス製五連ケージ（給餌器とともに）の交換は 2週間に 1回行った。

動物飼育室の清掃および消毒は毎日行った。

飼料は入荷後 3カ月以内のオートクレーブ処理した固型飼料（CRF-1 、オリエンタル酵母工業株式会社）を給餌器に入れ、飲料水は水道水を給水瓶に入れ、いずれも自由に摂取させた。

飼料の補充および飲料水の交換は、 1週間に 2回以上行った。

2．群分け法

群分けは、体重を層別後、無作為抽出法により、各群の平均体重および分散がほぼ等しくなるように投与開始の前日に行った。 群分け後の残余動物は、初回投与日にエーテルで麻酔致死させた後、廃棄処分した。

3．個体識別法

個体識別は、予備飼育期間中では色素による染毛法と油性インクペンで尾に印をつけることにより、群分け後では色素による染毛法と耳パンチ法により行った。

各ケージには、予備飼育期間中では試験番号、入荷年月日および馴化動物番号を記入したラベルを、群分け後では試験番号、被験物質名、投与量および動物番号を記入した群毎の色別のラベルを取り付けた。

4．投与

(1) 投与経路とその選択理由

Dunaliella Bardawil Spray Dried Powderは食品であり、ラットを用いた当試験では経口投与を選択した。

(2) 投与法および投与液量

投与は、金属製経口ゾンデを装着したプラスチック製ディスポーザブル注射筒を用いて行った。 投与液量は、投与日に最も近い測定日の体重を基準として 10ml/kgで算出した。

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(3) 投与回数、投与期間および投与時刻

投与初日を投与 1日とし、 1日 1回（午前 9時～12時の間）連日28日間の投与とした。

なお、動物の初回投与日の週齢は 6週齢であり、体重範囲は雄で 174～190g、雌で 142～170gであった。

5．投与量および群構成、ならびに投与量の設定理由

投与量および群構成は、以下に示した。

群	試験群	投与量	ラベルの色	雄（動物番号）	雌（動物番号）
第 1群	対 照				
	(0.5%CMC-Na水溶液)	―	白 色	5(011～015)	5(061～065)
第 2群	Dunaliella Bardawil Spray Dried Powder				
		0.5g/kg	黄 色	5(111～115)	5(161～165)
第 3群	Dunaliella Bardawil Spray Dried Powder				
		2.5g/kg	赤 色	5(211～215)	5(261～265)

当試験の投与量は、試験委託者と協議して決定した。 すなわち、先に実施した Dunaliella Bardawil Spray Dried Powderのマウスにおける経口投与による急性毒性試験[2]において、投与液量および懸濁濃度から得られる投与可能なほぼ最大量の 10g/40ml/kg 投与によっても一般状態、体重推移および剖検には異常は認められなかった。 しかし、この投与量を非絶食下で連続投与することは困難と判断された。 したがって、当試験の投与量は、連続投与に一般的に用いられている投与液量の 10ml/kgで算出した投与可能なほぼ最大量の 2.5g/kgを高用量に、0.5g/kg を低用量に設定した。 また、対照として、媒体の 0.5%CMC-Na水溶液を被験物質投与群と同容量投与する群を別に設けた。

6．観察および検査項目

(1) 一般状態

一般状態および死亡の有無を 1日 2回（ただし、剖検日は 1回のみ）観察した。

(2) 体重

1週間に 2回測定した。

(3) 摂餌量

1週間に 1回（連続 2日間量を測定し、 1日量を算出）測定した。 なお、摂餌量の表示は残量の測定日とした。

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(4) 尿検査

投与期間終了前に採尿ケージを用い、絶食・給水下で 3時間採取した尿（ 3時間尿）と引き続き給餌・給水下で21時間採取した尿（合計24時間尿）について、以下の検査を実施した。

3時間尿について、色調は外観で判定した。 pH、潜血、蛋白、糖、ケトン体、ウロビリノーゲン、ビリルビンは得られた尿のうち 200μℓをエームスクリニテック用検査紙（マイルス・三共株式会社）に滴下したのちにエームス尿分析器（クリニテックSG、マイルス・三共株式会社）により検査した。 沈渣は、顕微鏡下で上皮細胞、赤血球および白血球を 400倍で 100視野、血液凝集、円柱および結晶を 100倍で全視野観察した。

21時間尿について、比重[S.G.]を屈折型比重計（ユリベット・日本光学工業株式会社）により測定した。

24時間尿について、 3時間と21時間で得られた合計の尿量[UV]を重量測定した。

(5) 眼科的検査

初回投与開始前および投与期間終了前に、散瞳剤（ミドリンP ・参天製薬株式会社）を用いて両眼を散瞳させたのちに、眼底カメラ（RC-2 Model 621・興和株式会社）により眼底を撮影し、眼底検査を行った。

(6) 血液学的検査

最終投与の翌日に、ペントバルビタールナトリウム（東京化成工業株式会社）の腹腔内投与による麻酔下で、腹大動脈からカニュレーションにより血液を採取し、以下の血液学的検査を行った。 なお、検査後の残余血液は当日に廃棄した。

プロトロンビン時間[PT]は、3.13%クエン酸ナトリウム（純正化学株式会社）で処理した血漿について、Quick 一段法により FibroSystem（BBL 、Div.of Becton 、Dickinson & Co. ）を用いて測定した。

赤血球数 [RBC]、白血球数 [WBC]、ヘモグロビン量 [HGB]、ヘマトクリット値 [HCT]および血小板数 [PLT]は、 EDTA-2Kコーティングのsysmexカップに採取した血液について自動血球計数装置（Sysmex E-2000 、東亜医用電子株式会社）を用いて測定した。

網状赤血球数 [RET]は、EDTA-2K 処理した血液を Brecher法により超生体染色（ 0.5%ニューメチレンブルーおよび 1.6%シュウ酸カリウム混合液）してスライドグラスに塗抹後、Giemsa染色した標本を作製し、顕微鏡下で赤血球1000個中の数を計数した。

白血球百分率 [L:リンパ球、N:好中球、E:好酸球、B:好塩基球、M:単球] は、EDTA-2K処理した血液をスライドグラスに塗抹後、May-Giemsa染色した標本を作製し、顕微鏡下で白血球 100個を分類計数した。

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(7) 血液生化学的検査

血液学的検査用の血液と同時期に採取した血液を、室温下で45～60分間放置したのちに冷却遠心器（20PR-52 、日立工機株式会社）により、15分間遠心分離（約 4℃、 3000rpm）し、得られた血清につき、以下の血液生化学的検査を行った。

なお、血清は測定時まで－80℃設定の冷凍庫（MDF-390AT 、三洋電気株式会社）に保管した。 測定後の血清は－20℃設定の冷凍庫（RS-70 、株式会社日立製作所）に保管し、最終報告書提出後に廃棄することとした。

GOTおよび GPTは Henry法、アルカリフォスファターゼ [ALP]は p-NPP基質法、クレアチニンフォスフォキナーゼ [CPK]はOliver法、乳酸脱水素酵素 [LDH]はWroblewski-La Due法、総コレステロール [T-CHO]はCOD+DAOS法、トリグリセライド[TG]はGPO+DAOS法、総蛋白[TP]はBiuret法、尿素窒素 [BUN]は酵素法、クレアチニン [CRE]は Jaffe法、総ビリルビン [T-BIL]はAzobilirubin法、ブドウ糖 [GLU]はグルコースデヒドロゲナーゼ法、Caは OCPC法、無機リン[Pi]は Molybdenum blue法により、いずれも自動分析装置（AU 500、オリンパス光学工業株式会社）を用いて測定した。

ナトリウム[Na]およびカリウム [K]は、炎光光度法により炎光光度計（FLAME-30C 、日本分光メディカル株式会社）を用いて測定した。

クロール[Cl]は、電量滴定法により自動分析装置（Chloride meter C-200AP、株式会社常光）を用いて測定した。

蛋白分画 [アルブミン（ALB)およびα_1-、α_2-、α_3-、β- 、γ- グロブリン] は、電気泳動法により自動電気泳動装置（オリンパスAES600、オリンパス光学工業株式会社）を用いて測定した。

A/G（アルブミン／グロブリン）比およびアルブミン量 [ALB]は、総蛋白量ならびに蛋白分画から算出した。

(8) 剖検

前述（6)および（7)の項で採血したのちに動物を更に放血致死させてから剖検し、肉眼的に器官・組織の観察を行い、所見を記録した。 観察時に、異常が認められた部位について代表例の写真撮影を行い、10%中性緩衝ホルマリン液に固定した。

(9) 器官重量の測定

剖検時に、以下の器官重量（絶対重量）を測定した（対器官は一括秤量）。 また、剖検前の体重を基準とした器官重量の体重比（相対重量）も算出した。

心臓、肺（気管支を含む）、胸腺、肝臓、脾臓、腎臓、副腎、雄：精巣、雌：卵巣。

なお、器官重量の測定後は、10%中性緩衝ホルマリン液に固定し、保存した。

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(10)病理組織学的検査

肝臓、腎臓および副腎について、10%中性緩衝ホルマリン液に固定し、常法に従いパラフィン包埋後、Hematoxylin-Eosin 染色組織標本を作製し、病理組織学的検査を行った。その他、異常が認められた部位についても実施した。　また、代表例の写真撮影を行った。

7．統計学的方法

体重、摂餌量、血液学的検査、血液生化学的検査、器官重量（絶対重量および体重に対する相対重量）、尿量および尿比重は、各群で平均値および標準偏差を算出した。

投与各群と対照群との有意差検定には、多重比較検定を用い、危険率 5%未満（$P<0.05$）を有意とした。　すなわち、Bartlett法による等分散性の検定を行い、等分散の場合には一元配置法による分散分析を行い、有意ならば対照群との群間比較は Dunnett法（例数が等しい場合）を用いて行った。　一方、等分散と認められなかった場合は、順位を利用した一元配置法による分析（Kruskal-Wallisの検定）を行い、有意ならば対照群との群間比較は順位を利用した Dunnett法（例数が等しい場合）を用いて行った。

試験日程

当試験の日程は、以下の通りであった。

動物入荷日：平成元年 2月 9日
群分け日：平成元年 2月21日
投与開始日：平成元年 2月22日
投与終了日：平成元年 3月21日
剖検日：平成元年 3月22日
標本観察終了日：平成元年 6月30日

なお、当試験の実施期間中に、試験の信頼性に影響を及ぼす疑いのある予期し得なかった事態は認められなかった。

試験成績

1．途中死亡および一般状態

対照群を含めた各群の雌雄とも、死亡発現はなく、一般状態の異常も認められなかった。

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2．体重

体重推移を、雄は Fig.1および Table 1（Appendix 1-1～ 1-3）に、雌は Fig.2および Table 2（Appendix 2-1～ 2-3）に示した。

投与各群の雌雄とも、体重の推移は対照群とほぼ同様であり、いずれの測定日にも有意差は認められなかった。

3．摂餌量

摂餌量推移を、雄は Fig.3および Table 3（Appendix 3-1～ 3-3）に、雌は Fig.4および Table 4（Appendix 4-1～ 4-3）に示した。

0.5g/kg群において、雄の投与10日および雌の投与17日に対照群に比べて有意な高値を示した。　2.5g/kg群では、雌雄とも摂餌量の推移は対照群とほぼ同様であり、いずれの測定日にも有意差は認められなかった。

4．尿検査

尿検査成績のうち尿量および比重を、雄は Table 5（ Appendix 5-1 ～ 5-3）に、雌は Table 6（ Appendix 6-1 ～ 6-3）に示した。

雄の投与各群および雌の 2.5g/kg群では、尿量ならびに比重のいずれも対照群とほぼ同様な値であり、有意差は認められなかった。　雌の 0.5g/kg群では、比重の値は対照群とほぼ同様であったが、尿量は対照群に比べて有意な高値を示した。

その他の雌雄の尿検査成績を、色調、ｐＨ、潜血、蛋白、糖、ケトン体、ウロビリノーゲンおよびビリルビンは Table 7（ Appendix ：雄； 7-1～ 7-3、雌； 8-1～ 8-3）に、沈渣は Table 8（ Appendix ：雄； 9-1～ 9-3、雌；10-1～10-3）に示した。

投与各群の雌雄とも、いずれの検査項目も対照群とほぼ同程度であった。

5．眼科的検査

対照群を含めた各群の雌雄とも、眼底に異常は認められなかった。

6．血液学的検査

血液学的検査成績を、雄は Table 9-1（ Appendix 11-1～11-3）および Table 9-2（ Appendix 12-1～12-3）に、雌はTable 10-1（ Appendix 13-1～13-3）および Table 10-2（ Appendix 14-1～14-3）に示した。

白血球［WBC］数は、 0.5g/kg群の雄および 2.5g/kg群の雌の値が対照群に比べて有意な高値を示した。　その他の検査項目では、投与各群の雌雄とも対照群とほぼ同程度であり、有意差は認められなかった。

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7．血液生化学的検査

血液生化学的検査成績を、雄はTable 11-1（Appendix 15-1～15-3）、Table 11-2（Appendix 16-1～16-3）、Table 11-3（Appendix 17-1～17-3）およびTable 11-4（Appendix 18-1～18-3）に、雌はTable 12-1（Appendix 19-1～19-3）、Table 12-2（Appendix 20-1～20-3）、Table 12-3（Appendix 21-1～21-3）およびTable 12-4（Appendix 22-1～22-3）に示した。

ナトリウム[Na]値およびクロール[Cl]値は、2.5g/kg群の雄の値が対照群に比べて有意な低値を示したが、軽度な差であった。その他の検査項目では、投与各群の雌雄とも対照群とほぼ同程度であり、有意差は認められなかった。

8．剖検

雌雄の剖検所見を、Table 13に示した。

対照群では、胸腺出血が雌雄の各1例に、腎臓の一側に白色結節が雌の1例（No.065）に認められた。

Dunaliella Bardawil Spray Dried Powderの投与群では、肝臓に黄白色結節および被膜肥厚（一部）、ならびに脾臓に被膜肥厚（一部）が2.5g/kg群の雌の1例（No.264）に、胸腺出血が2.5g/kg群の雄の1例に、脾臓に腫大（No.215）が2.5g/kg群の雄の1例にそれぞれ認められた。

9．器官重量

雄の器官の絶対および相対重量をTable 14（Appendix 23-1～23-3）およびTable 15（Appendix 24-1～24-3）に、雌の器官の絶対および相対重量をTable 16（Appendix 25-1～25-3）およびTable 17（Appendix 26-1～26-3）に示した。

投与各群の雌雄とも、絶対重量ではいずれの器官も対照群とほぼ同様な値であり、有意差は認められなかった。一方、相対重量では2.5g/kg群において、雄の腎臓で有意な高値が認められた。

10．病理組織学的検査

病理組織学的検査を、雄はTable 18（Appendix 27-1～27-3）に、雌はTable 19（Appendix 28-1～28-3）に示した。

対照群では、肝臓に炎症細胞浸潤が雄の1例と雌の全例に、腎臓の間質に炎症細胞浸潤が雌雄の各1例に、胸腺出血が雌雄の各1例に認められたが、いずれもごく軽度～軽度の変化であった。この他、雌の1例（No.065）では腎臓の一側に腎芽細胞腫が認められた。

Dunaliella Bardawil Spray Dried Powderの投与群では、肝臓に炎症細胞浸潤が雌雄の投与各群で1～3例に、異物性肉芽腫が0.5g/kg群の雌で1例に、被膜肥厚および肉芽腫が2.5g/kg群の雌の1例（No.264）にごく軽度～軽度に認められた。腎臓では、間質に

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炎症細胞浸潤、嚢胞がそれぞれ 2.5g/kg群の雌雄の各 1例に、尿細管上皮好塩基性化、繊維化巣がそれぞれ 0.5g/kg群の雌雄の各 1例にごく軽度〜軽度に認められた。 その他、胸腺に出血が 2.5g/kg群の雄の 1例に、脾臓に髄外造血（No.215）および被膜肥厚（No.264）がそれぞれ 2.5g/kg群の雌雄の 1例に、いずれも軽度に認められた。

考　察

Dunaliella Bardawil Spray Dried Powderの 0.5および 2.5g/kgをSprague-Dawley [Crj:CD(SD)] 系ラットの雌雄に28日間経口投与してその毒性について検討し、以下の成績を得た。

投与各群の雌雄とも、死亡発現はなく、一般状態および眼底の異常も認められなかった。また、体重も対照群とほぼ同様な推移を示した。

摂餌量では、 0.5g/kg群の雌雄で観察期間の中頃に摂取量の増加傾向が一過性にみられたが、用量依存性のない変動であった。

尿検査、血液学的検査および血液生化学的検査では、投与各群の雌雄の一部の検査項目で対照群に比べて有意な高値あるいは低値傾向がみられたが、いずれの変動も用量依存性は乏しく、かつ、必ずしも雌雄で共通しているものではなかったことから、Dunaliella Bardawil Spray Dried Powderの投与による影響ではないと考えられた。

剖検では、胸腺出血が 2.5g/kg群の雄の 1例にみられたが、この変化は対照群でも認められており、Dunaliella Bardawil Spray Dried Powderの投与による影響ではないと考えられた。 その他、 2.5g/kg群において、雄の 1例で（No.215）脾臓の腫大が、雌の 1例（No.264）で肝臓に黄白色結節、肝臓および脾臓に被膜肥厚が認められたが、いずれもごく限られた個体にのみ出現しており、偶発的な変化と推定された。

器官の絶対重量では、投与各群の雌雄ともいずれの器官も対照群とほぼ同様な値であった。 一方、相対重量において 2.5g/kg群で、雄の腎臓で有意な高値が認められたが、雌雄で共通した変動ではないこと、絶対重量では差がなかったことから、Dunaliella Bardawil Spray Dried Powder の投与による影響ではないと考えられた。

病理組織学的検査では、胸腺に出血、肝臓および腎臓の間質に炎症細胞浸潤がみられたが、対照群でも認められており、用量依存性のない変化であった。 その他、肝臓に異物性肉芽腫、腎臓に尿細管上皮好塩基性化、嚢胞および繊維化巣が認められたが、いずれの所見もその程度は弱く、一部の例でのみ認められた変化であり、用量依存性もないことから、Dunaliella Bardawil Spray Dried Powderの投与による影響ではないと考えられた。また、剖検で、肝臓に黄白色結節および被膜肥厚、ならびに脾臓に被膜肥厚を認めた雌の 1例（No.264）では肝臓に被膜肥厚および肉芽腫、ならびに脾臓に被膜肥厚がみられ、脾臓に腫大を認めた雄の 1例（No.215）では髄外造血が認められたが、これらの変化はいず

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れも剖検時の肉眼的観察とほぼ一致した。

なお、剖検で対照群の雌の 1例（No.065）の腎臓に認められた白色結節は腎芽細胞腫であった。

以上の結果から、雌雄のラットに対してDunaliella Bardawil Spray Dried Powderによる毒性兆候を示唆する成績は得られなかった。 したがって、当試験条件下では連続投与可能なほぼ最大量と考えられたDunaliella Bardawil Spray Dried Powderの 2.5g/kgは、毒性学的無影響量と推定された。

資　料

1．株式会社日健総本社から提供された被験物質の特性および安定性に関する資料

2．有賀文彦ら；試験番号：4087「Dunaliella Bardawil Spray Dried Powderのラットにおける経口投与による急性毒性試験」、株式会社日本バイオリサーチセンター　羽島研究所、昭和62年 8月25日～昭和62年12月 7日

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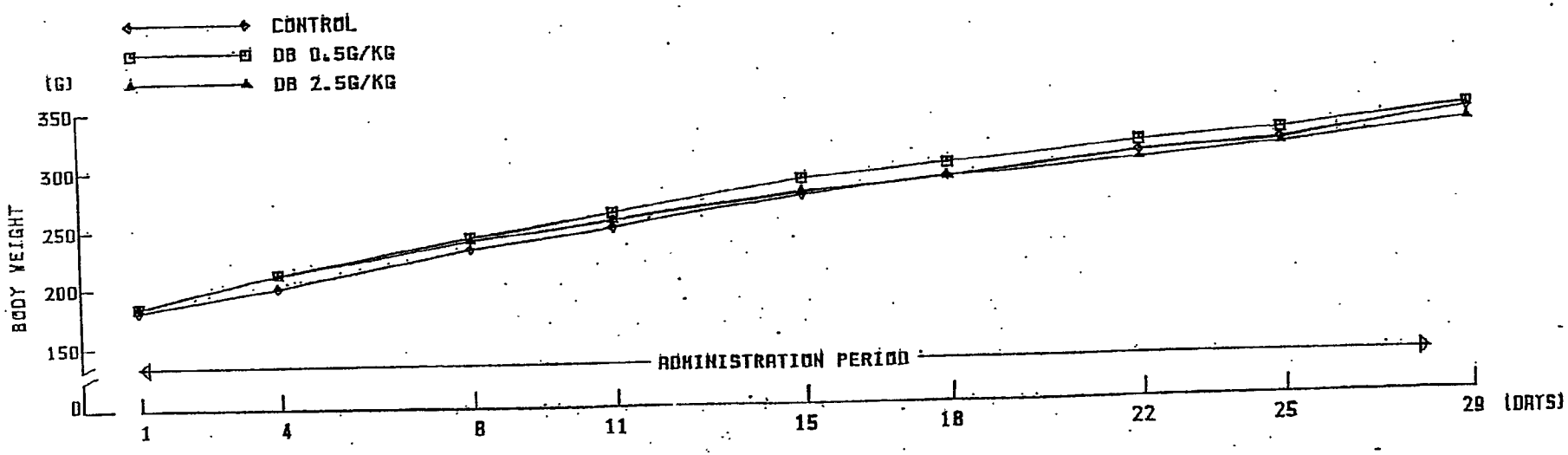

Fig. 1 Body weights of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

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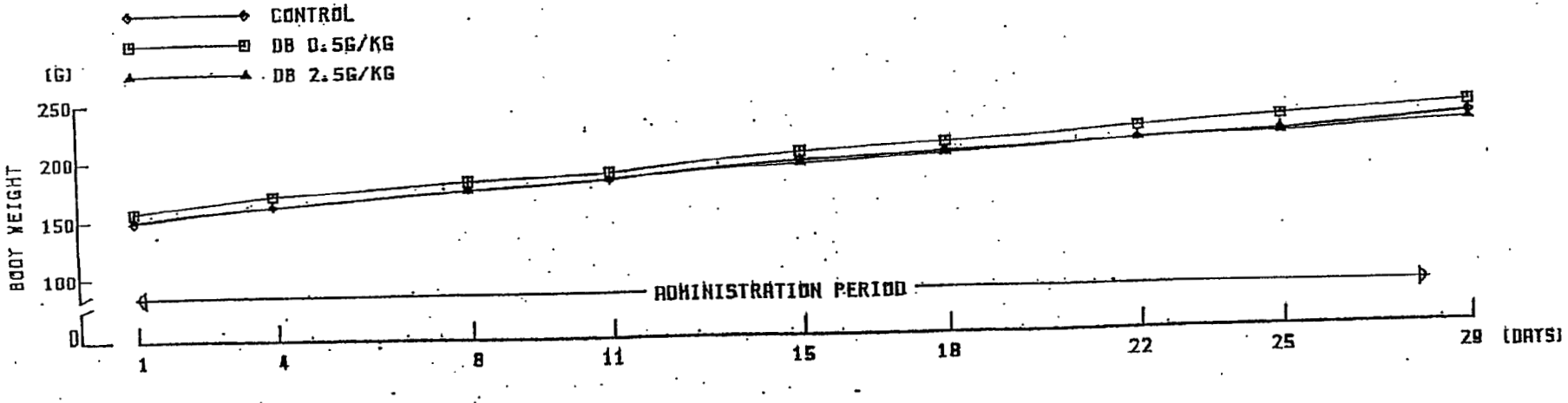

Fig. 2 Body weights of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

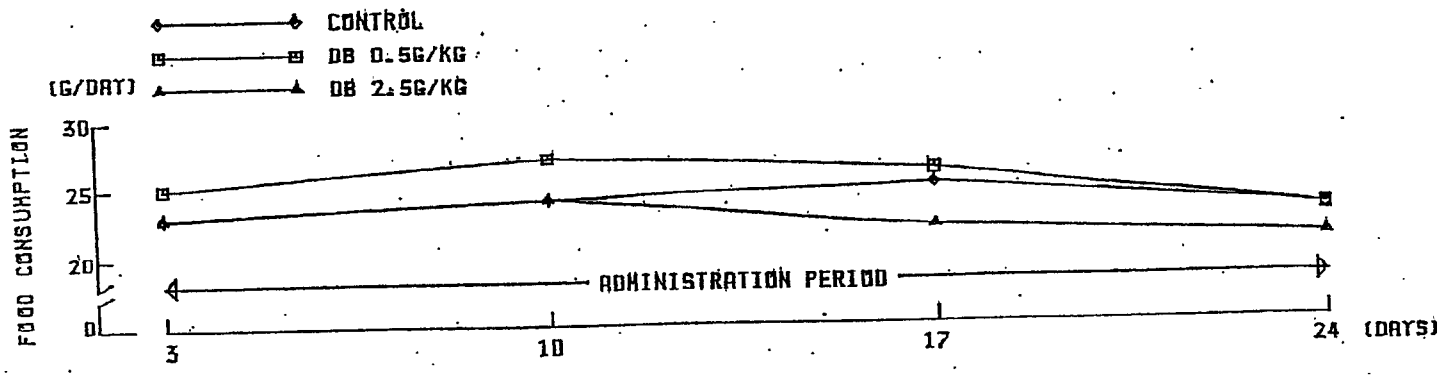

Fig. 3 Food consumptions by male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

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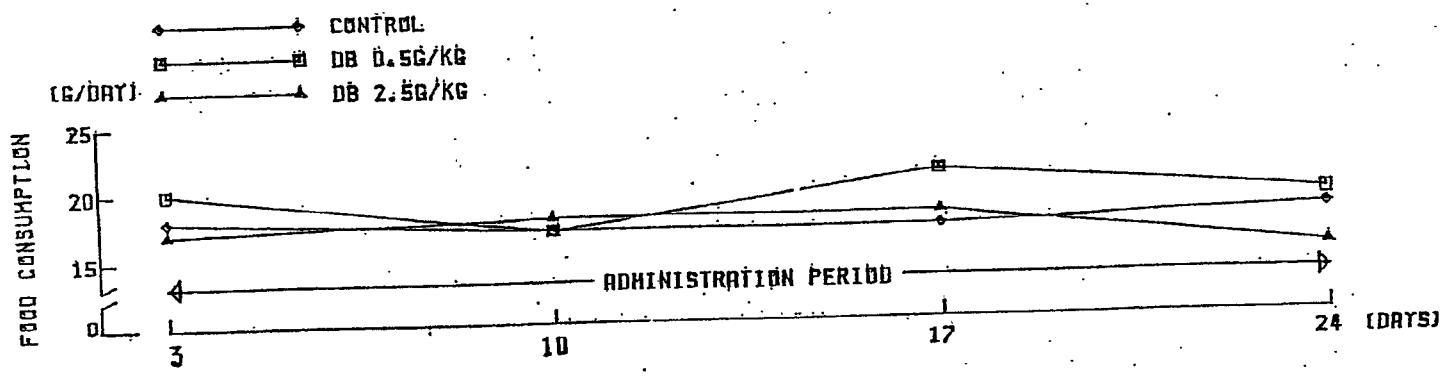

Fig. 4 Food consumptions by female rats treated orally with Duhallella Bardawil Spray Dried Powder for 28 days

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Table 1 Body weights of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

GROUP		1	4	8	11	15	18	22	25	29 (DAYS)
CONTROL	MEAN	180	199	230	248	273	287	305	314	337
	S.D.	7.1	7.3	14.2	15.3	20.2	22.8	24.6	23.7	26.0
	(N)	5	5	5	5	5	5	5	5	5
DB 0.5G/KG JUDGE	MEAN	184	210	241	260	287	298	314	322	341
	S.D.	6.2	8.2	8.8	7.9	5.1	7.0	8.7	8.5	12.5
	(N)	5	5	5	5	5	5	5	5	5
DB 2.5G/KG JUDGE	MEAN	184	209	238	254	276	286	299	310	328
	S.D.	5.8	5.9	4.0	8.3	10.4	13.5	17.9	19.0	25.2
	(N)	5	5	5	5	5	5	5	5	5

UNIT : G

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Table 2 Body weights of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

GROUP	1	4	8	11	15	18	22	25	29 (DAYS)
CONTROL									
MEAN	149	161	174	180	194	199	208	213	226
S.D.	3.7	8.8	9.8	11.7	12.0	12.8	12.5	16.4	16.0
(N)	5	5	5	5	5	5	5	5	5
DB 0.5G/KG									
JUDGE MEAN	157	170	180	186	201	207	218	226	236
S.D.	8.2	12.3	12.6	10.7	8.8	9.7	10.8	14.1	15.5
(N)	5	5	5	5	5	5	5	5	5
DB 2.5G/KG									
JUDGE MEAN	151	161	173	181	191	197	207	211	220
S.D.	7.6	11.3	11.2	16.1	17.0	15.3	16.7	16.3	18.5
(N)	5	5	5	5	5	5	5	5	5

UNIT : G

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Table 3 Food consumptions by male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

GROUP	3	10	17	24 (DAYS)
CONTROL				
MEAN	23	24	25	23
S.D.	1.1	2.5	3.1	2.6
(N)	5	5	5	5
DB 0.5G/KG				
JUDGE		*		
MEAN	25	27	26	23
S.D.	2.3	1.7	2.2	1.6
(N)	5	5	5	5
DB 2.5G/KG				
JUDGE				
MEAN	23	24	22	21
S.D.	1.6	0.7	1.5	1.9
(N)	5	5	5	5

* : SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP ($P<0.05$)
UNIT : G/DAY

Table 4 Food consumptions by female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

GROUP	3	10	17	24 (DAYS)
CONTROL				
MEAN	18	17	17	18
S.D.	2.3	2.9	2.3	1.5
(N)	5	5	5	5
DB 0.5G/KG				
JUDGE			*	
MEAN	20	17	21	19
S.D.	2.6	1.5	1.6	2.2
(N)	5	5	5	5
DB 2.5G/KG				
JUDGE				
MEAN	17	18	18	15
S.D.	1.5	0.9	1.5	1.9
(N)	5	5	5	5

* : SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP ($P<0.05$)
UNIT : G/DAY

Table 5. Urinalyses of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days --- (I)

GROUP		UV g	S.G.
CONTROL			
	MEAN	14.2	1.052
	S.D.	3.20	0.0099
	(N)	5	5
DB 0.5G/KG			
	JUDGE		
	MEAN	14.6	1.052
	S.D.	5.36	0.0081
	(N)	5	5
DB 2.5G/KG			
	JUDGE		
	MEAN	15.3	1.052
	S.D.	3.76	0.0108
	(N)	5	5

Table 6 Urinalyses of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days --- (I)

GROUP	UV g	S.G.
CONTROL		
MEAN	7.3	1.061
S.D.	1.62	0.0151
(N)	5	5
DB 0.5G/KG		
JUDGE	**	
MEAN	12.4	1.054
S.D.	2.43	0.0087
(N)	5	5
DB 2.5G/KG		
JUDGE		
MEAN	10.4	1.050
S.D.	2.80	0.0168
(N)	5	5

** : SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP (P<0.01)

Table 7 Urinalyses of male and female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (I)

Sex	Group	N[a]	Color	pH					Protein (mg/dl)					Glucose (g/dl)					Ketone body (mg/dl)					Bilirubin				Occult blood					Urobilinogen (E.U)				
				5	6	7	8	9	微量	痕跡	30	100	≧300	微量	痕跡	0.25	0.5	≧1	微量	痕跡	15	40	≧80	微量	+	±	++	微量	痕跡	+	±	++	0.1	1	2	4	8
Male	Control	5	Light yellow			2	3			3	2			5					2	2	1			5				5					3	2			
	DB 0.5 g/kg	5	Light yellow				5			3	1	1		5						2	3			4	1			5					3	2			
	DB 2.5 g/kg	5	Light yellow			2	3		2	3				5						4	1			5				5					4	1			
Female	Control	5	Light yellow				4	1	1	1	2	1		5					2	3				2	3			5					2	3			
	DB 0.5 g/kg	5	Light Yellow			2	3		3	1	1			5					4	1				4	1			5					3	2			
	DB 2.5 g/kg	5	Light yellow			1	4		1	4				5					4	1				3	2			5					2	3			

a) Number of samples tested.
E.U: Ehrlich Unit

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Table 8 Urinalyses of male and female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (Ⅲ)

Sex	Finding		Epithelial cells					Erythrocytes (RBC)					Leukocytes (WBC)					Blood coa.		Casts		Crys-tals	
	Group	N a)	20 ~ 0	100 ~ 21	200 ~ 101	V 201	Coagulation	20 ~ 0	100 ~ 21	200 ~ 101	500 ~ 201	V 501	20 ~ 0	100 ~ 21	200 ~ 101	500 ~ 201	V 501	−	+	−	+	−	+
Male	Control	5	5					5					5					5		5		5	
	DB 0.5 g/kg	5	5					5					5					5		5		4	1
	DB 2.5 g/kg	5	5					5					5					5		5		4	1
Female	Control	5	5					5					4	1				5		5		5	
	DB 0.5 g/kg	5	5					5					5					5		5		4	1
	DB 2.5 g/kg	5	5					5					4	1				5		5		4	1

coa. : coagulation

a) Number of samples tested.

Table 9 - 1 Hematological findings of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (I)

GROUP		RBC $10^4/mm^3$	HGB g/dl	HCT %	PLT $10^4/mm^3$	RET %	PT Sec.
CONTROL	MEAN	801	14.2	46.6	88.2	30	14.1
	S.D.	35.5	0.35	1.15	10.48	6.7	0.33
	(N)	5	5	5	5	5	5
DB 0.5G/KG	JUDGE MEAN	811	14.4	46.7	95.5	26	14.0
	S.D.	36.4	0.49	1.49	16.31	4.7	0.16
	(N)	5	5	5	5	5	5
DB 2.5G/KG	JUDGE MEAN	786	14.2	46.3	86.5	27	14.1
	S.D.	37.2	0.62	1.70	21.49	4.0	0.25
	(N)	5	5	5	5	5	5

Table 9 - 2 Hematological findings of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (I)

GROUP		WBC $10^3/mm^3$	L %	N %	E %	B %	M %
CONTROL	MEAN	55	87	12	0	0	0
	S.D.	8.1	5.0	5.5	0.9	0.0	0.9
	(N)	5	5	5	5	5	5
DB 0.5G/KG	JUDGE	*					
	MEAN	92	94	6	0	0	0
	S.D.	21.7	3.1	3.0	0.0	0.0	0.5
	(N)	5	5	5	5	5	5
DB 2.5G/KG	JUDGE						
	MEAN	81	93	7	0	0	0
	S.D.	27.2	3.8	3.8	0.9	0.0	0.4
	(N)	5	5	5	5	5	5

* : SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP ($P<0.05$)

Table 10 - 1 Hematological findings of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (I)

GROUP		RBC 10^4/mm³	HGB g/dl	HCT %	PLT 10^4/mm³	RET %	PT Sec.
CONTROL							
	MEAN	754	13.7	44.5	99.6	31	14.0
	S.D.	38.8	0.45	1.66	11.26	6.5	0.22
	(N)	5	5	5	5	5	5
DB 0.5G/KG							
	JUDGE						
	MEAN	735	13.3	43.5	106.2	26	14.1
	S.D.	39.0	0.52	1.23	21.53	4.3	0.33
	(N)	5	5	5	5	5	5
DB 2.5G/KG							
	JUDGE						
	MEAN	749	13.3	43.2	109.3	24	13.9
	S.D.	32.3	0.61	2.16	15.46	6.6	0.35
	(N)	5	5	5	5	5	5

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Table 10 - 2 Hematological findings of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (I)

GROUP		WBC $10^3/\mu l$	L %	N %	E %	B %	M %
CONTROL	MEAN	42	88	11	0	0	1
	S.D.	10.9	5.7	5.9	0.0	0.0	1.1
	(N)	5	5	5	5	5	5
DB 0.5G/KG	JUDGE						
	MEAN	44	89	9	1	0	0
	S.D.	6.4	3.8	3.4	1.2	0.0	0.5
	(N)	5	5	5	5	5	5
DB 2.5G/KG	JUDGE	*					
	MEAN	59	89	9	0	0	1
	S.D.	9.0	4.4	4.7	0.5	0.0	0.0
	(N)	5	5	5	5	5	5

* = SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP ($P<0.05$)

Table 11 - 1 Blood biochemical analyses of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days··· (I)

GROUP		GOT IU/ℓ	GPT IU/ℓ	ALP IU/ℓ	LDH IU/ℓ	CPK IU/ℓ
CONTROL	MEAN	80.8	34.1	256.2	1381.7	389.2
	S.D.	22.50	9.11	32.86	542.50	140.74
	(N)	5	5	5	5	5
DB 0.5G/KG	JUDGE MEAN	74.6	34.0	295.0	1080.6	377.7
	S.D.	4.98	7.24	33.97	309.04	66.16
	(N)	5	5	5	5	5
DB 2.5G/KG	JUDGE MEAN	78.8	36.9	238.0	1126.5	349.4
	S.D.	12.72	4.22	25.54	674.88	139.81
	(N)	5	5	5	5	5

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Table 11 - 2 Blood biochemical analyses of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (II)

GROUP		TP g/dl	ALB g/dl	A/G	T-BIL mg/dl	BUN mg/dl	CRE mg/dl	GLU mg/dl	T-CHO mg/dl	TG mg/dl
CONTROL	MEAN	5.4	3.24	1.49	0.05	17.9	0.42	153.9	52.2	92.2
	S.D.	0.21	0.154	0.202	0.007	0.85	0.022	7.93	4.64	28.49
	(N)	5	5	5	5	5	5	5	5	5
DB 0.5G/KG	JUDGE MEAN	5.6	3.21	1.36	0.05	19.2	0.45	165.9	55.9	113.8
	S.D.	0.14	0.196	0.214	0.019	1.04	0.011	7.61	5.34	33.25
	(N)	5	5	5	5	5	5	5	5	5
DB 2.5G/KG	JUDGE MEAN	5.4	2.96	1.21	0.05	18.9	0.43	159.7	51.9	121.0
	S.D.	0.25	0.186	0.087	0.004	1.25	0.015	8.91	6.62	42.55
	(N)	5	5	5	5	5	5	5	5	5

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Table 11 - 3 Blood biochemical analyses of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days··· (m)

GROUP		Na mEq/ℓ	K mEq/ℓ	Cl mEq/ℓ	Ca mg/dℓ	Pi mg/dℓ
CONTROL						
	MEAN	142.3	4.43	107.3	10.2	7.6
	S.D.	0.57	0.239	1.12	0.19	0.82
	(N)	5	5	5	5	5
DB 0.5G/KG						
	JUDGE					
	MEAN	140.9	4.44	106.2	10.4	7.9
	S.D.	0.56	0.246	1.24	0.23	0.74
	(N)	5	5	5	5	5
DB 2.5G/KG						
	JUDGE	**		*		
	MEAN	139.9	4.72	105.1	10.2	7.2
	S.D.	1.44	0.087	1.13	0.19	0.69
	(N)	5	5	5	5	5

* : SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP ($P<0.05$)
** : SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP ($P<0.01$)

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Table 11 - 4 Blood biochemical analyses of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (II)

GROUP	alb %	α-1 %	α-2 %	α-3 %	β %	γ %
CONTROL						
MEAN	59.7	16.3	5.5	6.0	10.8	1.7
S.D.	3.23	2.99	0.82	0.39	0.73	0.33
(N)	5	5	5	5	5	5
DB 0.5G/KG						
JUDGE						
MEAN	57.4	17.0	6.1	5.9	11.4	2.1
S.D.	3.72	3.46	0.43	0.30	0.66	0.38
(N)	5	5	5	5	5	5
DB 2.5G/KG						
JUDGE						
MEAN	54.9	20.1	6.3	6.4	10.4	1.9
S.D.	1.73	1.26	0.52	0.76	0.80	0.57
(N)	5	5	5	5	5	5

000269

Table 12 - 1 Blood biochemical analyses of female bats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days··· (I)

GROUP		GOT IU/ℓ	GPT IU/ℓ	ALP IU/ℓ	LDH IU/ℓ	CPK IU/ℓ
CONTROL	MEAN	59.7	25.9	152.0	826.1	271.1
	S.D.	8.13	3.12	30.59	276.92	69.51
	(N)	5	5	5	5	5
DB 0.5G/KG JUDGE	MEAN	58.1	28.8	162.2	691.2	231.0
	S.D.	1.88	2.62	19.59	267.36	49.40
	(N)	5	5	5	5	5
DB 2.5G/KG JUDGE	MEAN	63.8	29.1	161.4	810.8	269.6
	S.D.	13.55	7.92	30.49	387.77	110.41
	(N)	5	5	5	5	5

000270

Table 12 - 2 Blood biochemical analyses of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (II)

GROUP		TP g/dl	ALB g/dl	A/G	T-BIL mg/dl	BUN mg/dl	CRE mg/dl	GLU mg/dl	T-CHO mg/dl	TG mg/dl
CONTROL	MEAN	5.9	3.76	1.74	0.06	17.9	0.43	161.2	68.5	57.8
	S.D.	0.31	0.198	0.072	0.013	3.63	0.025	10.75	3.73	9.65
	(N)	5	5	5	5	5	5	5	5	5
DB 0.5G/KG	JUDGE MEAN	6.1	3.74	1.61	0.05	18.8	0.43	166.7	69.6	66.8
	S.D.	0.33	0.346	0.240	0.008	2.84	0.041	11.94	10.94	31.06
	(N)	5	5	5	5	5	5	5	5	5
DB 2.5G/KG	JUDGE MEAN	5.9	3.75	1.72	0.05	20.2	0.42	159.9	63.4	81.4
	S.D.	0.37	0.245	0.103	0.004	3.25	0.019	10.01	14.15	16.18
	(N)	5	5	5	5	5	5	5	5	5

Table 12 - 3 Blood biochemical analyses of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days--- (M)

GROUP		Na mEq/ℓ	K mEq/ℓ	Cl mEq/ℓ	Ca mg/dℓ	Pi mg/dℓ
CONTROL						
	MEAN	140.5	3.98	107.8	10.5	7.5
	S.D.	1.93	0.142	1.60	0.36	0.68
	(N)	5	5	5	5	5
DB 0.5G/KG						
	JUDGE					
	MEAN	139.7	4.12	106.5	10.6	7.1
	S.D.	1.34	0.135	1.94	0.43	0.52
	(N)	5	5	5	5	5
DB 2.5G/KG						
	JUDGE					
	MEAN	139.2	4.32	106.9	10.8	6.7
	S.D.	2.27	0.287	1.08	0.39	0.80
	(N)	5	5	5	5	5

000272

Table 12 - 4 Blood biochemical analyses of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days (x)

GROUP	alb %	α-1 %	α-2 %	α-3 %	β %	γ %
CONTROL						
MEAN	63.6	14.3	4.2	5.5	10.3	2.1
S.D.	1.02	1.54	0.72	0.63	0.70	0.67
(N)	5	5	5	5	5	5
DB 0.5G/KG						
JUDGE						
MEAN	61.6	15.3	3.9	6.0	10.7	2.6
S.D.	3.77	3.11	0.40	0.70	0.69	0.66
(N)	5	5	5	5	5	5
DB 2.5G/KG						
JUDGE						
MEAN	63.3	13.7	4.5	5.5	10.5	2.4
S.D.	1.50	1.24	1.40	0.15	0.68	1.27
(N)	5	5	5	5	5	5

000273

Table 13 Necropsy findings of male and female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days.

		male			female		
	Group	Control	DB		Control	DB	
			0.5 g/kg	2.5 g/kg		0.5 g/kg	2.5 g/kg
Findings	Number of animals	5	5	5	5	5	5
Liver							
Yellowish white nodule		0	0	0	0	0	1 (264)
Thickening of capsule (partially)		0	0	0	0	0	1 (264)
Thymus							
Hemorrhage		1 (013)	0	1 (211)	1 (062)	0	0
Spleen							
Swelling		0	0	1 (215)	0	0	0
Thickening of capsule (partially)		0	0	0	0	0	1 (264)
Kidney							
White nodule (left)		0	0	0	1 (065)	0	0

( ) : Animal number

000274

Table 14 Absolute organ weights of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

GROUP	BODY WEIGHT (G)	THYMUS (G)	LUNGS (G)	HEART (G)	LIVER (G)	SPLEEN (G)	KIDNEYS (G)	ADRENALS (MG)	TESTES (G)
CONTROL									
MEAN	337	0.45	1.11	1.05	12.59	0.66	2.31	46.6	2.97
S.D.	26.0	0.097	0.075	0.119	1.283	0.034	0.249	5.54	0.175
(N)	5	5	5	5	5	5	5	5	5
DB 0.5G/KG JUDGE									
MEAN	341	0.52	1.13	1.06	12.94	0.65	2.38	48.2	2.77
S.D.	12.5	0.088	0.079	0.039	1.041	0.038	0.070	3.11	0.145
(N)	5	5	5	5	5	5	5	5	5
DB 2.5G/KG JUDGE									
MEAN	328	0.38	1.13	1.07	12.07	0.80	2.46	48.8	2.87
S.D.	25.2	0.042	0.100	0.077	1.090	0.425	0.211	7.95	0.205
(N)	5	5	5	5	5	5	5	5	5

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Table 15 Relative organ weights of male rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

GROUP	BODY WEIGHT (G)	ORGAN WEIGHT (G OR MG) PER 100G OF BODY WEIGHT							
		THYMUS G(%)	LUNGS G(%)	HEART G(%)	LIVER G(%)	SPLEEN G(%)	KIDNEYS G(%)	ADRENALS MG(%)	TESTES G(%)
CONTROL									
MEAN	337	0.13	0.33	0.31	3.73	0.20	0.68	13.8	0.88
S.D.	26.0	0.032	0.016	0.020	0.105	0.017	0.027	0.68	0.090
(N)	5	5	5	5	5	5	5	5	5
DB 0.5G/KG JUDGE									
MEAN	341	0.15	0.33	0.31	3.79	0.19	0.70	14.1	0.81
S.D.	12.5	0.023	0.022	0.017	0.194	0.007	0.026	0.44	0.026
(N)	5	5	5	5	5	5	5	5	5
DB 2.5G/KG JUDGE									
MEAN	328	0.12	0.34	0.33	3.68	0.24	0.75**	14.8	0.88
S.D.	25.2	0.018	0.027	0.022	0.219	0.114	0.030	1.62	0.069
(N)	5	5	5	5	5	5	5	5	5

** : SIGNIFICANTLY DIFFERENT FROM CONTROL GROUP (P<0.01)

Table 16 Absolute organ weights of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

GROUP	BODY WEIGHT (G)	THYMUS (G)	LUNGS (G)	HEART (G)	LIVER (G)	SPLEEN (G)	KIDNEYS (G)	ADRENALS (MG)	OVARIES (MG)
CONTROL									
MEAN	226	0.52	0.97	0.86	8.89	0.49	1.80	59.8	88.2
S.D.	16.0	0.108	0.073	0.093	0.972	0.067	0.205	5.40	14.37
(N)	5	5	5	5	5	5	5	5	5
DB 0.5G/KG JUDGE									
MEAN	236	0.48	1.00	0.88	9.81	0.58	1.85	63.5	94.0
S.D.	15.5	0.046	0.042	0.097	1.328	0.128	0.123	4.53	20.49
(N)	5	5	5	5	5	5	5	5	5
DB 2.5G/KG JUDGE									
MEAN	220	0.51	0.94	0.82	9.06	0.52	1.75	59.7	88.8
S.D.	18.5	0.075	0.087	0.130	1.227	0.115	0.207	11.75	16.24
(N)	5	5	5	5	5	5	5	5	5

Table 17 Relative organ weights of female rats treated orally with Dunaliella Bardawil Spray Dried Powder for 28 days

GROUP	BODY WEIGHT (G)	ORGAN WEIGHT (G OR MG) PER 100G OF BODY WEIGHT							
		THYMUS G(%)	LUNGS G(%)	HEART G(%)	LIVER G(%)	SPLEEN G(%)	KIDNEYS G(%)	ADRENALS MG(%)	OVARIES MG(%)
CONTROL									
MEAN	226	0.23	0.43	0.38	3.93	0.22	0.79	26.5	39.0
S.D.	16.0	0.040	0.027	0.030	0.178	0.019	0.045	1.62	5.14
(N)	5	5	5	5	5	5	5	5	5
DB 0.5G/KG									
JUDGE									
MEAN	236	0.20	0.43	0.37	4.15	0.25	0.78	27.0	39.9
S.D.	15.5	0.013	0.029	0.034	0.341	0.052	0.011	0.22	7.90
(N)	5	5	5	5	5	5	5	5	5
DB 2.5G/KG									
JUDGE									
MEAN	220	0.23	0.43	0.37	4.10	0.24	0.80	27.0	40.3
S.D.	18.5	0.029	0.042	0.031	0.230	0.053	0.063	3.73	6.15
(N)	5	5	5	5	5	5	5	5	5

Table 18 Histopathological findings of male rats treated orally with Dunaliella Bardawil spray Dried Powder for 28 days

Findings	Group	control					DB 0.5g/kg					DB 2.5g/kg				
	Number of animals	5					5					5				
	Grade	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++
Liver																
Cellular infiltration		4	1	0	0	0	3	2	0	0	0	4	0	1	0	0
Kidney																
Cellular infiltration(interstitial)		4	1	0	0	0	5	0	0	0	0	4	1	0	0	0
Basophilic change of renal tubules		5	0	0	0	0	4	1	0	0	0	5	0	0	0	0
Cyst		5	0	0	0	0	5	0	0	0	0	4	0	1	0	0
Thymus*																
Hemorrhage		0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
Spleen*																
Extramedullary hematopoiesis		0	0	0	0	0	0	0	0	0	0	0	0	1	0	0

−: No remarkable chenges ±: Minimal +: Slight ++: Moderate +++: Marked

* : Abnomal findings were observed at necropsy

No remarkable changes in Adrenal gland

Table 19 Histopathological findings of female rats treated orally with Dunaliella Bardawil spray Dried Powder for 28 days

Findings	Group	control					DB 0.5g/kg					DB 2.5g/kg				
	Number of animals	5					5					5				
	Grade	−	±	+	++	+++	−	±	+	++	+++	−	±	+	++	+++
Liver																
Cellular infiltration		0	2	3	0	0	3	2	0	0	0	2	3	0	0	0
Foreign body granuloma		5	0	0	0	0	4	0	1	0	0	5	0	0	0	0
Thickening of capsule		5	0	0	0	0	5	0	0	0	0	4	0	1	0	0
Granuloma		5	0	0	0	0	5	0	0	0	0	4	0	1	0	0
Kidney																
Cellular infiltration(interstitial)		4	1	0	0	0	5	0	0	0	0	4	1	0	0	0
Cyst		5	0	0	0	0	5	0	0	0	0	4	0	1	0	0
Focal fibrosis		5	0	0	0	0	4	0	1	0	0	5	0	0	0	0
Nephroblastoma#		4	−	1	−	−	5	−	0	−	−	5	−	0	−	−
Thymus*																
Hemorrhage		0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Spleen*																
Thickening of capsule		0	0	0	0	0	0	0	0	0	0	0	0	1	0	0

−: No remarkable changes ±: Minimal +: Slight ++: Moderate +++: Marked

* : Abnomal findings were observed at necropsy

No remarkable changes in Adrenal gland

: This finding is indicated by only + or −

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試験計画書

表　題

Dunaliella Bardawil Spray Dried Powderのラットにおける28日間経口投与による亜急性毒性試験

試験委託者

~~日健製薬工業株式会社~~ 株式会社 日健総本社

~~岐阜県羽島市福寿町平方 4 - 41~~ 岐阜県羽島市福寿町茂平 1丁目32番地

試験施設

株式会社日本バイオリサーチセンター羽島研究所

岐阜県羽島市福寿町間島 6 - 104

試験目的

Dunaliella Bardawil Spray Dried Powderの安全性に関する試験の一環として、Dunaliella Bardawil Spray Dried Powderをラットに28日間連続経口投与し、その安全性について検討する。

試験関係者名

試験責任者　古橋忠和

試験担当責任者　宇土研二

試験日程

試験開始日（運営管理者の署名捺印日）	昭和64年 1月 6日
動物入荷日	昭和64年 2月 9日
群分け日	昭和64年 2月21日
投与開始日	昭和64年 2月22日
投与終了日	昭和64年 3月21日
剖検日	昭和64年 3月22日
標本観察終了日	昭和64年 7月31日
報告書（案）提出予定日	昭和64年 9月30日
試験終了日（最終報告書提出予定日）	昭和64年10月30日

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署名・捺印

試験責任者

(b) (6) 印　昭和64年1月5日

所　属：株式会社日本バイオリサーチセンター羽島研究所

試験計画書の承認

竹中 裕行　平成元年1月17日

試験委託者

(b) (6) 印　~~昭和64年1月7日~~

所　属：~~日健製薬工業株式会社~~
株式会社日健総本社

運営管理者

(b) (6) 印　昭和64年1月5日

所　属：株式会社日本バイオリサーチセンター羽島研究所

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1. 被験物質

（1）被験物質

被験物質名：Dunaliella Bardawil Spray Dried Powder（略称：DB）

ロット番号：~~(入手後記載)~~ NSK-1207

性　　状：赤褐色の粉末で、水に溶けない

安 定 性：原末は窒素置換後、密封保存で 1年以上安定であり、冷蔵・遮光あるいは冷凍保存で最低 2ヵ月間安定。 0.5%カルボキシメチルセルロースナトリウム水溶液中では、冷蔵（約 5℃）・遮光保存で 5および25%の濃度は 5時間まで安定。

入 手 日：~~昭和~~平成元年2月2日 ~~(入手後記載)~~

入 手 量：~~(入手後記載)~~ 416.0 g

保管場所：試験施設の被験物質保管室

保管条件：遮光、~~冷蔵~~冷凍 ~~(約 5℃)~~ -20

提 供 源：~~日健製薬工業株式会社~~ 株式会社日健総本社

（2）残余被験物質の返却

投与終了後に使用記録（写）を添え、~~試験委託者に返却する。~~

2. 投与検体の調製法

被験物質に必要量の0.5%カルボキシメチルセルロースナトリウム水溶液 ~~[CMC-Na、~~ [CMC-Na；ロット番号；109E5046，製造元；日本化学工業株式会社，注射用蒸留水のロット番号；8F8 ~~注射用蒸留水のロット番号および製造元は購入後記載~~ を加え、所定濃度となるよう ~~製造元；株式会社大塚製薬工場]~~ 懸濁または溶解する。 調製は投与終了時まで要する時間が調製後 5時間を越えない範囲で行う。 なお、使用後の残余液は廃棄する。

3. 試験動物

（1）動物種、系統および選択理由

1)動物種、系統：ラット(SPF)　Crj:CD(SD)

2)選 択 理 由：ラットは毒性試験に一般的に用いられている動物種で、当試験に使用するラットはその系統維持が明らかであり、集積データも揃っているため。

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（2）入荷時の週齢

4週齢

（3）入荷日、性別および入荷匹数

平成元年2月9日 雄21匹
~~昭和64年 2月 9日 雄 20匹~~
平成元年2月9日 雌21匹
~~昭和64年 2月 9日 雌 20匹~~

（4）入荷翌日の体重範囲

雄：55～ 105g 、雌：55～ 105g

（5）供給源

日本チャールス・リバー株式会社

（6）検疫および馴化

入荷したラットは、約 2週間の予備飼育期間を設け、この間 2回以上の体重測定と連日に外見および一般状態の観察を行い、検疫・馴化とする。

（7）群分け法

予備飼育期間中の体重推移、外見および一般状態に異常の見られない約 6週齢の動物を選び群分けに用いる。 群分けは、投与開始の前日に体重を層別後、無作為抽出法により、各群の平均体重および分散がほぼ等しくなるように行う。群分け後の残余動物は、初回投与日に麻酔死処分する。

（8）個体識別法

予備飼育期間中は油性インクペンで尾に印をつけることにより、群分け後は色素による染毛法および耳パンチ法の併用により識別する。 予備飼育期間中の各ケージには試験番号、入荷年月日および馴化動物番号を記入したラベルを、群分け後の各ケージには試験番号、被験物質名、投与量、動物番号などを記入した色別のラベルを装着する。

（9）環境条件

室温20～24℃、湿度40～70%、明暗各12時間（蛍光灯による照明時間：午前 6時～午後 6時）、換気回数12回／時間（フィルターにより除塵された新鮮空気）に設定された動物飼育室（C棟4号室）で飼育する。

飼育ケージは、予備飼育期間中は床敷（チップ、千葉アニマル資材株式会社）

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を入れたプラスチック製ケージ（800×855×175mm）を用いて 1ケージ当たり 5匹までの群飼いとし、群分け後はステンレス製五連ケージ（755×210×170mm）を用いて個別飼育する。 プラスチック製ケージの交換、ステンレス製五連ケージの受け皿交換、ならびに給水瓶交換は 1週間に 2回以上、ステンレス製五連ケージ（給餌器とも）交換は 2週間に 1回以上行う。

(10) 飼料

入荷後 3ヵ月以内の固型飼料（CRF-1、オリエンタル酵母工業株式会社）を、給餌器を用いて自由に摂取させる。 使用した飼料中の汚染物質の分析結果は、当該試験期間中に用いたロットについて財団法人日本食品分析センターで実施した成績を製造業者から入手する。

(11) 飲料水

上水道水をポリカーボネート製給水瓶を用いて自由に摂取させる。 飲料水の水質検査は、ほぼ 3ヵ月毎に財団法人岐阜県公衆衛生検査センターで実施した成績を入手する。

(12) 床敷

床敷の汚染物質等の分析結果は、ほぼ 1年毎に財団法人東京顕微鏡院で実施した成績を入手する。

4．投与法および投与区分

(1) 投与経路およびその選択理由

DBは食品であるため、ラットを用いた当試験では経口投与による亜急性毒性について検討する。

(2) 投与方法および投与液量

投与日に最も近い測定日の体重を基準とした投与液量10ml/kgで、金属製経口ゾンデを用いて強制経口投与する。

(3) 投与回数および投与期間

投与初日を投与 1日と起算し、 1日 1回（原則として午前 8時から午前12時までの間）の連日の28回投与する。

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（4）投与量および群構成

群	試験群	投与量	ラベルの色	雄（動物番号）	雌（動物番号）
第1群	対照(0.5%CMC-Na水溶液)	－	白　色	5(011～015)	5(061～065)
第2群	DB	0.5g/kg	黄　色	5(111～115)	5(161～165)
第3群	DB	2.5g/kg	赤　色	5(211～215)	5(261～265)

（5）投与量設定の理由

先に実施されたDBのマウスによる急性毒性試験（絶食下）の結果を参考にし、試験委託者と協議して決定した。　すなわち、投与可能な最大量と考えられる10g/40ml/kg投与によっても一般状態、体重推移および剖検で何ら異常が認められなかったが、この条件下では非絶食での連続投与は困難と判断された。したがって、本試験の投与量は一般的に用いられている投与液量の10ml/kgで換算し、高用量群に2.5g/kgを、低用量群に0.5g/kgを設定する。　対照群には、0.5%CMC-Na水溶液を被験物質投与群と同容量投与する。

5．観察．検査項目

（1）一般状態

試験期間中の全動物について、一般状態および死亡の有無を 1日 2回（但し、剖検日は 1回のみ）観察する。　死亡動物は発見後可及的速やかに剖検し、器官・組織の肉眼的な観察を行い、可能な限り生存例の剖検時におけると同様に、病理組織学的検査を行う。　また死期の迫りが明白と考えられた動物（瀕死例）については採血後速やかに剖検し、可能な限り剖検時におけると同様に血液学的検査、血液生化学的検査、器官重量の測定、病理組織学的検査を行う。

（2）体重測定

1週間に 2回測定する。

（3）摂餌量測定

1週間に 1回（連続 2日間量を測定し 1日量を算出する）測定する。

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（4）尿検査

全例について投与期間終了前に絶食・摂水下で採尿ケージに入れ、 8時間で採取した尿（ 8時間尿）と引き続いて摂餌・摂水下で21時間で採取した尿（合計24時間尿）について、以下の検査を実施する。

検査項目

尿量……重量測定（24時間尿）

色調……外観判定（ 8時間尿）*

比重……屈折計　（21時間尿、屈折型比重計：ユリベット・日本光学工業株式会社）*

pH、潜血、蛋白、糖、ケトン体、ウロビリノーゲン、ビリルビン……（ 8時間尿、エームスクリニテック用検査紙：マイルス・三共株式会社）*

沈渣……鏡検（ 8時間尿）*

* 8時間（検体投与後のほぼ午前10時～午後1時）で採尿できない場合は当該項目は検査しない。

（5）眼科的検査

初回投与開始前および投与期間終了前に全例について散瞳剤（ミドリンP・参天製薬株式会社）で散瞳させたのちに眼底カメラ（RC-2 Model 621・興和株式会社）により眼底を検査し、両眼の眼底撮影を行う。

（6）血液学的検査

最終投与の翌日に、ペントバルビタールナトリウムの腹腔内投与による麻酔下で腹大動脈からEDTA-2Kコーティングの Sysmexカップに血液を採取し、以下の血液学的検査を原則として全例について行う（残余血液は廃棄する）。 但しプロトロンビン時間は、3.13%クエン酸ナトリウム処理した血漿を用いる。

1)赤血球数、白血球数、ヘモグロビン量、ヘマトクリット値、血小板数……Sysmex E-2000 （東亜医用電子株式会社）

2)プロトロンビン時間……FibroSystem (BBL、Div. of Becton, Dickinson & Co.)

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3)網状赤血球……目算法

4)白血球百分率……目算法

（7）血液生化学的検査

最終投与の翌日にペントバルビタールナトリウムの腹腔内投与による麻酔下で腹大動脈から採取した血液から得た血清につき、以下の血液生化学的検査を原則として全例について行う。 血清は、測定時までは-80℃設定のディープフリーザーに保管し、測定後は最終報告書提出時まで-20℃設定のフリーザーに保管する。 その後の保管は試験委託者と協議して決定する。

ＧＯＴ、ＧＰＴ、ＡＬＰ、ＣＰＫ、ＬＤＨ、総コレステロール、トリグリセライド、総蛋白、尿素窒素、クレアチニン、総ビリルビン、ブドウ糖、Ｃａ、無機リン……AU 500（オリンパス光学工業株式会社）

Ｎａ、Ｋ……FLAME-80C（炎光光度計、日本分光メディカル株式会社）

Ｃｌ　……Chloride meter C-200AP（電量滴定法、株式会社常光）

蛋白分画（アルブミン量も含む）、A/G比……AES600（電気泳動法、オリンパス光学工業株式会社）

（8）剖検

最終投与の翌日に、（6）、（7）の項で採血した動物を更に放血致死後、器官・組織の肉眼的観察を行い、所見を記録する。 異常の認められた器官・組織およびその比較となる同部位の対照群の器官・組織について写真撮影（代表例）を行い、10%中性ホルマリン液に固定・保存する。

（9）器官重量の測定

最終投与の翌日の剖検時に全例について以下の器官重量を測定する（対器官は一括秤量する）。 更に剖検前の体重を基準として器官重量の体重比（相対重量）を算出する。 なお、測定後以下の器官は10%ホルマリン液に固定・保存する。

心臓、肺（気管支を含む）、胸腺、肝臓、脾臓、腎臓、副腎、♂：精巣、♀：卵巣。

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(10) 病理組織学的検査

全例の肝臓、腎臓および副腎について10%中性ホルマリン液に固定し、常法に従いパラフィン包埋後、ＨＥ染色組織標本を作製し、病理組織学的検査を行う。その他、剖検で異常が認められた部位についても実施する。　代表例について写真撮影する。

6. 統計学的方法

体重、摂餌量、血液学的検査、血液生化学的検査、器官重量、相対重量、尿量および尿比重は、各群で平均値および標準偏差を算出する。

対照群とDB投与群との有意差検定は多重解析(ASSIT解析；添付の用紙 1、 2参照）を用い、危険率 5%未満を有意とする。

7. 試験計画書の修正

試験受託契約の締結後に、試験計画書の重要な内容変更の必要性が生じた場合には、試験委託者と試験施設の両者間で協議して合意の上で変更し、変更箇所、変更内容、変更理由を記した試験計画書修正書を作成して試験計画書とともに保管する。

8. 資料および標本の保管

この試験に関するすべての資料および標本は、株式会社日本バイオリサーチセンターの資料および標本保管室に保管する。　保管期間は別途契約による。

（1）試験計画書、主計画表、試験計画書修正書、契約書

（2）被験物質に関する記録

1)受領・返却記録

2)特性および安定性に関する記録（試験委託者から入手したもの）

3)使用記録

4)保管記録

（3）環境条件および汚染物質分析記録

1)飼育室内環境記録

2)飼育管理記録

3)飼料中の汚染物質分析記録

4)飲料水の水質検査記録

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5)床敷の汚染物質分析記録

（4）試験動物に関する記録

1)動物受入記録

2)検疫および馴化記録

3)動物群分け記録

4)動物の移動および処理記録

5)ケージラベル

（5）投与に関する記録

1)投与液量算出記録

2)投与記録

（6）観察・検査記録

1)経日死亡記録

2)一般状態観察記録

3)体重測定記録

4)摂餌量測定記録

5)尿検査記録

6)眼科的検査記録

7)血液学的検査記録

8)血液生化学的検査記録

9)器官重量測定記録、相対重量記録

10)剖検所見記録

11)器官確認記録

12)病理組織標本作製記録

13)病理組織学的検査記録

14)写真撮影記録

（7）標本

1)ホルマリン固定標本およびパラフィン包埋標本

2)病理組織標本

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3)血液塗抹標本

4)血清（最終報告書提出まで）

5)写真およびネガフィルム

（8）報告書に関する保管資料

1)報告書作成用に整理した図、表

2)統計処理データ

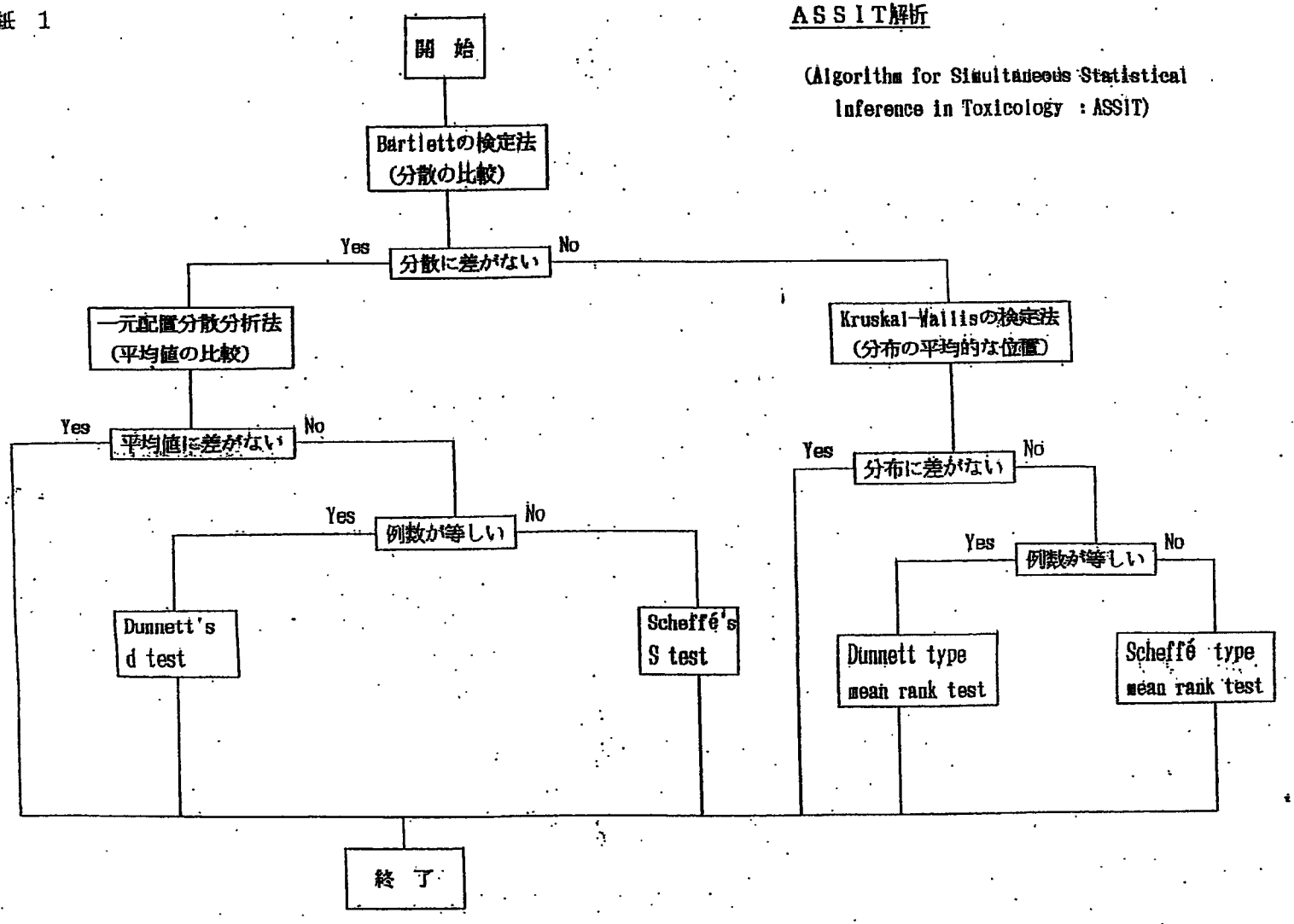
3)報告書（案）

4)最終報告書の写し

別紙 1

ASSIT解析

(Algorithm for Simultaneous Statistical Inference in Toxicology : ASSIT)



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別紙 2

検定結果は、Appendixの最下段に以下の記号で表示する。

ANH0 : 一元配置分散分析法で有意差なし
ANH1 : 一元配置分散分析法で有意差あり

KWH0 : Kruskal-Wallisの検定法で有意差なし
KWH1 : Kruskal-Wallisの検定法で有意差あり

DUH0 : Dunnett's d testで有意差なし
DUH1 : Dunnett's d testで有意差あり

SCH0 : Scheffé's S testで有意差なし
SCH1 : Scheffé's S testで有意差あり

DTH0 : Dunnett type mean rank test で有意差なし
DTH1 : Dunnett type mean rank test で有意差あり

STH0 : Scheffé type mean rank test で有意差なし
STH1 : Scheffé type mean rank test で有意差あり

試験計画書の修正書（No.1）

試験番号：5118

表　題：Dunaliella Bardawil Spray Dried Powderのラットにおける28日間経口投与による亜急性毒性試験

修正箇所、修正理由：

p.1　試験委託者

日健製薬工業株式会社

↓

株式会社日健総本社

p.2　試験計画書の承認

試験委託者　白石照雄　印　昭和64年 1月 7日

所　属：日健製薬工業株式会社

↓

竹中祐行 印　平成 元年 1月 17日

所　属：株式会社日健総本社

上記アンダーラインの部分を下段の如く修正する。

修正理由：試験委託者側担当者の変更があったため。

修　正

試験責任者 (b) (6) 印　平成 元年 1月 17日

承　認

運営管理者 (b) (6) 印　平成 元年 1月 17日

試験委託者 (b) (6) 印　平成 元年 1月 17日

000294

試験計画書の修正書（No.2）

試験番号：5118

表　題：Dunaliella Bardawil Spray Dried Powderのラットにおける28日間経口投与による亜急性毒性試験

修正箇所、修正理由：

p.3　1. 被験物質

（1）被験物質

ロット番号：（入手後記載）　→　NSK-1207

入 手 日：昭和　年　月　日（入手後記載）　→　平成元年 2月 2日

入 手 量：（入手後記載）　→　416.0g

保管条件：遮光、冷蔵（約 5℃）　→　遮光、冷凍（約－20℃）

上記アンダーラインの部分を右記の如く修正する。

修正理由：入手後に記載することとしていたため。　また、保管条件については試験委託者の指示による。

p.4　（8）個体識別法

予備飼育期間中は油性インクペンで尾に印をつけることにより、----------

↓

予備飼育期間中は色素による染毛法と油性インクペンで尾に印をつけることにより、----------

上記アンダーラインの部分を下段の如く修正する。

修正理由：標準操作手順書（ＳＯＰ）の改訂に伴う。

修　正

試験責任者 (b) (6) 印　平成 元 年 2 月 6 日

承　認

運営管理者 (b) (6) 印　平成 元 年 2 月 6 日

試験委託者 (b) (6) 印　平成 元 年 2 月 8 日

000295

試験計画書の修正書（No.3）

試験番号：5118

表　題：Dunaliella Bardawil Spray Dried Powderのラットにおける28日間経口投与による亜急性毒性試験

修正箇所、修正理由：

p.3　1．被験物質

(1) 被験物質

提 供 源：日健製薬工業株式会社　→　株式会社日健総本社

上記アンダーラインの部分を右記の如く修正する。

修正理由：社名の修正わすれのため。

p.3　2．投与検体の調製法

被験物質に必要量の 0.5%カルボキシメチルセルロースナトリウム水溶液［CMC-Na：ロット番号；109E5046、製造元；関東化学工業株式会社、注射用蒸留水：ロット番号；8F82N 、製造元；株式会社大塚製薬工場］を加え、所定濃度となるよう懸濁または溶解する。-------

上記アンダーラインの部分を追記する。

修正理由：購入後に記載することとしていたため。

p.4　(3) 入荷日、性別および入荷匹数

昭和64年 2月 9日　雄　20匹　→　平成元年 2月 9日　雄　21匹

昭和64年 2月 9日　雌　20匹　→　平成元年 2月 9日　雌　21匹

上記アンダーラインの部分を右記の如く修正する。

修正理由：動物納入業者の都合により上記匹数の動物が入荷したため。　また、元号については実情に合わせる。

000296

修 正

試験責任者 (b) (6) 印 平成 元年 9 月 25 日

承 認

運営管理者 (b) (6) 印 平成 元年 9 月 26 日

試験委託者 (b) (6) 印 平成 元年 9 月 28 日

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CERTIFICATE OF TRANSLATED CONTENTS

Dated: 8th February 2007

We, the undersigned, hereby certify that the documents attached are the original manuscript text of the following as well as a matching translation of the relevant text faithfully rendered into English.

Text details:	1. ヒトにおけるβ-カロテン長期間投与時の血中動態および効果に関する研究 2. 報告書：Dunaliella Bardawil Paste の変異原性試験（細菌を用いる復帰変異試験） 3. 最終報告書：Dunaliella Bardawil Spray Dried Powder のラットにおける28日間経口投与による亜急性毒性試験 <Total pages: 31>

Translation details:	1. Bioavailability of Synthesized and Naturally-occurring β-Carotene in Humans 2. Study Report: Mutagenecity Study of Dunaliella Bardawil Paste (Reversion test with bacteria) 3. Final Report: Subacute Toxicity Study of Dunaliella Bardawil Spray-Dried Powder by 28-Day Oral Administration in Rats <Total pages: 33>

(b) (6)

Ikuo HIGASHI

President

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000299

株式会社日本バイオリサーチセンター

Study No.9137

STATEMENT

Study No. : 9137

Title : Mutagenecity Study of Dunaliella Bardawil Paste (Reversion test with bacteria)

This study was performed incompliance with the Good Laboratory Practice Standard for Safety Studies on Drugs (Notification No.313 of the Pharmaceutical Affairs Bureau, Japanese Ministry of Health and Welfare, March 31, 1982)

January 16, 2007

Hashima Laboratory, Nihon Bioresearch Inc.

Study Director : (b) (6)

Fumihiko Aruga

000300

Correction

Dated: 20th February 2007

Due to a mistake in the original translation, the correction for "Test Protocol: Mutagenicity Test of Domalicilla Bordeaux Paste" is stated below.

Original translation:	2. Growth inhibition was recognized at 5000 μg/plate for TA1000 and at 2500 and 5000 μg/plate for TA1537 without S9Mix; and at 5000 μg/plate for TA1537 with S9Mix. <page 6>

Correction:	2. Growth inhibition was recognized at 5000 μg/plate for TA100 and at 2500 and 5000 μg/plate for TA1537 without S9Mix; and at 5000 μg/plate for TA1537 with S9Mix. <page 6>

(b) (6)

Terukisa Tsumoda
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Tokyo 105-0021, JAPAN

000301

Mutagenecity Study of Dunaliella Bardawil Paste

(Reversion test with bacteria)

Study Report

Submitted on February 5, 1988

Hashima Laboratory
Nihon Bioresearch Inc.

000302

Summary of the study (Study No.9137)

Title	**Mutagenecity Study of Dunaliella Bardawil Paste** **(Reversion test with bacteria)**
Study No.	9137
Name of test substance	Dunaliella Bardawil paste
Test system	Salmonella typhimurium;TA100,TA98,TA1535,TA1537 E.coli;WP2uvrA$^-$
Sponsor	NIKKEN SOHONSHA 1-32, Asahira, Fukuju-cho, Hashima-shi, Gifu-ken
Study site	Hashima Laboratory, Nihon Bioresearch Inc. 4-41, Hirakata, Fukuju-cho, Hashima-shi, Gifu-ken
Purpose of the study	A reversion test with bacteria was conducted using the Ames method,[1),2)] as part of the safety study of Dunaliella Bardawil Paste intended to be used as a dietary supplement. The test was carried out in accordance with the "Guideline for toxicity studies of drugs" (Yakushin No. 118, February 15, 1984), to examine the potential mutagenicity of Dunaliella Bardawil Paste.
~~Study period~~	~~August 25, 1987 to February 5, 1988~~
Storage of data and samples	Hashima Laboratory, Nihon Bioresearch Inc. (All data and samples obtained for the implementation of the study shall be stored for five years in the data storeroom and sample storeroom in Hashima Laboratory, Nihon Bioresearch Inc. after submission of the final report. Subsequent storage shall be decided by discussion with the sponsor.)

Signed and seal-stamped by the persons engaged in the study (Study No.9137)

Title	Mutagenicity study of Dunaliella Bardawil Paste (Reversion test with bacteria)
Study No.	9137

Study director:	Fumihiko Ariga	February 5, 1988
Study managing director:	Yoshiko Nagasawa	February 4, 1988
Person in charge of the study:	Mari Hiki	February 4, 1988

February 5, 1988

Facility manager: Kenichi Nakagawa

Persons engaged in the study and job assignment (Study No.9137)

Study director:	Supervision over implementation of the study, preparation of the protocol and of the report	Fumihiko Ariga
Study managing director:	Implementation of the study, preparation of samples and summary of data	Yoshiko Nagasawa
Person in charge of the study:	Tabulation	Mari Hiki

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Introduction

A reversion test with bacteria was conducted using the Ames method,[1), 2)] as part of the safety study of Dunaliella Bardawil Paste intended to be used as a dietary supplement. The test was carried out in accordance with the "Guideline for toxicity studies of drugs" (Yakushin No. 118, February 15, 1984), to examine the potential mutagenicity of Dunaliella Bardawil Paste.

The study was scheduled as follows:

1. Preliminary test

Preincubation of bacteria:	December 15, 1987
Implementation of the study:	December 16, 1987
Date of evaluation:	December 18, 1987

2. Main test

Preincubation of bacteria:	December 22, 1987
Implementation of the study:	December 23, 1987
Date of evaluation:	December 25, 1987

Test substance and positive control substances

Dunaliella Bardawil Paste (DB) is a brownish yellow paste with 60% of water.[3)] DB (lot No.NK-1010), provided by Nikken Seiyaku Kogyo (net:7.5g) was stored in a refrigerator, in a test substance storage room of the testing facility protected from light.

The test substance was dissolved or suspended in distilled water (Wako Junyaku, lot No.P1071), and the remaining sample solution was discarded after use. The volume of distilled water needed to prepare the sample solution was determined considering the water content in the test substance (60%), and the concentration of the active substance needed for the test. The stability of DB in distilled water will be confirmed by the sponsor. In this study, the sample solution was prepared just before the test and used within three hours.

Commercialized products were used as positive control substances. Table 1 shows their name, manufacturer, lot No., concentration used and solvent. Since their stability was unknown, the positive control substances were also prepared just before use.

Test method

1. Strain used in the study

The name, origin, and storing method of the strain are shown in Table 2. One month before implementation of the study, the features of the test strain (presence/absence of amino acid requirement, ultraviolet sensitivity, rfa mutation or R-factor plasmid, drug resistance factor) were examined according to the "New guidebook for mutagenicity study with bacteria",[4] and the strain compatibility was confirmed (Table 3). 0.07 mL of dimetyl sulphoxide (DMSO) sterilized by filtration (Millipore FH type) and 0.8 mL of the bacteria suspension was filled in a screw tube for sterilization, frozen with dry ice-acetone mixture, and stored in a deep freezer (Sanyo, type: MDF-190AT) set at –80°C. For preincubation of the test strain, 100 mL of distilled water was added to 2.5g of NUTRIENT BROTH No.2 (OXOID) and sterilized in an autoclave for 20 minutes at 121°C. Then, 10 mL-aliquots were dispensed in L-shape tubes sterilized by dry heat for one hour at 181°C. The cryopreserved bacteria were scraped, inoculated and incubated with shaking in a water bath incubator (Taiyo Kagaku Co., Ltd., type: M-100^{N}) at 37±0.5°C for sixteen hours to obtain the bacteria suspension. The bacteria suspension was placed in ice before use.

2. S9Mix

For the S9Mix, and according to the conditions listed in Table 4, products manufactured by Oriental Yeast Co., Ltd. on September 4, 1987 were purchased on September 18 the same year and stored in a deep freezer set at -80°C until use.

The composition of the S9Mix is shown in Table 5. The Cofactor for S9Mix (Oriental Yeast Co., Ltd., Lot No.702) was dissolved with distilled water, sterilized by filtration (Millipore HA type), and added to S9 before use.

3. Medium

Minimal glucose agar plates were prepared with 30 mL each of minimal Vogel-Bonner E medium containing 2wt% of glucose and 2wt% of agar powder (Shoei, Lot No.K-151) in γ-sterilized petri dishes (90 mm Ø, Falcon # 1001). For the top overlay, agar containing 0.6wt% of Bacto-Agar (DIFCO) and 0.5wt% of sodium chloride was prepared and mixed with an amino acid solution at the ratio of 10:1. As the amino acid solutions, an aqueous solution of 0.5mM L-histidine and 0.5mM biotin was used for each strain of *Salmonella typhimurium* and a 0.5mM L-tryptophan aqueous solution sterilized by filtration (Millipore FH type) was used for E.coli.

4. Sterility test

2 mL of top agar was added to 0.1 mL each of the sample solution containing 5000 μg of the test substance per 0.1 mL and of S9Mix, spread over minimal glucose agar plates, and incubated upside down at 37±0.5°C for 48 hours to confirm sterility. Two plates were used, respectively, and sterility was confirmed.

000308

5. Test method

(1) Preliminary test

A preliminary test was conducted to choose the concentrations to be used in the study and the test methods.

The test was conducted by the plate incorporation and preincubation methods, under the conditions shown in Table 6, using two plates for each concentration, with or without the presence of S9Mix.

As for the concentrations, according to the "Guideline for toxicity studies of drugs"(Yakushin No.118, February 15 in 1984), 5000 μg/plate was the maximum concentration, followed by 1000, 500 and 100 μ/plate and solvent control (distilled water).

The plates were examined to assess the inhibitory effect of the test substance under an inverted microscope at a 100-fold magnification (Olympus, CK); after macroscopic observation of the precipitate on the plate, the number of colonies were counted.

As a result, no growth inhibition of any strain was observed by the plate incorporation method in the presence or absence of S9Mix (Tables 7 and 8). On the other hand, the growth of TA100, TA1535, TA98 and TA1537 strains, was inhibited when cultured in the presence of 5000 μg of the test substance without S9Mix; whereas, the growth of TA100 and TA1537 strains was inhibited at this concentration in the presence of S9Mix.

A precipitate was recognized on the plates either by the incorporation method or preincubation method at concentrations of 1000 μg or more with or without S9Mix.

No increases in the number of reversed colonies/plate were recognized by the incorporation method or preincubation method for any strain, regardless of the presence of S9Mix, compared to the solvent control.

(3) Main test

Although there was no increase in the number of reversed colonies with either method, a bigger inhibitory tendency was recognized with the preincubation method than with the plate incorporation method.

Therefore, the preincubation method was adopted for the study, with and without metabolic activation; that is, in the presence and absence of S9Mix, respectively. In the absence of metabolic activation, 0.1 mL of sample solution, 0.5 mL of 0.1M phosphate buffer (pH7.4) sterilized by autoclave and 0.1 mL of the strain suspension were added in sequence to a test tube sterilized by dry heat (12.5×75mm, THERMO Corporation, clean test tube Larbo) and preincubated at 37±0.5°C for 20 minutes. Then, the bacteria were plated and 2 mL of top agar at 45°C was added and incubated in a thermostat bath (YAMATO SCIENTIFIC CO., LTD., type IA-81) at 37±0.5°C for 48 hours, with the plate turned upside down. In the presence of metabolic activation, the 0.5 mL 0.1M phosphate buffer was replaced by 0.5 mL of S9Mix. Regarding the concentration of DB, since the inhibitory effect was recognized only at the concentration of 5000 μg/plate in the preliminary test, this was the maximum concentration used for all strains, followed by 2500, 1250, 625, 312.5 μg/plate. In addition, for all strains, the test with the solvent control (distilled water) or positive control was conducted with or without S9Mix. Three plates were used at each concentration. The plates were examined under an inverted microscope at a 100-fold magnification to assess the effect of the test substance. After macroscopic observation of the precipitate on the plate, the reversed colonies were counted and the mean number obtained.

000309

(3) Criteria

Cases were considered positive when the number of reversed colonies was more than twice the number obtained with the solvent control, and a concentration-dependent increase was recognized.

Storage of the data

The data will be stored in the data storeroom of the testing facility for the time being. Future storage will be discussed and decided with the sponsor.

000310

Test results and conclusion

The results are shown in Tables 9 and 10 and in figure 1. The growth of TA100 strain was inhibited at the concentration of 5000 μg/plate and that of TA1537 strain was inhibited at 2500/5000 μg /plate in the absence of S9Mix; whereas, the growth of TA1537 strain was also inhibited at 5000 μg/plate with S9Mix.The precipitate on the plate was recognized at concentrations of 625 μg/plate or more for any strain, with or without S9Mix.

The number of reversed colonies/plate of any strain was less than twice the number observed in the control with or without the metabolic activator; no concentration-dependent increases were recognized either.

The used positive control substances showed an obvious increase in the number of colonies; contamination was not recognized in the sterility test; and the number of reversed colonies in the solvent control or positive control was within the acceptable range, according to the background data of our facility. In addition, no environmental factors affecting the reliability of the data were recognized during the study period.

Based on the results, it is considered that DB is not capable of inducing gene mutation under the conditions of this study.

References

1) Ames BN, McCann J, Yamasaki E. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat Res 1975; 31: 347-64.

2) McCann J, Choi E, Yamasaki E, Ames BN. Detection of carcinogens as mutagens in the Salmonella/microsome test: assay of 300 chemicals. Proc Natl Acad Sci U S A 1975; 72: 5135-9.

3) Information from Nikken Seiyaku Kogyo.

4) "New guidebook for mutagenicity test with bacteria" edited by Chemical Substances Investigation Division, Industrial Safety and Health Department, Labor Ministry. Issued by Japan Industrial Safety and Health Association on April 30, 1986.

000311

Summary

The potential mutagenicity of Dunaliella Bardawil Paste was examined using five strains: four of *Salmonella typhimurium* (TA100, TA98, TA1535, TA1537) and one of E.coli (WP2uvrA-).

1. Based on the results of the preliminary test, the study was conducted using the preincubation method with or without metabolic activation. The concentrations of the test substance were 5000, 2500, 1250, 625 and 312.5 μg/plate for all strains. 2. Growth inhibition was recognized at 5000 μg/plate for TA100 and at 2500 and 5000 μg/plate for TA1537 without S9Mix; and at 5000 μg/plate for TA1537 with S9Mix.
3. The precipitate on the plate was recognized at concentrations of 625 μg/plate or more with or without S9Mix.
4. Neither in the presence nor in the absence of S9Mix, the number of reversed colonies/plate was twice or more than that observed with the control for any strain: no concentration-dependent increases were recognized either.
5. The sterility test showed no contamination of Dunaliella Bardawil Paste or S9Mix, and each positive control showed an obvious double or higher increase in the number of reversed colonies of the tested strain, compared to that observed with the solvent control.

Based on the above, it is considered that Dunaliella Bardawil Paste is not capable of inducing gene mutation under the conditions of this study.

000312

Table 1 Positive Control

	Strains	Name	Lot No.	Concentration (μg/plate)	Vehicle
−S9 Mix	TA100	2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2)	STJ0788 a)	0.01	DMSO
	TA98			0.1	
	TA1535	Sodium azide (Na-Azide)	M6M9272 b)	0.5	H_2O
	TA1537	9-Aminoacridine hydrochloride (ACR)	091097 c)	80	DMSO
	WP2uvrA⁻	N-Ethyl-N'-nitro-N-nitrosoguanidine (ENNG)	V4A7444 b)	2	DMSO
+S9 Mix	TA100	2-Aminoanthracene (2AA)	M5A3387 b)	1.0	DMSO
	TA98			0.5	
	TA1535			2	
	TA1537			2	
	WP2uvrA⁻			20	

Obtained from;

a): Wako Pure Chemical Industries, LTD.

b): Nakarai Chemicals, LTD.

c): Aldrich Chemicals Company, Inc.

Table 2 Test Strains History

Strains / History	Salmonella typhimurium TA100, TA98, TA1535, TA1537	Escherichia coli WP2uvrA⁻
Obtained from	National Institute of Hygienic Science	Institute of Medical Science, University of Tokyo
Date obtained	April 1, 1980	November 15, 1980
Storage temperature	-80 °C	
Composition	Bacterial suspension 0.8 ml + DMSO 0.07 ml	

000313

000313

Table 8 Specificity of tester strains

		TA100	TA98	TA1535	TA1537	WP2uvrA⁻
Amino acid requirement	His⁻	0 a) 0 0	0 0 0	0 0 0	0 0 0	–
	Trp⁻	– b)	–	–	–	1 3 3
Sensitivity to UV radiation		+	+	+	+	+
rfa specificity		16 mm c) 16 16	14 mm 15 15	17 mm 17 17	16 mm 16 16	0 mm 0 0
R-factor presence		0 mm d) 0 0	0 mm 0 0	24 mm 24 24	26 mm 26 27	24 mm 24 24

a) : Number of revertant colonies / plate
b) : Not done
c) : Diameter of inhibition zone induced by Crystal violet (10μg / 10μl)
d) : Diameter of inhibition zone induced by Ampicillin (80μg / 10μl)

Table 4 Preparation of S9

Animal used		Inducing substance	
Species, Strain	Crj : CD(SD) rat	Name	Phenobarbital (PB) 5,6-Benzoflavone (BF)
Sex	Male		
Age (in week)	7 weeks	Administration method	i.p.
Weight	202.0±10.0g* (n=83)	Administration period and amount	day 1 PB 30 mg/kg day 2,3,4 PB 60 mg/kg day 3 BF 80 mg/kg

*: Mean ± S.D.

000314

Table 5 Composition of S9 Mix

Constituents	Amount in 1ml S9 Mix	Constituents	Amount in 1ml S9 Mix
S9	0.1 ml	NADPH	4 μmol
$MgCl_2$	8 μmol	NADH	4 μmol
KCl	33 μmol	Na - phosphate buffer (pH 7.4)	100 μmol
Glucose - 6 phosphate	5 μmol		

Table 6 Test condition

Test Condition \ Method		Plate method	Pre-incubation method
Test substance volume		0.1 ml	0.1 ml
Bacterial suspension		0.1 ml	0.1 ml
Na-phosphate buffer (in case of non-metabolic activation method)		0.5 ml	0.5 ml
S9Mix (in case of metabolic activation method)		0.5 ml	0.5 ml
Pre-incubation	Temperature	— a)	37 °C
	Time	—	20 minutes
Top agar solution		2 ml	2 ml
Incubation	Temperature	37 °C	37 °C
	Time	48 hours	48 hours

a) Not done

000315

Table7 Reversion test of Dunaliella Bardawil Paste (DB) in bacterial strains
— In case of non -metabolic activation method —
(preliminary)

Method	Test substance	Concentration (μg/plate)	With(+) or Without (−) S9Mix	Number of revertant colonies / plate				
				Base-pair substitution type			Frameshift type	
				TA100	TA1535	WP2uvrA⁻	TA98	TA1537
Plate method	Vehicle Control	—	—	136 162	11 12	19 27	15 20	6 10
	DB	100	—	148 157	14 14	11 27	11 11	5 5
		500	—	141 162	7 14	23 30	12 15	7 7
		1000	—	126★ 146★	14★ 15★	16★ 16★	9★ 19★	8★ 9★
		5000	—	197★ 207★	8★ 19★	18★ 23★	24★ 27★	5★ 7★
Pre-incubation method	Vehicle Control	—	—	159 165	10 11	17 20	10 11	4 7
	DB	100	—	125 163	9 10	17 25	9 15	5 7
		500	—	144 149	16 17	20 21	12 14	5 6
		1000	—	131★ 140★	11★ 14★	20★ 29★	15★ 15★	2★ 4★
		5000	—	185★★ 204★★	8★★ 18★★	38★ 43★	14★ 27★	5★★ 8★★

Vehicle : D.Water
* : killing
★ : observed crystalline matter

000316

Table8 Reversion test of Dunaliella Bardawil Paste (DB) in bacterial strains
— In case of metabolic activation method —
(preliminary)

Method	Test substance	Concentration (μg/plate)	With(+) or Without (−) S9Mix	Number of revertant colonies / plate				
				Base-pair substitution type			Frameshift type	
				TA100	TA1535	WP2uvrA⁻	TA98	TA1537
Plate method	Vehicle Control	—	+	189 197	17 24	17 21	20 20	11 16
	DB	100	+	162 176	8 12	20 21	17 22	8 10
		500	+	154 174	13 15	11 26	17 22	11 12
		1000	+	161* 190*	14* 23*	21* 27*	14* 17*	7* 10*
		5000	+	212* 212*	19* 19*	23* 23*	18* 24*	10* 12*
Pre-incubation method	Vehicle Control	—	+	174 192	16 16	17 21	25 27	13 17
	DB	100	+	144 165	13 14	13 21	23 29	11 17
		500	+	156 161	14 19	14 19	15 22	10 15
		1000	+	170* 182*	18* 19*	26* 27*	26* 30*	5* 9*
		5000	+	184** 201**	12* 17*	24* 32*	18* 24*	12** 14**

Vehicle : D.Water
* : killing
* : observed crystalline matter

000317

Table 9 Reversion test of Dunaliella Bardawil Paste (DB) in bacterial strains

— In case of non-metabolic activation method —

Test substance	Concentration (μg/plate)	With(+) or Without(−) S9Mix	Number of revertant colonies / plate				
			Base-pair substitution type			Frameshift type	
			TA100	TA1535	WP2uvrA⁻	TA98	TA1537
Vehicle Control	—	—	141 144 149 (145)	14 17 18 (16)	14 16 18 (16)	13 16 17 (15)	4 4 6 (5)
DB	312.5	—	134 152 171 (152)	8 12 15 (12)	20 25 26 (24)	13 13 21 (16)	4 7 10 (7)
	625	—	128* 138* 143* (136)	9* 14* 16* (13)	20* 20* 25* (22)	10* 15* 20* (15)	3* 6* 8* (5)
	1250	—	154* 155* 155* (155)	7* 11* 14* (11)	13* 14* 21* (16)	17* 22* 26* (22)	6* 6* 6* (6)
	2500	—	135*☆ 150* 179* (155)	11* 12* 13* (12)	18* 19* 35* (24)	15* 20* 22* (19)	2*☆ 3*☆ 5*☆ (3)
	5000	—	197*☆ 203*☆ 206*☆ (202)	10* 12* 17* (13)	24* 28* 32* (28)	15* 22* 29* (22)	6*☆ 8*☆ 15*☆ (10)
AF-2	0.01	—	328 333 346 (336)				
	0.1	—				212 224 241 (226)	
Na-Azide	0.5	—		166 191 237 (198)			
ENNG	2.0	—			1614 1854 1956 (1808)		
ACR	80.0	—					499 535 564 (533)

( ) : Average number of colonies / plate

* : killing

☆ : observed crystalline matter

Vehicle : D.Water

000318

Table10 Reversion test of Dunaliella Bardawil Paste (DB) in bacterial strains
— In case of metabolic activation method —

Test substance	Concentration (μg/plate)	With(+) or Without (−) S9Mix	Number of revertant colonies / plate				
			Base-pair substitution type			Frameshift type	
			TA100	TA1535	WP2uvrA⁻	TA98	TA1537
Vehicle Control	—	+	137 148 157 (147)	10 12 12 (11)	17 20 22 (20)	17 24 30 (24)	13 13 13 (13)
DB	312.5	+	137 141 149 (142)	9 10 16 (12)	14 17 20 (17)	19 21 25 (22)	9 13 20 (14)
	625	+	115* 147* 152* (138)	10* 12* 13* (12)	21* 22* 23* (22)	24* 24* 36* (28)	7* 10* 19* (12)
	1250	+	114* 131* 124* (123)	11* 13* 15* (13)	13* 22* 24* (20)	19* 21* 25* (22)	15* 15* 18* (16)
	2500	+	138* 142* 165* (148)	16* 17* 17* (17)	13* 15* 22* (17)	19* 16* 28* (21)	8* 11* 16* (12)
	5000	+	197* 219* 238* (218)	14* 15* 21* (17)	16* 16* 20* (17)	25* 28* 35* (29)	9*☆ 14*☆ 19*☆ (14)
2AA	0.5	+				475 490 493 (486)	
	1.0	+	2154 2070 2592 (2272)				
	2.0	+		250 276 277 (268)			215 226 240 (227)
	20.0	+			474 486 465 (475)		

( ) : Average number of colonies / plate
* : killing
☆ : observed crystalline matter

Vehicle : D.Water

000319

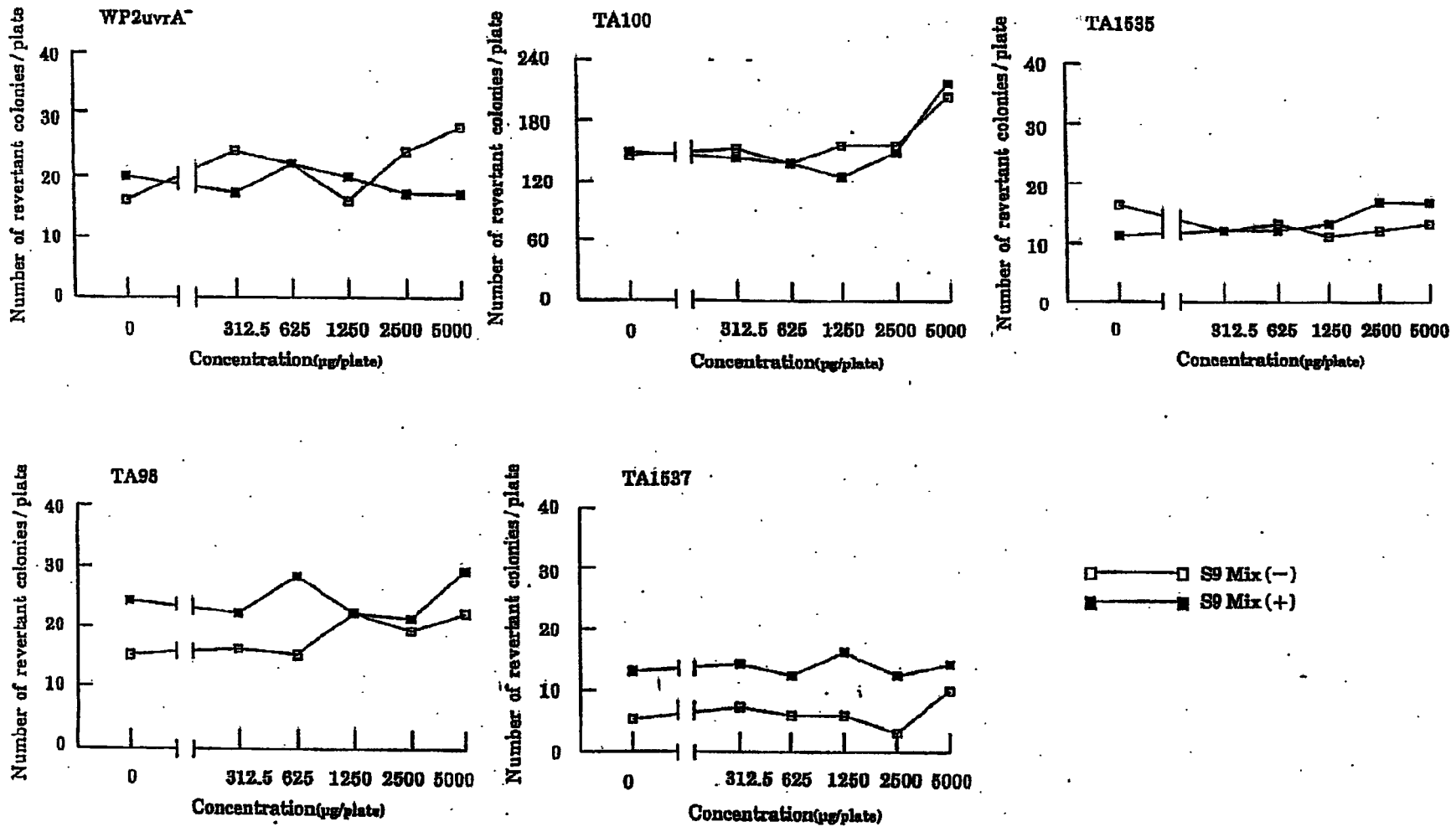

Fig.1 Reversion test of Dunaliella Bardawil Paste (DB) in bacterial strains

予備試験結果表

(2 /) AHS/17-01/RS-01

試験番号 9137　　実施日 62. 9. 2　　実施者 永澤

試験の種類 予備試験　予備追加試験 (−S9Mix・+S9Mix)　　判定日 62. 9. 4　　判定者 永澤

被験物質名 D. B.　　Lot.No. NK-1001

検体濃度 (ug/plate)	復帰変異コロニー数/plate									
	プレート法					プレインキュベーション法				
	塩基置換型			フレームシフト型		塩基置換型			フレームシフト型	
	TA100	TA1535	WP2uvrA	TA98	TA1537	TA100	TA1535	WP2uvrA	TA98	TA1537
溶媒対照 (DMSO)	134	9	26	15	8	127	10	26	10	8
	149	11	28	27	12	147	13	27	11	15
100	138	8	31	16	11	114	11	18	13	14
	143	18	28	24	19	139	15	20	14	17
500	A147	A 3	A 17	A 11	A 7	A 121	A 9	A 23	A 7	A 7
	A100	A 12	A 25	A 17	A 18	A 122	A 15	A 25	A 16	A 8
1000	A105	A 5	A 18	A 16	A 13	A 79	A 6	A 8	A 13	A 5
	A128	A 17	A 29	A 20	A 18	A 114	A 16	A 13	A 20	A 13
5000	A 80	A 6	A 10	A 15	A 7	A 58	A 6	A 7	A 5	A 9
	A104	A 17	A 30	A 18	A 16	A 126	A 11	A 35	A 13	A 11

A: 析出物

<無菌試験結果>

S9 Mix	(o)(o)
	( )( )
	( )( )

<O.D.値>

TA98	1,545	TA1535	1,575	WP2uvrA	1,545
TA100	1,465	TA1537	1,235		

0003321

予備試験結果表

(/ /) AHS/17-01/RS-01

試験番号 9137　　実施日 62. 9. 2　　実施者 永澤

試験の種類 予備試験　予備追加試験 (−S9Mix・+S9Mix)　　判定日 62. 9. 4　　判定者 永澤

被験物質名 DB　　Lot.No. NK-1001

検体濃度 (ug/plate)	復帰変異コロニー数/plate									
	プレート法					プレインキュベーション法				
	塩基置換型			フレームシフト型		塩基置換型			フレームシフト型	
	TA100	TA1535	WP2uvrA	TA98	TA1537	TA100	TA1535	WP2uvrA	TA98	TA1537
溶媒対照 (DMSO)	130 138	9 14	20 24	10 10	2 5	115 139	8 17	19 20	11 14	5 9
100	A 127 A 133	A 14 A 18	A 26 A 33	A 6 A 15	A 5 A 12	A 136 A 152	A 16 A 27	A 17 A 33	A 11 A 12	A 4 A 9
500	A 120 A 145	A 2 A 11	A 24 A 29	A 9 A 12	A 3 A 7	A 89 A 95	A 6 A 10	A 15 A 31	A 16 A 16	A 2 A 8
1000	A 95 A 111	A 9 A 13	A 22 A 31	A 17 A 18	A 2 A 10	A 124 A 167	A 6 A 6	A 22 A 25	A 4 A 10	A 3 A 6
5000	A 35 A 115	A 8 A 12	A 24 A 26	A 9 A 12	A 4 A 3	A 112 A 126	A 5 A 8	A 18 A 30	A 8 A 12	A 3 A 5
5										

A = 析出物

<無菌試験結果>

5000 μg/plate	(有)	(有)
	( )	( )
	( )	( )

① カビ 4, 雑菌 18
② カビ 7 / 雑菌 25

<O.D.値>

TA98	1.545	TA1535	1.575	WP2uvrA	1.545
TA100	1.465	TA1537	1.235		

000322

000322

SOP/TSB/07/RS-02

Dunaliella Bardawil (Spray Dried Powder) 使用記録用紙 (No. 2)

試験番号: 9137

LOT No.: NK-1001

入手年月日: 昭和 62年 9月 17日

使用期限: 昭和 年 月 日

正味量: 2.5g × 2本

風袋込み量: ① 10.5333g ② 11.2378g

保管条件: 冷凍, 遮光,

保管形態: 褐色瓶,

保管庫 No.: 冷凍庫 No. 6

試験責任者: 有賀文子

使用記録

年月日	採取量(使用量) (mg)	風袋込み残量(使用後) (g)	使用者	調製指示書 No.
62. 9 17	100mg	① 10.4328g	永澤	無菌試験
		残量確認 62. 10. 28	永澤	
		使用量 100.5mg		

000323

000323

SOP/TSB/07/RS-02

Dunaliella Bardawil
(Spray Dried Powder) 使用記録用紙 (No. 1)

試験番号: 9137
LOT No.: NK-1001
入手年月日: 昭和 62年 9月 1日
使用期限: 昭和 年 月 日

正味量: 2.5g × 2本
風袋込み量: ① 10.7113g ② 10.7611g
保管条件: 冷凍, 遮光
保管形態: 褐色瓶
保管庫 No.: 冷凍庫 No. 6

試験責任者 [illegible]

使用記録

年月日	採取量(使用量) (mg)	風袋込み残量(使用後) (g)	使用者	調製指示書 No.
62.9.2	400.1mg	① 10.3108	永澤	予備試験
		残量確認		
		62.10.28	永澤	
		使用量 400.5mg		

000324

000324

SOP/TSB/07/RS-02

（ DB ）使用記録用紙 （No. 1 ）

試験番号： 4087
LOT No.： NK－1001
入手年月日： 昭和 62年 9 月 1 日
使用期限： 昭和 ~~年 月 日~~

正味量： 20g
風袋込み量： 157.65g
保管条件： 冷凍
保管形態： 密栓ガラス瓶
保管庫 No.： 冷凍庫 No. 6

試験責任者 有賀文子 （印）

使用記録

年月日	採取量（使用量）（ g ）	風袋込み残量（使用後）（ g ）	使用者	調製指示書 No.
62.9.18	17.5	140.14	加藤	1
	使用量 17.5g	残量 140.14g		
		0.25±0.01g 確認	62.9.18	加藤

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Test Protocol

Test substance: DB (abbreviation)

Title

Mutagenicity Test of Dunaliella Bardawil Paste (Reverse Mutation Test Using Bacteria)

Sponsor

Nikken Pharmaceutical Industry Co., Ltd.

Address: 4-41 Hirakata, Fukuju-cho, Hashima-shi, Gifu Prefecture

Testing laboratory

Nihon Bioresearch Inc. Hashima Research Center (Management: Kenichi Nakagawa)

Address: 6-104 Majima, Fukuju-cho, Hashima-shi, Gifu Prefecture

Objective

To test Dunaliella Bardawil Paste for reverse mutagenicity using bacteria in compliance with the Guidelines for Toxicity Studies of Drugs as specified in the Notification No. 118 from the Evaluation and Licensing Division, Pharmaceutical and Food Safety Bureau, Ministry of Health and Welfare (dated February 15, 1984) to estimate its potential to induce gene mutation

Test personnel

Study director: Fumihiko Aruga

Study operator: Yoshiko Nagasawa

Quality Assurance Department

Person in charge: Kenji Okada

Start of test (date of signature and seal by management):	August 25, 1987
Test period	
Pre-cultivation of bacteria for preliminary test:	December 15, 1987
Final decision:	December 25, 1987
Submission of report (draft):	January 31, 1988
Scheduled date of submission of final report:	February 29, 1988

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Signature and seal

Study director

Name: Fumihiko Aruga Date: August 25, 1987

Address: Nishikawate Bldg. 312, 8-40, Nishikawate, Gifu-shi, Gifu

Position: Nihon Bioresearch Inc. Hashima Research Center

Approval of test protocol

Sponsor: Teruo Shiraishi Date: August 25, 1987

Address: 4-41, Hirakata, Fukuju-machi, Hashima-shi, Gifu

Position: President, Nikken Pharmaceutical Industry Co., Ltd.

Management: Kenichi Nakagawa Date: August 25, 1987

Position: Nihon Bioresearch Inc. Hashima Research Center

Confirmation

Quality Assurance Department

Person in charge: Kenji Okada Date: August 25, 1987

Position: Nihon Bioresearch Inc. Hashima Research Center)

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1. Test and positive control substances

(1) Test substance

Name:	Dunaliella Bardawil Paste (Abbreviation; DB)
Lot No.:	NK-1010
Properties:	Yellowish brown paste with a water content of 60%
Stability:	The stability of the test substance in distilled water has not been established. It will be confirmed by the sponsor and added to the present protocol as soon as it will become available.
Date of delivery:	December 14, 1987
Quantity delivered:	7.5 g
Storage conditions:	This substance should be kept refrigerated in a light-shielded place.
Storage place:	Test substance cabinet (refrigerator) at testing laboratory
Supplier:	Nikken Pharmaceutical Industry Co., Ltd.
Medium:	Distilled water (Wako Pure Chemical Industries, Lot No.: P1071)

(2) Positive control substances

Substance	Manufacturer	Lot No.
2-Aminoanthracene (2AA)	Nakarai Kagaku	M5A3387
Sodium azide (Na-Azide)	Nakarai Kagaku	M6M9272
9-Aminoacridine hydrochloride (ACR)	Aldrich C.	091097
N-Ethyl-N'-nitro-N-nitrosoguanidine (ENNG)	Nakarai Kagaku	V4A7444
2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2)	Wako Pure Chemical Industries, Ltd.	STJ0738

(3) Records

The date of delivery, lot No., quantity delivered, and usage details of the test substance shall be recorded in a specified form.

2. Test systems

(1) Type of test strains and justification

Salmonella typhimurium: TA98, TA100, TA1535, and TA1537

Escherichia coli: WP2uvrA$^-$

These strains were selected according to the Guidelines for Toxicity Studies of Drugs as specified in the Notification No. 118 from the Evaluation and Licensing Division, Pharmaceutical and Food Safety Bureau, Ministry of Health and Welfare (dated February 15, 1984).

(2) Suppliers and date of delivery

S. typhimurium: National Institute of Health Sciences (April 1, 1980)

E. coli: The Institute of Medical Science, the University of Tokyo (November 15, 1980)

(3) Storage method of strains

Each suspension of the *S. typhimurium* and *E. coli* strains shall be mixed with DMSO at a ratio of 0.8 ml to 0.07 ml and each mixture kept in a deep freezer at -80°C (Sanyo Ultradeep Freezer, Model: MDF-190AT).

(4) Characteristic test of strains

Bacterial strains that meet the following characteristic test criteria (SOP/AMS/07-04) shall be used. The characteristic test shall be performed within one month before the present test is started.

		TA100	TA98	TA1535	TA1537	WP2uvrA⁻
Amino acid requirement[a]	His⁻	0	0	0	0	-
	Trp⁻	-[b]	-	-	-	<5
Ultraviolet (UV) sensitivity[c]		+	+	+	+	+
Membrane mutation: rfa characteristics[d]		>12 mm	>12 mm	>10 mm	>13 mm	0
Presence/absence of drug resistance factor (R factor)[e] plasmid		0 mm	0 mm	>20 mm	>20 mm	>20 mm

a) Reverse mutation colony count/plate

b) Not performed

c) +: Sensitive

d) Diameter of growth inhibition circle as determined by paper disk method (diameter: 8 mm) including crystal violet (10 μg/10 μL)

e) Diameter of growth inhibition circle as determined by paper disk method (diameter: 8 mm) including ampicillin sodium (80 μg/10 μL)

(5) Identification of test systems

The test tubes and plates for different test strains shall be color coded with solvent inks.

3. S9 mix

(1) S9

Lot No.:	87090412
Date of manufacture:	September 4, 1987
Date of purchase:	September 18, 1987
Manufacturer:	Oriental Yeast Co., Ltd.
Storage method:	Deep freezer at testing laboratory (Sanyo Ultradeep Freezer, Model: MDF-190AT)
Induction method:	(quoted from the test methods specified by Oriental Yeast Co., Ltd.)

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To induce S9, intraperitoneally administer 30 mg/kg of phenobarbital as an inducer on Day 1 and 60 mg/kg on Days 2 to 4 as well as 80 mg/kg of 5,6-benzoflavone on Day 3 in 83 seven-week-old male rats (Crj: CD (SD)) (body weight: 202±10.0 g).

(2) Composition of S9 Mix (quantity per ml)

S9	0.1 mL	
$MgCl_2$	8 μmol	Cofactor (Lot No.: 702, Oriental Yeast Co., Ltd.)
KCl	33 μmol	
Glucose-6-phosphate	5 μmol	
NADPH	4 μmol	
NADH	4 μmol	
Sodium-phosphate buffer (pH 7.4)	100 μmol	

4. Preparation of test substance

Dissolve or suspend the test substance in distilled water to prepare the test solution at the highest concentration. Dilute it to prepare the solutions at lower concentrations. Prepare them based on the concentration of the drug substance of the test substance because the test substance contains water at 60%. Prepare the solutions as required. Discard any remainder of the solutions at the end of the test.

5. Pre-culture of test strains

Scrape each of the frozen strains into 10 ml of a sterilized nutrient broth (Nutrient Broth No. 2, Oxoid) and shake-culture it at 37°C for 16 hours. Keep the bacterial suspension obtained from the culture on ice before use.

6. Culture medium

(1) Composition of minimum glucose agar plate

Agar (powder agar)	75 g	Agar (Lot No. K-329, Shoei Kanten Co., Ltd.)
Distilled water	4.5 L	
Medium E (X20)	250 mL	
40% glucose	250 mL	

Thirty ml is dispensed per plate.

(2) Composition of Medium E (X20)

$MgSO_4 \cdot 7H_2O$	4 g
Citric acid $\cdot H_2O$	40 g
K_2HPO_4	200 g
$NaNH_4HPO_4 \cdot 4H_2O$	70 g

Distilled water is added to make 1 L.

7. Sterility test

The test solutions and S9 Mix shall be tested for sterility using the plate method at the

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time of the preliminary test. Two plates shall be used for each test.

8. Test methods

(1) Preliminary test

The preliminary test is designed for investigating the effect of the test substance on the strains (growth inhibition or bactericidal effect) and the dissolution of the test substance on the plate to select an appropriate test method (plate or pre-incubation method) and concentration.

1) Test method

Perform the preliminary test with the plate or pre-incubation method with/without S9 Mix under the following conditions

		Pre-incubation method	Plate method
Composition	Bacterial suspension	0.1 mL	0.1 mL
	Test substance solution	0.1 mL	0.1 mL
	Sodium-phosphate buffer (without metabolic activation)	0.5 mL	0.5 mL
	S9 Mix (with metabolic activation)	0.5 mL	0.5 mL
	Soft agar solution	2 mL	2 mL
Pre-incubation	Temperature	37°C	-
	Period	20 min.	-
Incubation	Temperature	37°C	37°C
	Period	48 hrs	48 hrs

2) Test concentration

In principle, perform the preliminary test using the test solutions at four concentrations of 5000, 1000, 500, and 100 µg/plate and solvent control (distilled water).

When bacterial growth is inhibited at 100 µg/plate, set lower concentrations including 50, 10, 5, and 1 µg/plate or lower to specify the lowest concentration at which no bacterial growth inhibition is detected, and perform the test.

Use two plates for each concentration of the test solutions or solvent control.

(2) Main test

1) Test method

Perform the main test using either the plate or pre-incubation method with/without S9 Mix based on the preliminary test results. Select the test method associated with increased reverse mutation colony count. When the two methods are comparable for reverse mutation colony count, select the method associated with higher bacterial growth inhibition. When they are comparable for both reverse mutation colony count and bacterial growth inhibition, select the pre-incubation method.

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2) Test concentration

Perform the main test using the test solution at five concentrations, solvent control, and positive control. To set the five concentrations of the test solution, use the minimum concentration at which bacterial growth inhibition is detected or 5000 μg/plate as the highest concentration and set four lower concentrations using a common ratio of 2.

3) Number of plates

Use three plates for each combination of the test strains, presence/absence of metabolic activation, and concentration levels.

4) Positive control substances

(a) Concentration

Positive control substance requiring S9 Mix

Test strain	Chemical substance	Concentration (μg/plate)
TA98	2AA	0.5
TA100	2AA	1.0
TA1535	2AA	2.0
TA1537	2AA	2.0
WP2uvrA⁻	2AA	20.0

Positive control substances requiring no S9 Mix

Test strain	Chemical substance	Concentration (μg/plate)
TA98	AF-2	0.1
TA100	AF-2	0.01
TA1535	Na-Azide	0.5
TA1537	ACR	80.0
WP2uvrA⁻	ENNG	2.0

(b) Solvent and preparation method

Dissolve Na-Azide in sterilized distilled water and 2AA, ACR, AF-2, and ENNG in DMSO. Prepare them as required.

(c) Stability

No reliable information is available for the stability of these substances. Therefore, the test strains are considered to have normal reactivity and the positive controls have biologically sufficient activity when the test results of the positive control substances do not markedly deviate from their previous results (SOP/AMS/20-01).

5) Observation items

Presence/absence of deposits on plates: Macroscopic observation

Bacterial growth inhibition: Microscopic observation at a magnification of 100 times

Reverse mutation colony count: Visual counting

Indication of test results: Indicate the actual counts and calculated mean

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values of reverse mutation colonies. Indicate the mean values using figures.

9. Decision criteria

The test is considered valid when the sterility test demonstrates no bacterial contamination and the reverse mutation colony counts of the solvent and positive controls are within the range of their respective background data. The test substance is considered positive when its reverse mutation colony count concentration-dependently increases to a level more than double that of the solvent control.

10. Amendment to test protocol

When it becomes necessary to make an important change to the test protocol after the test agreement is concluded, the change shall be agreed on by the sponsor and testing laboratory and an amendment to the test protocol including the location, description, and reason of the change issued and retained with the test protocol by both parties.

11. Archive

All the data and documents pertaining to the present test will be stored at the archive of the Nihon Bioresearch Center. The storage period shall be agreed on separately.

The data and documents to be stored are listed below.

(1) Test protocol, amendment to test protocol, agreement, and main schedule

(2) Records of test substance

1) Records of receipt and return of test substance

2) Records of properties and stability of test substance (supplied by sponsor)

3) Usage details of test substance

4) Records of preparation of test substance

(3) Records of analyses and environmental control

1) Records of operating procedures

2) Copies of characteristic test results of bacterial strains (records of tests performed within one month for bacterial strains used)

3) Copies of S9 certificate (attached to S9)

(4) Records of test results

(5) Report and related documents to be stored

1) Tables and figures used for preparing report

2) Manuscript of report

3) Copy of final report

12. Return of any residual test substance

Any residual test substance at the end of the test shall be returned to the sponsor with the usage details (copy).

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Amendment to test protocol (No. 1)

Test No.: 9137

Title: Mutagenicity Test of Dunaliella Bardawil Paste (Reverse Mutation Test Using Bacteria)

Location, description, and reason of amendment

Page 1: 1. Test and positive control substances

(1) Test substance

Date of delivery: December 14, 1987

Quantity delivered: 7.5 g

Medium: Distilled water (Wako Pure Chemical Industries, Lot No.: P1071)

The underlined parts are added:

Reason: Because it was decided that the parts would be entered after the test substance and medium would be purchased.

Page 5: 3. S9 Mix

(1) S9 Mix

Lot No.: 87090412

Date of manufacture: September 4, 1987

Date of purchase: September 18, 1987

Induction method: 83 seven-week-old male rats (Crj: CD (SD)) (body weight: 202.0±10.0 g)

(2) Composition of S9 Mix (quantity per ml)

Cofactor (Lot No. 702: Oriental Yeast Co., Ltd.)

The underlined parts are added.

Reason: Because it was decided that the parts would be entered after S9 and Cofactor would be purchased

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Study director: Fumihiko Aruga Amend date: December 17, 1987

Approval

Management: Kenichi Nakagawa Date: December 17, 1987

Sponsor: Teruo Shiraishi Date: December 28, 1987

Address: 4-41, Hirakata, Fukuju-machi, Hashima-shi, Gifu

Position: President, Nikken Pharmaceutical Industry Co., Ltd.

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株式会社日本バイオリサーチセンター

試験番号：9137

陳　述　書

試験番号：９１３７

試験表題：Dunaliella Bardwil Paste の変異原性試験（細菌を用いる復帰変異試験）

当試験は、薬発第 313 号厚生省薬務局長通知（昭和 57 年 3 月 31 日付）「医薬品の安全性試験の実施に関する基準について」に従って実施されたものである。

2007年 1月 16日

株式会社日本バイオリサーチセンター　羽島研究所

試験責任者 (b) (6)

000336

Dunaliella Bardawil Pasteの変異原性試験
（細菌を用いる復帰変異試験）

報　告　書

提出日　昭和６３年　２月　５日

株式会社日本バイオリサーチセンター
羽島研究所

ＮＢＲ－１

000337

信頼性保証部門陳述書

試験番号：9187

表　　題：Dunaliella Bardawil Paste の変異原性試験（細菌を用いる復帰変異試験）

上記の試験に関して、昭和62年12月16日から昭和63年2月5日にかけて検閲を実施し、その結果は運営管理者および試験責任者に報告されている。　この検閲記録は、㈱日本バイオリサーチセンター羽島研究所QAU室にすべて保管されている。

この最終報告書は、試験で得られた生データを正確に反映している事をここに保証する。

昭和63年2月5日

㈱日本バイオリサーチセンター　羽島研究所

信頼性保証部門責任者 [illegible]

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試験概要（試験番号：9137）

表　　題	Dunaliella Bardawil Pasteの変異原性試験（細菌を用いる復帰変異試験）
試験番号	９１３７
被験物質名	Dunaliella Bardawil Paste
試　験　系	Salmonella typhimurium；TA100,TA98,TA1535,TA1537 E.coli；WP2uvrA⁻
試験委託者	日健製薬工業株式会社 岐阜県羽島市福寿町平方 4-41
試験実施場所	岐阜県羽島市福寿町間島 6-104 株式会社日本バイオリサーチセンター羽島研究所
試験目的	薬審第118号（昭和59年2月15日付）「医薬品のための毒性試験法ガイドライン」に基づいてDunaliella Bardawil Pasteの細菌を用いる復帰変異試験を行い、それの遺伝子突然変異誘発性を推定する。
試験実施期間	自　昭和６２年１２月１５日（菌の前培養実施日） 至　昭和６２年１２月２５日（最終判定日）
試験期間	昭和６２年　８月２５日　～　昭和６３年　２月　５日
資料保管場所	岐阜県羽島市福寿町間島 6-104 株式会社日本バイオリサーチセンター羽島研究所

試験従事者の署名・捺印（試験番号：9137）

表　　題	Dunaliella Bardawil Pasteの変異原性試験（細菌を用いる復帰変異試験）
試験番号	9137

試験責任者 (b) (6) 昭和63年2月5日

試験担当責任者 (b) (6) 昭和63年2月4日

試験担当者 (b) (6) 昭和63年2月4日

昭和63年2月5日

運営管理者 (b) (6)

NBR－3

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試験従事者および業務分担（試験番号：9137）

試験責任者：（試験実施の指示、試験計画書の作成、報告書の作成）
有賀文彦

試験担当責任者：（試験の実施、検体調製、データのまとめ）
永澤佳子

試験担当者：（表の作成）
白木マリ

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緒　言

今回、食品としての使用が予定されているDunaliella Bardawil Pasteの安全性に関する試験の一環として、薬審第118号（昭和59年2月15日付）「医薬品のための毒性試験法ガイドライン」に基づき、Amesの方法[1),2)]により細菌を用いた復帰変異試験を行い、Dunaliella Bardawil Pasteの遺伝子突然変異誘発性の有無を検討したので、その結果を報告する。

当試験の日程は下記の通りであった。

1．予備試験
　菌の前培養：昭和62年12月15日
　試験の実施：昭和62年12月16日
　判　定　日：昭和62年12月18日

2．本試験
　菌の前培養：昭和62年12月22日
　試験の実施：昭和62年12月23日
　判　定　日：昭和62年12月25日

被験物質および陽性対照物質

被験物質Dunaliella Bardawil Paste（以下、DBと略す）は、黄褐色のペーストで、水分を60%含む[3)]。DB（ロット番号：NK-1010）は昭和62年12月14日に、日健製薬工業株式会社より提供（正味量：7.5g）されたものを、試験実施機関の被験物質保管庫（冷蔵庫内）に遮光下で保管した。

被験物質は蒸留水（和光純薬工業株式会社、ロット番号：P1071）を用いて溶解または懸濁し、使用後の残余検体液は廃棄した。また、検体液の調製に際して、被験物質には60%の水分が含まれているため、原体量が試験濃度になるよう、含まれている水分量を加味して蒸留水を加えた。なお、DBの蒸留水中での安定性は試験委託者において確認される予定であるが、今回の試験における検体液の調製は用時に行い、調製後3時間以内に使用した。

一方、陽性対照物質は、市販品を購入して用いた。その名称、製造元、ロット番号、使用濃度および溶媒はTable 1に示した。なお、陽性対照物質は溶媒中での安定性が不明なため、いずれも用時調製した。

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試験方法

1. 試験に用いた菌株

菌株の名称、入手先、入手年月日および保存方法はTable 2にまとめて示した。 すなわち、millipore filter (FH type) で濾過滅菌したジメチルスルホキシド (以下DMSO) 0.07mlと菌懸濁液0.8mlを滅菌スクリュー管に入れ、ドライアイスアセトンで凍結し、-80℃設定のディープフリーザー(サンヨー、型式 MDF-190AT) に保管した。 試験菌株は、菌株の特性を「新・微生物を用いる変異原性試験ガイドブック」[4]に従い、アミノ酸要求性、紫外線感受性、膜変異rfa特性および薬剤耐性因子R-factor プラスミドの有無について、試験の実施1か月以内に検査して適合していることを確認した後、試験に用いた。検査結果をTable 3に示した。

試験菌株の前培養は、NUTRIENT BROTH No.2 (OXOID) 2.5gに蒸留水100mlを加え、高圧蒸気滅菌 (121℃、20分、以下同様) したものを乾熱滅菌 (181℃、1時間、以下同様) したL字管に10ml宛分注し、保存凍結菌を掻き取り移植、ウォーターバスインキュベーター (大洋科学、型式 M-100^N) で37±0.5℃、16時間振盪培養し、菌懸濁液とした。 使用時まで菌懸濁液は氷中に保存した。

2. S9Mix

S9はTable 4の条件により、昭和62年9月4日にオリエンタル酵母工業 (株) で製造されたもの (Lot No.87090412) を昭和62年9月18日に購入し、用時まで-80℃設定のディープフリーザーに保管した。

S9MixはTable 5の組成を成しており、S9Mix用のCofactor (オリエンタル酵母工業、Lot No.702) を蒸留水で溶解し、millipore filter (HA type) で濾過滅菌した後、使用直前にS9を加えて調製した。

3. 培地

最少グルコース寒天平板培地は、Vogel-Bonner最少培地Eにグルコース2wt%、粉末寒天 (松栄寒天、Lot No.K-151) 1.5wt%の割合になるように調製した。 これを30mlづつ直径90mmのγ線滅菌されたシャーレ (Falcon、1001type) に分注し、固化させて平板とした。

トップアガーは、Bact-Agar (DIFCO) 0.6wt%、塩化ナトリウム0.5wt%の割合で調製したものに、アミノ酸溶液を10:1の割合で混合して用いた。 アミノ酸溶液は、Salmonella typhimuriumの各菌株の場合は0.5mM L-ヒスチジンと0.5mM ビオチンの水溶液を、E.coli

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の場合は0.5mM L-トリプトファン水溶液をmillipore filter (FH type) で濾過滅菌したものを用いた。

4．無菌試験

5000μg/0.1ml濃度の検体液とS9Mixのそれぞれ0.1mlにトップアガー 2mlを加え、最少グルコース寒天平板培地上にひろげ、37±0.5℃で48時間、プレートを転倒して培養し、コロニーの出現を指標とした無菌試験を行った。 プレート数は各2枚を用い、予備試験の際に実施した。

その結果、検体液およびS9Mixともにコロニーの出現は認められなかった。

5．試験方法

(1)予備試験

本試験の濃度設定ならびに試験方法の選択のために、予備試験を行った。

方法は、プレート法およびプレインキュベーション法により、Table 6に示した試験条件で濃度毎に2枚のプレートを用い、S9Mix添加および無添加で行った。

試験濃度は、薬審第118号（昭和59年2月15日付）「医薬品のための毒性試験法ガイドライン」に従い、5000μg/plateを最高濃度とし、以下1000、500、100μg/plateと溶媒対照（蒸留水）について実施した。

試験菌株の生育阻害を100倍の培養顕微鏡（オリンパス、CK）下でlawnの生育状態を観察することにより調べ、プレート上での析出物を肉眼で観察した後、復帰変異により出現したコロニー数を計数した。

その結果（Table 7，8）、菌の生育阻害は、プレート法では、S9Mix添加および無添加のいずれにおいても全菌株とも認められなかった。 一方、プレインキュベーション法では、S9Mix無添加でTA100、TA1535、TA98、TA1537の5000μg/plateに、S9Mix添加でTA100およびTA1537の5000μg/plateに菌の生育阻害が認められた。

プレート上での析出物は、プレート法およびプレインキュベーション法とも、S9Mix添加および無添加のいずれにおいても1000μg/plate以上で認められた。

復帰変異コロニー数／plateは、プレート法およびプレインキュベーション法ともS9Mix添加および無添加において、いずれの菌株とも溶媒対照に比して増加を認めなかった。

(2)本試験

予備試験において、プレート法およびプレインキュベーション法とも復帰変異コロニー数の増加は認められなかったものの、菌の生育阻害はプレート法に比べプレインキュベーション法において強い傾向を示した。

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したがって、本試験の試験方法にはプレインキュベーション法を採用し、代謝活性化によらない場合（S9Mix無添加）および代謝活性化による場合（S9Mix添加）で行った。　すなわち、代謝活性化による場合は、乾熱滅菌した試験管（12.5×75mm、テルモ、清浄試験管ラルボ）に①検体液0.1ml、②高圧蒸気滅菌した0.1Mリン酸緩衝液（pH7.4）0.5ml、③菌懸濁液0.1mlの順に加えたのち、37±0.5℃、20分間プレインキュベーションし、これに45℃に保温したトップアガー2mlを加えて、37±0.5℃の恒温器（ヤマト科学、型式 IA-81）で48時間、プレートを転倒して培養した。　一方代謝活性化による場合は、さきの②0.1Mリン酸緩衝液をS9Mix 0.5mlに換え、他の操作はすべて同様に行った。

DBの濃度は、予備試験において菌の生育阻害がみられないか、あるいは菌の生育阻害がみられても5000μg/plateのみであったことから、本試験においてはいずれの菌株とも5000μg/plateを最高用量とし、以下公比2により2500、1250、625、312.5μg/plateとした。　また、いずれの菌株とも溶媒対照（蒸留水）および陽性対照をS9Mix添加および無添加の場合で実施した。　プレート数は濃度毎に3枚を使用した。　観察は、菌の生育阻害を100倍の培養顕微鏡下でlawnの生育状態を観察することにより調べ、プレート上での析出物を肉眼で観察した後、復帰変異コロニー数を計数し平均を算出した。

(3)判定基準

被験物質の復帰変異コロニー数が、溶媒対照の2倍以上の値を示し、さらに濃度に依存して増加が認められた場合を陽性とした。

資料の保管

当試験の実施により得られた資料は、報告書提出後1年間は試験実施機関の資料保管室に保管する。　その後は試験委託者と協議の上、決定する。

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試験成績および考察

本試験の結果をTable 9, 10およびFig.1に示した。

菌の生育阻害は、S9Mix無添加ではTA100の5000μg/plateとTA1537の2500および5000μg/plateに認められ、S9Mix添加ではTA1537の5000μg/plateに認められた。

プレート上での析出物は、S9Mix添加および無添加のいずれにおいても625μg/plate以上の濃度で認められた。

復帰変異コロニー数／plateは、S9Mix添加および無添加において、いずれの菌株とも溶媒対照に比して2倍以上の値を示さず、濃度に依存した増加も認められなかった。

なお、用いた陽性対照物質はそれぞれの菌株に対していずれにおいても明らかなコロニー数の増加を示し、無菌試験では雑菌の混入を認めず、溶媒対照および陽性対照の復帰変異コロニー数は当施設のバックグランドデータの範囲内を示した。　さらに、当試験の実施期間中にデータの信頼性に影響を与える環境因子は認められなかった。

以上の結果から、当試験の条件下ではDBには遺伝子突然変異誘発性はないと考えられた。

文　献

1)Ames,B.N.,McCann,J.& Yamasaki,E.:Methods for detecting carcinogens and mutagens with the salmonella/mammalian-microsome mutagenicity test,Mutation Res.,31;347～364(1975)

2)McCann,J.,Choi,E.,Yamasaki,E.& Ames,B.N.:Detection of carcinogens as mutagens in the salmonella/microsome test;Assay of 300 chemicals,Proc.Nat.Acad.Sci.,72;5135～5139(1975)

3)日健製薬工業株式会社からの情報

4)労働省安全衛生部化学物質調査課編「新・微生物を用いる変異原性試験ガイドブック」，中央労働災害防止協会，昭和61年4月30日発行

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要　約

Dunaliella Bardawil Pasteの遺伝子突然変異誘発性を、Salmonella typhimurium TA100、TA98、TA1535、TA1537およびE.coli WP2uvrA⁻の5菌株を用いて検討した。

1．予備試験の結果から、試験方法はプレインキュベーション法を用い、代謝活性化によらない場合（S9Mix無添加）と代謝活性化による場合（S9Mix添加）で実施した。試験濃度は、全菌株ともS9Mix添加および無添加のいずれにおいても5000、2500、1250、625、312.5μg/plateとした。

2．菌の生育阻害は、S9Mix無添加ではTA100の5000μg/plateとTA1537の2500および5000μg/plateに認められ、S9Mix添加ではTA1537の5000μg/plateに認められた。

3．プレート上での析出物は、S9Mix添加および無添加のいずれにおいても625μg/plate以上の濃度で認められた。

4．復帰変異コロニー数／plateは、S9Mix添加および無添加において、いずれの菌株とも溶媒対照に比して2倍以上の値を示さず、濃度に依存した増加も認められなかった。

5．無菌試験ではDunaliella Bardawil PasteおよびS9Mixに雑菌の混入を認めず、各陽性対照ともそれぞれの菌株に対し溶媒対照の2倍以上の明らかな復帰変異コロニー数の増加を示した。

以上のことから、当試験の条件下ではDunaliella Bardawil Pasteには遺伝子突然変異誘発性はないと考えられた。

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Table 1 Positive Control

	Strains	Name	Lot No.	Concentration (μg/plate)	Vehicle
−S9 Mix	TA100	2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2)	STJ0738 a)	0.01	DMSO
	TA98			0.1	
	TA1535	Sodium azide (Na-Azide)	M6M9272 b)	0.5	H_2O
	TA1537	9-Aminoacridine hydrochloride (ACR)	091097 c)	80	DMSO
	WP2uvrA⁻	N-Ethyl-N'-nitro-N-nitrosoguanidine (ENNG)	V4A7444 b)	2	DMSO
+S9 Mix	TA100	2-Aminoanthracene (2AA)	M5A3387 b)	1.0	DMSO
	TA98			0.5	
	TA1535			2	
	TA1537			2	
	WP2uvrA⁻			20	

Obtained from;

a): Wako Pure Chemical Industries, LTD.

b): Nakarai Chemicals, LTD.

c): Aldrich Chemicals Company., Inc.

Table 2 Test Strains History

Strains / History	Salmonella typhimurium TA100,TA98,TA1535,TA1537	Escherichia coli WP2uvrA⁻
Obtained from	National Institute of Hygienic Science	Institute of Medical Science, University of Tokyo
Date obtained	April 1, 1980	November 15, 1980
Storage temperature	-80 °C	
Composition	Bacterial suspension 0.8 ml + DMSO 0.07 ml	

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Table 3 Specificity of tester strains

		TA100	TA98	TA1535	TA1537	WP2uvrA⁻
Amino acid requirement	His⁻	0 a) 0 0	0 0 0	0 0 0	0 0 0	—
	Trp⁻	— b)	—	—	—	1 3 3
Sensitivity to UV radiation		+	+	+	+	+
rfa specificity		16 mm c) 16 16	14 mm 15 15	17 mm 17 17	16 mm 16 16	0 mm 0 0
R-factor presence		0 mm d) 0 0	0 mm 0 0	24 mm 24 24	26 mm 26 27	24 mm 24 24

a) : Number of revertant colonies / plate
b) : Not done
c) : Diameter of inhibition zone induced by Crystal violet (10μg / 10μl)
d) : Diameter of inhibition zone induced by Ampicillin (80μg / 10μl)

Table 4 Preparation of S9

Animal used		Inducing substance	
Species, Strain	Crj : CD(SD) rat	Name	Phenobarbital (PB) 5,6-Benzoflavone (BF)
Sex	Male		
Age (in week)	7 weeks	Administration method	i.p.
Weight	202.0 ± 10.0g * (n = 83)	Administration period and amount	day 1 PB 30 mg/kg day 2,3,4 PB 60 mg/kg day 3 BF 80 mg/kg

* : Mean ± S.D.

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Table 5 Composition of S9 Mix

Constituents	Amount in 1ml S9 Mix	Constituents	Amount in 1ml S9 Mix
S9	0.1 ml	NADPH	4 μmol
$MgCl_2$	8 μmol	NADH	4 μmol
KCl	33 μmol	Na-phosphate buffer (pH 7.4)	100 μmol
Glucose-6 phosphate	5 μmol		

Table 6 Test condition

Test Condition \ Method		Plate method	Pre-incubation method
Test substance volume		0.1 ml	0.1 ml
Bacterial suspension		0.1 ml	0.1 ml
Na-phosphate buffer (in case of non-metabolic activation method)		0.5 ml	0.5 ml
S9Mix (in case of metabolic activation method)		0.5 ml	0.5 ml
Pre-incubation	Temperature	— a)	37 °C
	Time	—	20 minutes
Top agar solution		2 ml	2 ml
Incubation	Temperature	37 °C	37 °C
	Time	48 hours	48 hours

a) Not done

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Table7 Reversion test of Dunaliella Bardawil Paste (DB) in bacterial strains
— In case of non-metabolic activation method —
(preliminary)

Method	Test substance	Concentration (μg/plate)	With(+) or Without(−) S9Mix	Number of revertant colonies / plate				
				Base-pair substitution type			Frameshift type	
				TA100	TA1535	WP2uvrA⁻	TA98	TA1537
Plate method	Vehicle Control	—	—	136 162	11 12	19 27	15 20	6 10
	DB	100	—	148 157	14 14	11 27	11 11	5 5
		500	—	141 162	7 14	23 30	12 15	7 7
		1000	—	126☆ 146☆	14☆ 15☆	16☆ 16☆	9☆ 19☆	8☆ 9☆
		5000	—	197☆ 207☆	8☆ 19☆	18☆ 23☆	24☆ 27☆	5☆ 7☆
Pre-incubation method	Vehicle Control	—	—	159 165	10 11	17 20	10 11	4 7
	DB	100	—	125 163	9 10	17 25	9 15	5 7
		500	—	144 149	16 17	20 21	12 14	5 6
		1000	—	131☆ 140☆	11☆ 14☆	20☆ 29☆	15☆ 15☆	2☆ 4☆
		5000	—	185*☆ 204*☆	8*☆ 18*☆	38☆ 43☆	14☆ 27☆	5*☆ 8*☆

Vehicle : D.Water
* : killing
☆ : observed crystalline matter

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Table8 Reversion test of Dunaliella Bardawil Paste (DB) in bacterial strains
— In case of metabolic activation method —
(preliminary)

Method	Test substance	Concentration (μg/plate)	With(+) or Without(−) S9Mix	Number of revertant colonies / plate				
				Base-pair substitution type			Frameshift type	
				TA100	TA1535	WP2uvrA⁻	TA98	TA1537
Plate method	Vehicle Control	—	+	189 197	17 24	17 21	20 20	11 16
	DB	100	+	162 176	8 12	20 21	17 22	8 10
		500	+	154 174	13 15	11 26	17 22	11 12
		1000	+	161☆ 190☆	14☆ 23☆	21☆ 27☆	14☆ 17☆	7☆ 10☆
		5000	+	212☆ 212☆	19☆ 19☆	23☆ 23☆	18☆ 24☆	10☆ 12☆
Pre-incubation method	Vehicle Control	—	+	174 192	16 16	17 21	25 27	13 17
	DB	100	+	144 165	13 14	13 21	23 29	11 17
		500	+	156 161	14 19	14 19	15 22	10 15
		1000	+	170☆ 182☆	18☆ 19☆	26☆ 27☆	26☆ 30☆	5☆ 9☆
		5000	+	184*☆ 201*☆	12☆ 17☆	24☆ 32☆	18☆ 24☆	12*☆ 14*☆

Vehicle : D.Water
* : killing
☆ : observed crystalline matter

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Table 9 Reversion test of Dunaliella Bardawil Paste (DB) in bacterial strains

— In case of non-metabolic activation method —

Test substance	Concentration (μg/plate)	With(+) or Without(−) S9Mix	Number of revertant colonies/plate				
			Base-pair substitution type			Frameshift type	
			TA100	TA1535	WP2uvrA⁻	TA98	TA1537
Vehicle Control	—	—	141 144 149 (145)	14 17 18 (16)	14 16 18 (16)	13 16 17 (15)	4 4 6 (5)
DB	312.5	—	134 152 171 (152)	8 12 15 (12)	20 25 26 (24)	13 13 21 (16)	4 7 10 (7)
	625	—	128* 138* 143* (136)	9* 14* 16* (13)	20* 20* 25* (22)	10* 15* 20* (15)	3* 6* 8* (6)
	1250	—	154* 155* 155* (155)	7* 11* 14* (11)	13* 14* 21* (16)	17* 22* 26* (22)	6* 6* 6* (6)
	2500	—	135☆ 150* 179* (155)	11* 12* 13* (12)	18* 19* 35* (24)	15* 20* 22* (19)	2*☆ 3*☆ 5*☆ (3)
	5000	—	197*☆ 203*☆ 206*☆ (202)	10* 12* 17* (13)	24* 28* 32* (28)	15* 22* 29* (22)	6*☆ 8*☆ 15*☆ (10)
AF-2	0.01	—	328 333 346 (336)				
	0.1	—				212 224 241 (226)	
Na-Azide	0.5	—		166 191 237 (198)			
ENNG	2.0	—			1614 1854 1956 (1808)		
ACR	80.0	—					499 535 564 (533)

( ): Average number of colonies/plate

* : killing

☆ : observed crystalline matter

Vehicle: D.Water

000354

Table10 Reversion test of Dunaliella Bardawil Paste (DB) in bacterial strains
— In case of metabolic activation method —

Test substance	Concentration (μg/plate)	With(+) or Without (−) S9Mix	Number of revertant colonies/plate				
			Base-pair substitution type			Frameshift type	
			TA100	TA1535	WP2uvrA⁻	TA98	TA1537
Vehicle Control	—	+	137 148 157 (147)	10 12 12 (11)	17 20 22 (20)	17 24 30 (24)	13 13 13 (13)
DB	312.5	+	137 141 149 (142)	9 10 16 (12)	14 17 20 (17)	19 21 25 (22)	9 13 20 (14)
	625	+	115* 147* 152* (138)	10* 12* 13* (12)	21* 22* 23* (22)	24* 24* 36* (28)	7* 10* 19* (12)
	1250	+	114* 131* 124* (123)	11* 13* 15* (13)	13* 22* 24* (20)	19* 21* 25* (22)	15* 16* 18* (16)
	2500	+	138* 142* 165* (148)	16* 17* 17* (17)	13* 15* 22* (17)	19* 16* 28* (21)	8* 11* 16* (12)
	5000	+	197* 219* 238* (218)	14* 15* 21* (17)	16* 16* 20* (17)	25* 28* 35* (29)	9*☆ 14*☆ 19*☆ (14)
2AA	0.5	+				475 490 493 (486)	
	1.0	+	2154 2070 2592 (2272)				
	2.0	+		250 276 277 (268)			215 226 240 (227)
	20.0	+			474 486 465 (475)		

( ) : Average number of colonies/plate
* : killing
☆ : observed crystalline matter

Vehicle : D.Water

000355

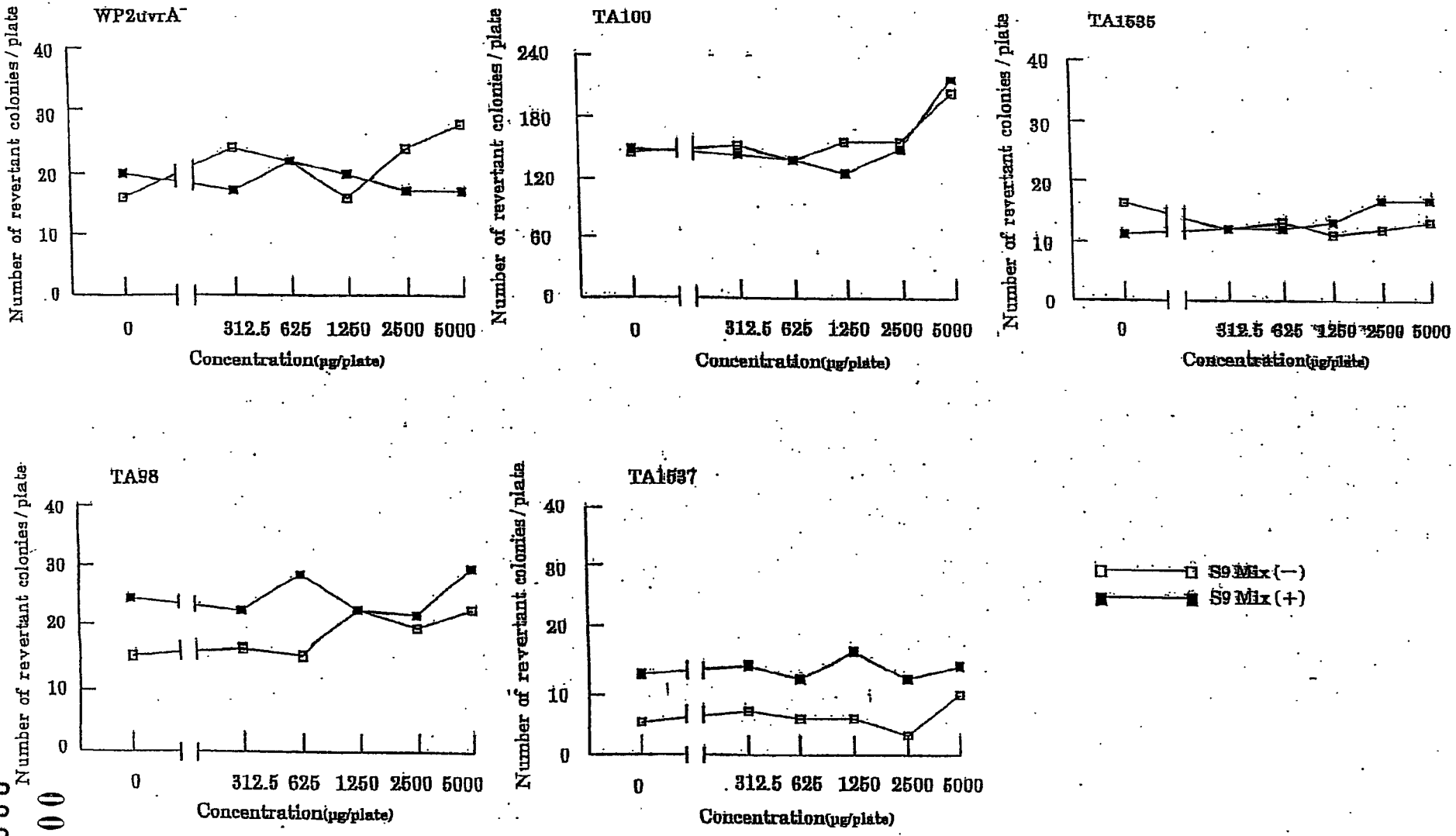

Fig.1 Reversion test of Dunaliella Bardawil Paste (DB) in bacterial strains

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予備試験結果表

(2 / ·) AHS/47-01/RS-01

試験番号 9137　　実施日 62. 9. 2　　実施者 永澤

試験の種類 予備試験　予備追加試験 (−S9Mix ・ +S9Mix)　　判定日 62. 9. 4　　判定者 永澤

被験物質名 D. B.　　Lot.No. NK-1001

検体濃度 (ug/plate)	復帰変異コロニー数/plate									
	プレート法					プレインキュベーション法				
	塩基置換型			フレームシフト型		塩基置換型			フレームシフト型	
	TA100	TA1535	WP2uvrA	TA98	TA1537	TA100	TA1535	WP2uvrA	TA98	TA1537
溶媒対照 (DMSO)	134	9	21	13	8	127	10	26	10	8
	149	11	28	27	12	147	13	27	11	15
100	138	8	21	16	11	114	11	18	13	14
	143	18	28	24	19	139	15	20	14	17
500	A147	A 3	A 17	A 11	A 7	A 121	A 9	A 23	A 7	A 7
	A100	A 12	A 25	A 17	A 18	A 122	A 15	A 25	A 16	A 8
1000	A105	A 5	A 18	A 16	A 13	A 79	A 6	A 8	A 13	A 5
	A128	A 17	A 29	A 20	A 18	A 114	A 16	A 13	A 20	A 13
5000	A 80	A 6	A 10	A 15	A 7	A 58	A 6	A 7	A 5	A 9
	A104	A 17	A 30	A 18	A 16	A 126	A 11	A 35	A 13	A 11

A: 析出物

<無菌試験結果>

S9 Mix	(0)	(0)
	( )	( )
	( )	( )

<O.D.値>

TA98	1,345	TA1535	1,575	WP2uvrA	1,545
TA100	1,465	TA1537	1,235		

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予備試験結果表

(/ /) AHS/A17-01XRS-01

試験番号 9137　　実施日 62. 9. 2　　実施者 永澤

試験の種類 予備試験　予備追加試験 (−S9Mix・+S9Mix)　　判定日 62. 9. 4　　判定者 永澤

被験物質名 D.B　　Lot.No. NK-1001

検体濃度 (ug/plate)	復帰変異コロニー数/plate									
	プレート法					プレインキュベーション法				
	塩基置換型			フレームシフト型		塩基置換型			フレームシフト型	
	TA100	TA1535	WP2uvrA	TA98	TA1537	TA100	TA1535	WP2uvrA	TA98	TA1537
	−	−	−	−	−	−	−	−	−	−
溶媒対照 (DMSO)	130 138	9 14	20 24	10 10	2 5	115 139	8 17	19 20	11 14	5 9
100	A 127 A 133	A 14 A 18	A 26 A 33	A 6 A 15	A 5 A 12	A 136 A 152	A 16 A 27	A 17 A 33	A 11 A 12	A 4 A 9
500	A 120 A 145	A 2 A 11	A 24 A 29	A 9 A 12	A 3 A 7	A 89 A 95	A 6 A 10	A 15 A 31	A 16 A 16	A 2 A 8
1000	A 95 A 111	A 9 A 13	A 22 A 31	A 17 A 18	A 2 A 10	A 124 A 167	A 6 A 6	A 22 A 25	A 4 A 10	A 3 A 6
5000	A 35 A 115	A 8 A 12	A 24 A 25	A 9 A 12	A 4 A 3	A 112 A 126	A 5 A 8	A 18 A 30	A 8 A 12	A 3 A 5
5										

A = 析出物

<無菌試験結果>

5000 ug/plate	(有)	(有)
	( )	( )
	( )	( )

① 培 4, 雑菌 18
② 培 7 / 雑菌 25

<O.D.値>

TA98	1.545	TA1535	1.575	WP2uvrA	1.545
TA100	1.465	TA1537	1.235		

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SOP/TSB/07/RS-02

Dunaliella Bardawil Spray Dried Powder 使用記録用紙 (No. 2)

試験番号: 9137
LOT No.: NK-1001
入手年月日: 昭和 62年 9月 17日
使用期限: 昭和 年 月 日

正味量: 3.5g × 2本
風袋込み量: ① 10.5333g ② 11.2378g
保管条件: 冷凍, 遮光
保管形態: 褐色瓶
保管庫 No.: 冷凍庫 No. 6

試験責任者 有賀文子

使用記録

年月日	採取量(使用量) (mg)	風袋込み残量(使用後) (g)	使用者	調製指示書 No.
62. 9 17	100mg	① 10.4328g	永澤	照射試験
		残量確認 62. 10. 28	永澤	
		使用量 100.5mg		

000359

SOP/TSB/07/RS-02

Dunaliella Bardawil
(Spray Dried Powder) 使用記録用紙 (No. 1)

試験番号: 9137
LOT No.: NK-1001
入手年月日: 昭和 62年 9月 1日
使用期限: 昭和 年 月 日

正味量: 2.5g × 2本
風袋込み量: ① 10.7113g ② 10.7611g
保管条件: 冷凍, 遮光
保管形態: 褐色瓶
保管庫 No.: 冷凍庫 No. 6

試験責任者: 有賀文方

使用記録

年月日	採取量(使用量) (mg)	風袋込み残量(使用後) (g)	使用者	調製指示書 No.
62.9.2	400mg	① 10.3108	永澤	予備試験
		残量確認		
		62.10.28	永澤	
		使用量 400.5mg		

000360

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SOP/TSB/07/RS-02

(Dunaliella Bardawil Spray Dried Powder) 使用記録用紙 (No. 2)

試験番号: 9137

L.O.T No.: NK-1001

入手年月日: 昭和 62年 9月 17日

使用期限: 昭和 年 月 日

正味量: 2.5g × 2本

風袋込み量: ① 10.5333g ② 11.2378g

保管条件: 冷凍 遮光

保管形態: 褐色瓶

保管庫 No.: 冷凍庫 No. 6

試験責任者: 有賀文子

使用記録

年月日	採取量(使用量) (mg)	風袋込み残量(使用後) (g)	使用者	調製指示書 No.
62. 9. 17	100mg	① 10.4328g	永澤	無菌試験
		残量確認 62. 10. 28	永澤	
		使用量 100.5mg		

000361

SOP/TSB/07/RS-02

Dunaliella Bardawil
(Spray Dried Powder) 使用記録用紙 (No. 1)

試験番号: 9137
LOT No.: NK-1001
入手年月日: 昭和 62年 9月 1日
使用期限: 昭和 年 月 日

正味量: 2.5g × 2本
風袋込み量: ① 10.7113g ② 10.7611g
保管条件: 冷凍, 遮光
保管形態: 褐色瓶
保管庫 No.: 冷凍庫 No. 6

試験責任者: 堀頃文彦

使用記録

年月日	採取量(使用量) (mg)	風袋込み残量(使用後) (g)	使用者	調製指示書 No.
62 9. 2	400.1mg	① 10.3108	永澤	予備試験
		残量確認 62.10.28	永澤	
		使用量 400.5mg		

000362

予備試験結果表

(2 /) AHS/17-01/RS-01

試験番号 9137　　実施日 62. 9. 2　　実施者 永澤

試験の種類 予備試験　予備追加試験 (−S9Mix・+S9Mix)　　判定日 62. 9. 4　　判定者 永澤

被験物質名 b. B.　　Lot.No. NK-1001

検体濃度 (ug/plate)	復帰変異コロニー数/plate									
	プレート法					プレインキュベーション法				
	塩基置換型			フレームシフト型		塩基置換型			フレームシフト型	
	TA100	TA1535	WP2uvrA	TA98	TA1537	TA100	TA1535	WP2uvrA	TA98	TA1537
溶媒対照 (DMSO)	134 149	9 11	26 28	15 27	8 12	127 147	10 13	26 27	10 11	8 15
100	138 143	8 18	21 28	16 24	11 19	114 139	11 15	18 20	13 14	14 17
500	A 147 A 100	A 3 A 12	A 17 A 25	A 11 A 17	A 7 A 18	A 121 A 122	A 9 A 15	A 23 A 25	A 7 A 16	A 7 A 8
1000	A 105 A 128	A 5 A 17	A 18 A 29	A 16 A 20	A 13 A 18	A 79 A 114	A 6 A 16	A 6 A 13	A 13 A 20	A 5 A 13
5000	A 80 A 104	A 6 A 17	A 10 A 30	A 15 A 18	A 7 A 16	A 58 A 126	A 6 A 11	A 7 A 35	A 5 A 13	A 9 A 11

A = 析出物

<無菌試験結果>

S9 Mix	(0)(0)
	( )( )
	( )( )

<O.D.値>

TA98	1.545	TA1535	1.575	WP2uvrA	1.545
TA100	1.465	TA1537	1.235		

0003363

0003363

予備試験結果表

(/ /) AMS/A7-01/RS-01

試験番号 9137　　実施日 62. 9. 2　　実施者 永澤

試験の種類 予備試験　予備追加試験 (−S9Mix・+S9Mix)　　判定日 62. 9. 4　　判定者 永澤

被験物質名 D B　　Lot.No. NK-1001

検体濃度 (ug/plate)	復帰変異コロニー数/plate									
	プレート法					プレインキュベーション法				
	塩基置換型			フレームシフト型		塩基置換型			フレームシフト型	
	TA100	TA1535	WP2uvrA	TA98	TA1537	TA100	TA1535	WP2uvrA	TA98	TA1537
溶媒対照 (DMSO)	130 138	9 14	20 24	10 10	2 5	115 139	8 17	19 20	11 14	5 9
100	A 127 A 133	A 14 A 18	A 26 A 33	A 6 A 15	A 5 A 12	A 136 A 152	A 16 A 27	A 17 A 33	A 11 A 12	A 4 A 9
500	A 120 A 145	A 2 A 11	A 24 A 29	A 9 A 12	A 3 A 7	A 89 A 95	A 6 A 10	A 15 A 31	A 16 A 16	A 2 A 8
1000	A 95 A 111	A 9 A 13	A 22 A 31	A 17 A 18	A 2 A 10	A 124 A 167	A 6 A 6	A 22 A 25	A 4 A 10	A 3 A 6
5000	A 35 A 115	A 8 A 12	A 24 A 26	A 9 A 12	A 4 A 3	A 112 A 126	A 5 A 8	A 18 A 30	A 8 A 12	A 3 A 5
5										

A = 析出物

<無菌試験結果>

5000 ug/plate	(有)	(有)
	( )	( )
	( )	( )

① カビ 4, 雑菌 18
② カビ 7 / 雑菌 25

<O.D.値>

TA98	1.545	TA1535	1.575	WP2uvrA	1.545
TA100	1.465	TA1537	1.235		

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試験番号：9157

試験計画書

被験物質名：DB（略称）

表　題

Dunaliella Bardawil Paste の変異原性試験（細菌を用いる復帰変異試験）

試験委託者

日健製薬工業株式会社

岐阜県羽島市福寿町平方 4 -41

試験機関

株式会社日本バイオリサーチセンター羽島研究所（運営管理者　仲川憲一）

岐阜県羽島市福寿町間島 6 - 104

試験目的

薬審第 118号（昭和59年 2月15日付）「医薬品のための毒性試験法ガイドライン」に基づいてDunaliella Bardawil Paste の細菌を用いる復帰変異試験を行い、それの遺伝子突然変異誘発性を推定する。

試験関係者

試験責任者　　有賀文彦

試験担当責任者　　永澤佳子

信頼性保証部門

担当責任者　　岡田謙司

試験開始日（運営管理者の署名捺印日）　　昭和62年 8月25日

試験実施期間

予備試験の菌の前培養実施日　　昭和62年12月15日

最終判定日　　昭和62年12月25日

報告書（案）提出日　　昭和63年 1月31日

最終報告書提出予定日　　昭和63年 2月29日

000365

試験番号：9137

署名捺印

試験責任者 (b) (6) [redacted]

氏　名：_____ 昭和62年8月25日

住　所：岐阜市西川手8-40 西川手ビル3F

所属先：株式会社日本バイオリサーチセンター羽島研究所

試験計画書の承認 岐阜県羽島市福寿町平方4丁目41番地
日健製薬工業株式会社
代表取締役 白石照雄

試験委託者 (b) (6) [redacted]

_____ 昭和62年8月25日

（日健製薬工業株式会社）

運営管理者 (b) (6) [redacted]

_____ 昭和62年8月25日

（株式会社日本バイオリサーチセンター羽島研究所）

確　認

信頼性保証部門

担当責任者 (b) (6) [redacted]

_____ 昭和62年8月25日

（株式会社日本バイオリサーチセンター羽島研究所）

000366

1．被験物質および陽性対照物質

（1）被験物質

被験物質名：Dunaliella Bardawil Paste （略称：DB）

ロット番号：NK-1010

性　　状：黄褐色のペーストで、~~水性~~水分を60%含む。

安 定 性：蒸留水中での安定性は現在不明であるが、試験委託者において確認される予定であるので、データ入手後追記する。

入 手 日：昭和62年12月14日 ~~（入手後記載）~~

入 手 量：75 g ~~（入手後記載）~~

保管条件：遮光下で冷蔵保存

保管場所：試験実施機関の被験物質保管庫（冷蔵庫）

提 供 元：日健製薬工業株式会社

媒　　体：蒸留水（和光純薬工業株式会社Lot No. P1071 ~~購入後記載）~~

（2）陽性対照物質

物質名	製造元	ロット番号
2-Aminoanthracene (2AA)	半井化学（株）	M5A3387
Sodium azide (Na-Azide)	半井化学（株）	M6M9272
9-Aminoacridine hydrochloride (ACR)	Aldrich C.	091097
N-Ethyl-N'-nitro-N-nitrosoguanidine(ENNG)	半井化学（株）	V4A7444
2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2)	和光純薬工業（株）	STJ0738

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（3）記録

被験物質の入手日、ロット番号、入手量、使用ごとの使用量を所定の用紙に記録する。

2．試験系

（1）試験菌株の種類および選択理由

Salmonella typhimurium

TA98、TA100 、TA1535、TA1537

E.coli

WP2uvrA⁻

薬審第 118号（昭和59年 2月15日付）「医薬品のための毒性試験法ガイドライン」に従い採用した。

（2）入手先および入手年月日

Salmonella typhimurium……国立衛生試験所（昭和55年 4月 1日）

E.coli ……東京大学医科学研究所（昭和55年11月15日）

（3）菌株の保存方法

Salmonella typhimuriumおよびE.coliとも菌懸濁液 0.8mℓに DMSO 0.07mℓの割合で加え、－80℃設定のディープフリーザー（サンヨー超低温フリーザー、型式MDF-190AT ）に保存する。

（4）菌株の特性検査

試験に用いる菌株は以下の特性検査基準（SOP/AMS/07-04 ）に適合しているものを用いる。 なお、この特性検査は当試験開始前 1ヵ月以内に実施する。

		TA100	TA98	TA1535	TA1537	WP2uvrA-
アミノ酸要求性[a]	His⁻	0	0	0	0	−
	Trp⁻	−[b]	−	−	−	5>
紫外線感受性[c]		+	+	+	+	+
膜変異 rfa特性[d]		12mm<	12mm<	10mm<	13mm<	0
薬剤耐性因子R-factor[e] プラスミドの有無		0mm	0mm	20mm<	20mm<	20mm<

a)復帰変異コロニー数/plate　b)実施しない　c)＋：感受性有り

d)Crystal violet (10μg／10μl) を含むpaper disk (8mm diameter) による生育阻止円の直径

e)Ampicillin Sodium (80μg／10μl) を含むpaper disk (8mm diameter) による生育阻止円の直径

(5) 試験系の識別方法

菌株毎に試験管およびプレートに油性インクで色分けし識別する。

3. S9Mix

(1) S9 ~~(購入後記載)~~

ロット番号：8709042

製　造　日：昭和62年9月4日

購　入　日：昭和62年9月18日

製　造　元：オリエンタル酵母工業株式会社

保 存 方 法：試験実施機関のディープフリーザー（サンヨー超低温フリーザー、型式 MDF-190AT）

誘 導 方 法：（オリエンタル酵母工業株式会社発行の検定書より）

7週齢の雄ラット（Crj:CD(SD)）約 匹（202.0 土 10.0g）に誘導物質としてPhenobarbital を 1日目は30mg／Kg、 2、 3、 4日目には60mg／Kgを腹腔内投与し、さらに 3日目には

000369

5,6-Benzoflavone 80mg／Kgを腹腔内投与して誘導する。

（2）S9Mix の組成（1mℓ中の量）

S9	0.1mℓ
$MgCl_2$	8 μmol
KCl	33 μmol
グルコース -6-リン酸	5 μmol
NADPH	4 μmol
NADH	4 μmol
Na- リン酸緩衝液(pH7.4)	100 μmol

Cofactor(Lot No.~~購入後記載~~ ラベル
オリエンタル酵母工業株式会社)

4．被験物質の調製方法

被験物質を蒸留水に溶解または懸濁して最高濃度溶液を調製し、以下の濃度は希釈法により作製する。 なお、被験物質には60%の水分が含まれているため、原体量が所定の濃度になるよう調製する。 また、調製は用時に行い、使用後の残液は廃棄する。

5．試験菌株の前培養

滅菌したニュートリエントブロス（NUTRIENT BROTH-No.2 、OXOID 社）10mℓに凍結菌株をかき取り移植して、37℃で16時間振盪培養する。 なお、培養終了後は使用時まで菌懸濁液は氷中に保存する。

6．培地

（1）最少グルコース寒天平板培地の組成

Agar（粉末寒天）	75g	Agar（Lot No.K-329、松栄寒天株式会社）
蒸留水	4.5ℓ	
Medium E (X20)	250mℓ	
40%Glucose	250mℓ	

1plate＝30mℓ分注

000370

（2）Medium E (X20)の組成

$MgSO_4 \cdot 7H_2O$	4g
Citric acid $\cdot H_2O$	40g
K_2HPO_4	200g
$NaNH_4HPO_4 \cdot 4H_2O$	70g

蒸留水にて全量を 1ℓ にする

7．無菌試験

検体溶液およびS9Mix の無菌試験をプレート法を用いて予備試験実施の際に行う。プレート数は各 2枚を用いる。

8．試験方法

（1）予備試験

被験物質の菌株に対する作用（生育阻害又は殺菌性）ならびにプレート上での溶解性を調べ、試験法（プレート法又はプレインキュベーション法）の選択および濃度設定をする目的で実施する。

1)試験方法

S9Mix 無添加および添加でプレート法およびプレインキュベーション法を以下の条件で行う。

		プレインキュベーション法	プレート法
組成	菌懸濁液	0.1 ml	0.1 ml
	被験物質溶液	0.1 ml	0.1 ml
	Na-リン酸緩衝液（代謝活性化によらない場合）	0.5 ml	0.5 ml
	S9Mix(代謝活性化法の場合）	0.5 ml	0.5 ml
	軟寒天溶液	2 ml	2 ml
プレインキュベーション	温　度	37 ℃	―
	時　間	20 分	―
インキュベーション	温　度	37 ℃	37 ℃
	時　間	48 時間	48 時間

2)試験濃度

原則として5000; 1000、 500、 100μg/plateの 4濃度および溶媒対照（蒸留水）について実施する。

なお、 100μg/plateにおいて菌の生育阻害が認められた場合は、濃度を50、10、5、 1、……μg/plateと下げ、菌の生育阻害が認められない濃度まで数段階を設定し、実施する。

使用プレート数は各 2枚を用いる。

（ 2）本試験

1)試験方法

予備試験の結果に基づき、プレート法又はプレインキュベーション法のどちらか一方によりS9Mix 無添加および添加で行う。

なお、試験方法の選択は、復帰変異コロニー数の増加が認められた方法を採用するが、復帰変異コロニー数の出現が同程度の場合は菌の生育阻害が強く認め

られた方法を、また復帰変異コロニー数および菌の生育阻害が同程度の場合は、プレインキュベーション法を採用する。

2)試験濃度

菌の生育阻害を認めた最小濃度、もしくは5000μg/plateを上限として、原則として公比 2で 5段階の濃度と溶媒対照および陽性対照について実施する。

3)使用プレート数

試験菌株、代謝活性化の有無および濃度段階の組合わせ毎に 3枚のプレートを用いる。

4)陽性対照物質

(a) 使用濃度

S9Mix を必要とする陽性対照

試験菌株の種類	化学物質の名称	濃度（μg/plate）
TA98	2AA	0.5
TA100	〃	1.0
TA1535	〃	2.0
TA1537	〃	2.0
WP2uvrA⁻	〃	20.0

S9Mix を必要としない陽性対照

試験菌株の種類	化学物質の名称	濃度（μg/plate）
TA98	AF-2	0.1
TA100	〃	0.01
TA1535	Na-Azide	0.5
TA1537	ACR	80.0
WP2uvrA⁻	ENNG	2.0

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(b) 溶媒および調製方法

Na-Azideは滅菌蒸留水に、2AA 、ACR 、AF-2およびENNGはDMSOに溶解する。調製は用時に行う。

(c) 安定性

これらの物質の安定性に関しては現在、正確な情報はない。 従って、試験結果がそれぞれの物質における過去の成績(SOP/AMS/20-01) から大きく逸脱していない場合、試験菌株が正常な反応性を有していたとともに、陽性物質も生物学的に充分な活性を有していたと判断する。

5)観察項目

プレート上での析出物の有無……肉眼的観察

菌の生育阻害…… 100倍の顕微鏡下で観察

復帰変異コロニー数……目算法

試験結果の表示……復帰変異コロニー数を実数で表示し、平均値を算出する。 また、その平均値を図示する。

9. 判定基準

無菌試験で雑菌の混入のないこと、溶媒対照、陽性対照の復帰変異コロニー数がバックグランドデータの範囲内の時試験は成立する。 試験の結果は、復帰変異コロニー数が溶媒対照の 2倍以上の値を示し、濃度に依存して増加した場合に陽性と判定する。

10. 試験計画書の修正

試験受託契約の締結後に、試験計画書の重要な内容変更の必要性が生じた場合には、試験委託者と試験機関の両者間で協議して合意の上で変更し、変更箇所、変更内容、変更理由を記した試験計画書修正書を作成して試験計画書とともに保管する。

11. 資料の保管

この試験に関するすべての資料は、株式会社日本バイオリサーチセンターの資料保管室に保管する。 保管期間は別途契約による。

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以下にそのリストを示す。

（1）試験計画書、試験計画書修正書、契約書、主計画表

（2）被験物質に関する記録

1)被験物質の受領および返却記録

2)被験物質の性状および安定性に関する記録（試験委託者から入手したもの）

3)被験物質の使用記録

4)被験物質の調製記録

（3）分析、環境制御記録

1)作業手順に関する記録

2)菌株の特性検査結果表（用いた菌株について 1ヵ月以内に実施したもの）の写し

3)S9検定書（S9に添付されたもの）の写し

（4）試験結果の記録

（5）報告書に関する保管資料

1)報告書作成に用いた図、表

2)報告書の原稿

3)最終報告書の写し

12．残余被験物質の返却

残余の被験物質は、試験終了後に使用記録（写）を添えて試験委託者に返却する。

000375

試験計画書の修正書（No.1）

試験番号：9137
表　題：Dunaliella Bardawil Pasteの変異原性試験（細菌を用いる復帰変異試験）

修正箇所、修正内容、修正理由

p.1　1．被検物質および陽性対照物質
(1) 被検物質
入手日：昭和62年12月14日
入手量：7.5g
媒　体：蒸留水（和光純薬工業株式会社、Lot No.P1071）

アンダーラン部分を追加する。
修正理由：被検物質入手後および媒体の購入後に記載することになっていたため。

p.5　3．S9Mix
(1) S9
ロット番号：87090412
製　造　日：昭和62年 9月 4日
購　入　日：昭和62年 9月18日
誘導方法：7週齢の雄ラット［Crj:CD(SD)］83匹（202.0±10.0g）に誘導～

(2) S9Mixの組成（1ml中の量）
Cofactor（Lot No.702、オリエンタル酵母工業株式会社）

アンダーラン部分を追加する。
修正理由：S9およびCofactor購入後に記載することになっていたため。

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(b) (6)

試験責任者 [redacted] 修正日 昭和62年12月17日

承認
運営管理者 [redacted] 昭和62年12月17日

試験委託者 [redacted] 昭和62年12月28日

岐阜県羽島市福寿町平方4丁目41番地
日健製薬工業株式会社
代表取締役 白石照雄

000377

試験計画書の修正書（No.2）

試験番号：9137
表　　題：Dunaliella Bardawil Pasteの変異原性試験（細菌を用いる復帰変異試験）

修正箇所、修正内容、修正理由

p.3　1．被験物質および陽性対照物質
　(1) 被験物質
　　性　　状：黄褐色のペーストで、水性を60%含む。
　　↓
　　水分

アンダーライン部分を修正する。
修正理由：タイプミス。

p.6　3．S9Mix
　(2) S9Mixの組成（1ml中の量）
　　Cofactor（Lot No.702、オリエンタル酵母工業株式会社）

アンダーライン部分を追加する。
修正理由：購入後記載することになっていたため。

試験責任者 (b) (6) 修正日　昭和63年2月1日

承認
運営管理者 (b) (6) 昭和63年2月1日

試験委託者 (b) (6) 昭和63年2月2日

000378

試験計画書の修正書（No.2）

試験番号：9137
表　題：Dunaliella Bardawil Pasteの変異原性試験（細菌を用いる復帰変異試験）

修正箇所、修正内容、修正理由

p.3　1．被験物質および陽性対照物質
　(1) 被験物質
　　性　　状：黄褐色のペーストで、水性を60%含む。
　　↓
　　水分

アンダーライン部分を修正する。
修正理由：タイプミス。

p.6　3．S9Mix
　(2) S9Mixの組成（1ml中の量）
　　Cofactor（Lot No.702、オリエンタル酵母工業株式会社）

アンダーライン部分を追加する。
修正理由：購入後記載することになっていたため。

試験責任者 (b) (6) 修正日　昭和 63 年 2 月 1 日

承認
運営管理者 (b) (6) 昭和 63 年 2 月 1 日

試験委託者 (b) (6) 昭和 63 年 2 月 2 日

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Dr. Jürgen E.W. Polle

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CURRICULUM VITAE

EDUCATION

Georg-August-University, Goettingen, Germany, 1994 - 1997
Doktor in Biology, 1997
Area of specialization: Plant Physiology and Biochemistry

Georg-August-University, Goettingen, Germany, 1989 - 1994
Diplom in Biology, 1994
Area of specialization: Plant Physiology and Biochemistry

University of Applied Financial Sciences, Rinteln, Germany, 1985 -1988
Diplom in Financial Sciences, 1988
Concentration: Tax Law

APPOINTMENTS

Associate Professor (tenured)

January 2007 - current: Department of Biology, Brooklyn College of the City University of New York, 2900 Bedford Ave, Brooklyn, NY, USA

December 2002 - current: Faculty in Biology (MCD subprogram) at the Graduate Center of the City University of New York

Assistant Professor

September 2002 – December 2006: Department of Biology, Brooklyn College of the City University of New York, 2900 Bedford Ave, Brooklyn, NY, USA

Associate Director

December 2005 - current: Associate Director of the Aquatic Research and Environmental Assessment Center at Brooklyn College of CUNY, Brooklyn, NY, USA

CONSULTANT

Consultant to companies in the area of biofuels from microalgae.

RESEARCH EXPERIENCE

Research performed in my laboratory centers on fundamental and applied aspects of cellular stress physiology in microalgae. Microalgae are the fastest growing plants on earth and are capable of producing biofuels or feedstock for biofuels. My laboratory focuses on the following research areas:

- Regulation of cellular stress physiology of hyper-saline organisms of the genus *Dunaliella* (Chlorophyta).
- Genomics of a model system for unicellular green algae, *Dunaliella salina.*
- Tool development for metabolic engineering of *Dunaliella salina.*
- Carotenoids as natural products from microalgae, example *Dunaliella salina.*
- Metabolism of microalgae for biofuels generation from microalgae.

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- Regulation of light-harvesting and improving solar energy conversion efficiency through photosynthesis of microalgae by investigating regulation of light harvesting in model systems such as the unicellular green alga *Chlamydomonas reinhardtii*, the marine diatom *Cyclotella sp.*, and the cyanobacterium *Synechococcus sp.* PCC6803.
- Engineering of new photobioreactors to tailor photonic input to photosynthesis for exceptional biomass productivity of microalgae.

Previous Research Experience

Postdoctoral Research

August 1997 – August 2002: *Dept. of Plant & Microbial Biology, University of California, Berkeley, USA* (advisor: Dr. A. Melis)

- Investigated the photosynthetic apparatus organization and performance of new strains of the green alga *Chlamydomonas reinhardtii* created by DNA insertional mutagenesis.
- Investigated biosynthesis of high-value carotenoids in microalgae.
- Investigated production of hydrogen by green algae.
- Analyzed the effect of different carbon sources on the photosynthetic apparatus organization and function.
- Studied acclimation and recovery of the photosynthetic apparatus to light stress.

Doctoral Research

August 1994 - July 1997: *Georg-August-University, Goettingen, Germany* (advisor: Dr. W. Wiessner)

- Studied changes in the chlorophyll antenna size and inactivation of photosystem II upon dark incubation of green algae.
- Investigated the effect of inhibitors on antenna and function of photosystem II.

Teaching Experience

Brooklyn College, Brooklyn, USA, 2002- present

Brooklyn College is a liberal arts institution within the system of the City University of New York enrolling both undergraduate and graduate students. PhD students are enrolled through the Graduate Center of the City University of New York. I taught classes at all levels. I created and developed the courses 'Comparative Physiology', 'Genomics & Beyond', and 'Biotechnology of Algae'. A list of courses I taught is provided below:

1. Comparative Physiology [Undergraduate, Bio34.1, currently about 300-500 students per year]: Every Semester 2004 - present
2. Genomics and Beyond [Undergradudate, Bio85.1, six students]: Fall 2008
3. Biotechnology of Algae [Undergraduate, Bio 59, about 15 to 25 students per year]: Spring Semesters 2004 - present
4. Biotechnology of Algae [Graduate, U711, about 10 students per year]: Spring Semesters 2004 - present
5. Field Botany [Undergraduate, Bio15, about 30 students]: Summer 2006
6. Special Topics: Model Systems in Biology, Lecture about green algae model systems

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[Graduate, Bio793.01]: Fall 2004, Fall 2007

7. Animal Physiology [Undergraduate, Bio 34, about 60 students]: Fall Semester 2003
8. Laboratory Research [Undergraduate and Graduate, currently about 10 undergraduate students and four graduate students: Fall 2002 - present
   Introduction and supervision of undergraduate and graduate students in the laboratory with teaching of molecular biology, biochemistry, and biophysical techniques.
9. Guest-Lectures on topics "Algal Viruses", "Genetically Modified Organisms", and 'Green Algae Model Systems'.

University of California, Berkeley, USA, 1998 - 2002
Introduction and supervision of undergraduate students in the laboratory.

University of California, Berkeley, USA, 2000 and 2001
Seminars on photosynthesis and plastid development, Graduate Course

SCHOLARSHIPS

- Education Abroad Program Fellowship (*University of California*), 1997 - 1998
- "Studienstiftung des Deutschen Volkes", German Honors Society, 1995 - 1997

SOCIETIES

- Project Kaleidoscope: Faculty for the 21st Century - 2006 to present
- International Society of Photosynthesis (ISP) - 2005 to present
- American Association for the Advancement of Sciences (AAAS) - 2004 to present
- Council for Undergraduate Research - 2004 to present
- Phycological Society of America (PSA) - 2000 to present
- American Society of Plant Biologists (ASPB) - 1998 to present
- German Botanical Society (DBG) - 1995 to 2003

SYNERGISTIC ACTIVITIES

- Member of the consortium for the *Dunaliella salina* genome sequencing project.
- Created *DUNALIELLA* CULTURE COLLECTION (DCCBC) a culture collection of unicellular green algae of the genus *Dunaliella*. This collection serves as a source facility providing *Dunaliella* algae for researchers worldwide. The collection can be accessed online at: *http://www.dunaliella.org/DCCBC*
- Set up a new database with information about saltwater algae of the genus *Dunaliella* (DUNABASE). This database is also already frequently accessed for information by users from numerous countries.
- Organizer and/or co-organizer for several regional research conferences.
- From 2003 to 2007, associated with the 'International Network on Biofixation of CO_2 and Greenhouse Gas Abatement with Microalgae'.
  Network manager is Dr. J.R. Benemann, Walnut Creek, CA, USA

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PUBLICATIONS

Accepted

Kim M, Park S, **Polle JEW**, and ES Jin. cDNA microarray analysis of the transcriptome of *Dunaliella* sp. (Chlorophyta) grown under different salinities. *Phycol Res*

Schubert BA, Timofeeff MN, **Polle JEW**, and TK Lowenstein. *Dunaliella* cells in fluid inclusions in halite: Significance for long-term survival of prokaryotes. *Geomicrobiology J*

Peer-Reviewed in Journals und Books

Tran D, Haven J, Qiu W-G, and **JEW Polle**. (2009) An update on carotenoid biosynthesis in algae: Phylogenetic evidence for the existence of two classes of phytoene synthase. *Planta*, 229 (3), pp. 723-729.

Ben-Amotz A, **Polle JEW**, and S Rao (Book Editors). (2009) The alga *Dunaliella*: Biodiversity, Physiology, Genomics & Biotechnology. Publisher: Science Publishers, New Hampshire, USA

Polle JEW, Tran D, and A Ben-Amotz. (2009) History, Distribution, and Habitats of Algae of the Genus *Dunaliella* TEODORESCO (Chlorophyceae). In: The alga *Dunaliella*: Biodiversity, Physiology, Genomics & Biotechnology. Publisher: Science Publishers, New Hampshire, USA

Polle JEW and S Qin. (2009) Development of Genetics and Molecular Tool Kits for Species of the Unicellular Green Alga *Dunaliella* (Chlorophyta). In: The alga *Dunaliella*: Biodiversity, Physiology, Genomics & Biotechnology. Publisher: Science Publishers, New Hampshire, USA

Gonzalez MA, Gomez PI, and **JEW Polle**. (2009) Taxonomy and Phylogeny of the Genus *Dunaliella*. In: The alga *Dunaliella*: Biodiversity, Physiology, Genomics & Biotechnology. Publisher: Science Publishers, New Hampshire, USA

Jin ES and **JEW Poll e**. (2009) Carotenoid Biosynthesis in *Dunaliella*. In: The alga *Dunaliella*: Biodiversity, Physiology, Genomics & Biotechnology. Publisher: Science Publishers, New Hampshire, USA

Polle JEW, Struwe L, and ES Jin. (2008) Identification and characterization of a new strain of the unicellular Green Alga *Dunaliella salina* (Teod.) from Korea. *J Microbiol Biotechnol* 18(5): 821-827.

Gordon J and **JEW Polle**. (2007) Ultrahigh bioproductivity from algae. *Appl Microbiol Biotechnol* 76: 969–975

Jin ES, Lee C-G, and **JEW Polle**. (2006) Secondary Carotenoid Accumulation in *Haematococcus* (Chlorophyceae): Biosynthesis, Regulation and Biotechnology. *J Microbiol Biotechnol* 16(6): 821-831

Park S, **Polle JEW**, Melis A, Lee TK, and ES Jin. (2006) Up-regulation of photoprotection and PSII-gene repair expression by irradiance in the unicellular green alga *Dunaliella salina*. *J Marine Biotechnol* 8(2): 120-128

Yokthongwattana K, Savchenka T, **Polle JEW**, and A Melis. (2005) Isolation and characterization of a xanthophyll-rich fraction from the thylakoid membrane of *Dunaliella salina* (green algae). *Photochem Photobiol Sci* 4(12): 1028-34.

Jin ES, **Polle JEW**, Hyun SM, Lee HK, and M Chang. (2003) Xanthophylls in microalgae: From biosynthesis to biotechnological mass production and application. *J Microbiol Biotechnol*

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13(2): 165-174

Polle JEW, Kanakagiri S, and A Melis. (2003) *TLA1*, a *Chlamydomonas reinhardtii* nuclear gene regulates the chlorophyll antenna size of photosynthesis. *Planta*, 217: 49-59

Jin ES, Yokthonkwattana K, **Polle JEW**, A Melis. (2003) Role of the reversible xanthophylls cycle in the photosystem-II damage and repair cycle in *Dunaliella salina* (green alga). *Plant Physiology*, 132: 352 - 364

Masuda T, **Polle JEW**, and A Melis. (2002) Biosynthesis and Distribution of Chl among the photosystems during recovery of the green alga *Dunaliella salina* from irradiance stress. *Plant Physiol*, 128: 603-614

Lokstein H, Tian L, **Polle JEW**, and D DellaPenna. (2002) Xanthophyll Biosynthetic Mutants of *Arabidopsis thaliana*: Altered Nonphotochemical Quenching of Chlorophyll Fluorescence is due to Changes in Photosystem II Antenna Size and Stability. *Biochim Biophys Acta*, 1553: 309-319

Polle JEW, Kanakagiri S, Jin ES, Masuda T, and A Melis. (2002) Truncated chlorophyll antenna size of the photosystems – A practical method to improve microalgal productivity and hydrogen production in mass culture. *Int J Hydrogen Energy*, 27: 1257-1264

Polle JEW, Niyogi KK and A Melis. (2001) Absence of lutein, violaxanthin and neoxanthin affects the functional chlorophyll antenna size of photosystem-II but not that of photosystem-I in the green alga *Chlamydomonas reinhardtii*. *Plant Cell Physiol* 42 (5): 482-491

Polle J, Kanakagiri S, Benemann JR, and A Melis. (2001) Maximizing photosynthetic efficiencies and hydrogen production by microalgal cultures. In, *Biohydrogen II: An Approach to Environmentally Acceptable Technology* (J Miyake, T Matsunanga and A San Pietro, Eds.), pp. 111-130. Pergamon Press, Oxford, UK

Jin ES, **Polle JEW**, and A Melis. (2001) Involvement of Zeaxanthin and of the Cbr protein in the repair of photosystem II from photoinhibition in the green alga *Dunaliella salina*. *Biochim Biophys Acta*, 1506 (3): 244-259

Polle JEW, Benemann JR, Tanaka A, and A Melis. (2000) Photosynthetic apparatus organization and function in wild type and a Chl *b*-less mutant of *Chlamydomonas reinhardtii*. Dependence on carbon source. *Planta* 211(3): 335-344

Doltchinkova VR, Georgieva K, Kapchina-Toveta V, and **JEW Polle**. (2000) Electrokinetic properties of thylakoids in *in vitro* cultured *Gyopsophila paniculata* plants. *Aust J Plant Physiol* 27 (11): 1085-1091

Polle JEW and A Melis. (1999) Recovery of the photosynthetic apparatus from photoinhibition during dark incubation of the green alga *Dunaliella salina*. *Aust J Plant Physiol* 26 (7): 679-686

Books Teaching

Polle JEW (2005) Comparative Physiology, Publisher: Thomson Brooks/Cole.

Polle JEW (2005) Instructor's Manual with Test Items. For: Plant Biology, Eds. Rost, Barbour, Stocking, and Murphy. Publisher: Thomson Brooks/Cole.

Polle JEW (2003) 34.1 Comparative Physiology. XanEdu Publishing Services, BRK129

Book Chapters

Contributed to a chapter: Renneberg R (2005) Biotechnologie für Einsteiger. Elsevier, Spectrum Akademischer Verlag, Heidelberg, Germany

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Conference Proceedings

Polle J, Belay A, Weissman J, Huesemann M, Pedroni P, and J Benemann. (2004) Microalgae Culture Maintenance for Greenhouse Gas Abatement and other Applications. *Proceedings 10th International Congress for Culture Collections*, Tsukuba, Japan

Polle JEW, Aksoy M, Stauffer G, Benemann J, and J Weissman. (2004) Genetic Improvements for Increasing Solar Energy Conversion Efficiency by Microalgae Cultures. *Proceedings 13th International Congress on Photosynthesis*, Eds. Van der Est & Bruce, Montreal, Canada, p. 1063-1065

Benemann J, **Polle J**, Huesemann M, Yu J, Brune D, Weissman J, and D Kyle. (2004) A Novel Photobiological Hydrogen Production Process. *Proceedings 13th International Congress on Photosynthesis*, Eds. Van der Est & Bruce, Montreal, Canada, p. 878-880

PATENTS

2008 - One patent application was filed for a novel type of photobioreactor.

2009 - Two provisional patent applications were filed for enzymes involved in isoprenoid and carotenoid biosynthesis.

INVITED SEMINARS AND PRESENTATIONS

Regularly invited to present research results at regional, national and international levels. A complete list with titles of talks can be provided upon request.

2009

1. NASA Ames, Moffet Field, CA, USA
2. AIChE Annual Meeting, Nashville, TN, USA
3. Algae Biomass Summit, San Diego, CA, USA
4. Lectures for the summer course 'Capturing the Sun', Massachusetts Institute of Technology, Boston, USA
5. 2nd Conference of the Intergovernmental Renewable Energy Organization, New York, USA
6. Scripps Institute of Oceanography, San Diego, CA, USA
7. Conference of the Institute of Biological Engineering, San Jose, CA, USA
8. University of California, San Diego, USA

2008

1. National Renewable Energy Laboratory, Golden, CO, USA
2. The Institute for Sustainable Cities, New York, NY, USA
3. Binghampton University of SUNY, NY, USA
4. International Conference on Salt Lake Research, Salt Lake City, Utah, USA
5. NREL-AFOSR Workshop: Algal Oil for Jet Fuel Production, Arlington, USA
6. Utah State University, Logan, Utah, USA

2007

1. 8th International Marine Biotechnology Conference, Eilat, ISRAEL
2. University of Nevada, Reno, NV, USA

2006

1. International Conference on Marine Algae and Global Warming, Seoul, SOUTH KOREA
2. University of Hanoi, University of Hue, and University of Ho Chi Minh City, VIETNAM

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3. 1st Pacific Rim Summit on Industrial Biotechnology, Honolulu, Hawaii, USA
4. University of New Hampshire, Durham, NH, USA

2005

1. University of Pennsylvania, Philadelphia, PA, USA
2. Hunter College of CUNY, Department of Biology, Manhattan, NY, USA

2004

1. Fairleigh Dickinson University, NJ, USA
2. Queens College of CUNY, Queens, NY, USA
3. 1st World Congress on Industrial Biotechnology and Bioprocessing, Orlando, FL, USA

2003

1. Korean Ocean Research and Development Institute, Ansan, Kung-Gi Do, SOUTH KOREA
2. Lehman College of CUNY, New York, NY, USA

2001

1. Tokyo Institute of Technology, Yokohama, JAPAN
2. Long Island University, Southampton College, Southampton, NY, USA
3. Biochemical Engineering Conference XII, Sonoma, CA, USA

2000

1. University of Nevada, Reno, NV, USA
2. Humboldt-University, Berlin, GERMANY
3. Georg-August University, Göttingen, GERMANY
4. University of California, Davis, CA, USA

CONFERENCES

Regular participation in regional, national, and international conferences. A complete list is available upon request.

(b) (6)

Jürgen Polle
Associate Professor

SUBMISSION END

SUBMISSION END

AM

From: Ruxanne Baines
To: Mosley, Sylvester;
cc: CK GUND;
Subject: Points for Clarification Regarding GRN 000351 (Dunaliella bardawil alga) (Nikken)
Date: Tuesday, October 12, 2010 2:41:07 PM
Attachments: Revised TOC - GRN 10-12-10.doc
Revised TOC Vol 2- GRN 10-12-10.doc

Dear Dr. Mosley,

Attached is the revised Table of Contents document regarding the above-mentioned subject.

Feel free to contact Dr. Gund if you have any questions.

Regards,

Ruxanne
Ruxanne Baines
Administrative Assistant
Phoenix Regulatory Associates, Ltd.
21525 Ridgetop Circle, Suite 240
Sterling, VA 20166
Phone: 1-703-406-0906
Fax: 1-703-406-9513
Email: phoenix@phoenixrising.com
Website: http://www.phoenixrising.com

Celebrating 19 Years of Service

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Japan Food Research Laboratories, Certificates of Analysis from Lots #0909, #1001, #1004 of *Dunaliella bardawil* powder for Iodine, Lead and Cadmium using Inductively coupled plasma mass spectrometry, June 17, 2010, 6pp; AminoLab, Ltd., Certificate of Analysis, *Dunaliella* algae Medium, As, Cd and Pb, dated 11/3/09, Method, Mercury by Cold Vapor and Metal scan by ICP, 1p; and Bactochem, Ltd., Report No.61/89122, *Dunaliella* Algae Medium for Iodine, 4/20/09, 1p.	000080	7
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AM

From: CK GUND
To: Mosley, Sylvester;
Subject: RE: Points for Clarification Regarding GRN 000351 (Dunaliella bardawil alga)
Date: Friday, October 15, 2010 1:55:13 PM

Hi--

I just left a message on your voice mail regarding the other two issues that you identified in our conversation on Tuesday.

1. Page 9 of 52: The revised sentence, 'This genus includes several ill-defined unicellular species that live in a wide range of marine habitats from seawater to hyper-saline waters. The words in red will replace 'fresh water.'

2. Page 23 of 52: After discussing the Lock, 1985 citation with Dr. Kraska, the paper itself is not available and the company is no longer in the Dunaliella business. He obtained this information from the summary of the JECFA report on Carotene from natural sources (algal and vegetable). This report looked at the biomass as well as the carotene and is discussed in the first paragraph on page 23 of 52. I recommend that we revised page 23 of 52 as follows:

Last paragraph, 2nd line, 'dose of 5g/kg and were observed for the subsequent 14 days (Lock, 1985; copy unavailable, summary available in WHO, 1993).

I will remove the citation in the reference list of Lock, 1985.

If this is agreeable with you, then I will forward the revised pages to you. Also do you want a copy of the JECFA report?

Best regards,

ck

AM

From: Ruxanne Baines
To: Mosley, Sylvester;
cc: CK GUND;
Subject: Points for Clarification Regarding GRN 000351 (Dunaliella bardawil alga)
Date: Tuesday, October 19, 2010 10:09:22 AM
Attachments: Nikken JECFA Report.pdf
Nikken revised GRN Pages.pdf

Dear Dr. Mosley,

Attached are the revised pages for the GRN Report and the JECFA Report.

Feel free to contact Dr. Gund if you have any questions.

Ruxanne

From: CK GUND
Sent: Tuesday, October 19, 2010 9:38 AM
To: 'Mosley, Sylvester'
Subject: RE: Points for Clarification Regarding GRN 000351 (Dunaliella bardawil alga)

I forgot to ask whether you want the pages that are revised to show today's date.

Best regards,

ck

From: Mosley, Sylvester [mailto:Sylvester.Mosley@fda.hhs.gov]
Sent: Tuesday, October 19, 2010 9:00 AM
To: CK GUND
Subject: RE: Points for Clarification Regarding GRN 000351 (Dunaliella bardawil alga)

The PDF copies should suffice.

Sincerely,
Sylvester

Sylvester L. Mosley, Ph.D.
Consumer Safety Officer

Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety-CFSAN
U.S. Food and Drug Administration
5100 Paint Branch Parkway (HFS-255)
College Park, MD 20740-3835
Phone: 301-436-1333
Fax: 301-436-2965
Email: Sylvester.Mosley@fda.hhs.gov

From: CK GUND [mailto:ckgund@phoenixrising.com]
Sent: Tuesday, October 19, 2010 9:01 AM
To: Mosley, Sylvester
Subject: RE: Points for Clarification Regarding GRN 000351 (Dunaliella bardawil alga)

Thanks so much Sylvester. I will have Ruxanne revise and send PDF copies of the pages. Do you want hard copies as well for the sets that were sent to you?

Best regards,

ck

From: Mosley, Sylvester [mailto:Sylvester.Mosley@fda.hhs.gov]
Sent: Tuesday, October 19, 2010 8:53 AM
To: CK GUND
Subject: RE: Points for Clarification Regarding GRN 000351 (Dunaliella bardawil alga)

Good morning CK,

I have spoken with the necessary reviewers regarding the issues in 1 and 2 and they are both in agreement with your amendments. Therefore, you can go ahead and send the amended pages. A copy of the JECFA report would also be helpful.

Sincerely,
Sylvester

Sylvester L. Mosley, Ph.D.
Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review

CAROTENES FROM NATURAL SOURCES (ALGAL AND VEGETABLE)

First draft prepared by
Dr G.J.A. Speijers
National Institute of Public Health
and Environmental Protection
Laboratory for Toxicology, Bilthoven, The Netherlands

1. EXPLANATION

Carotenes from natural sources were reviewed at the eighteenth, thirty-first and thirty-fifth meetings of the Committee (Annex 1, references 35, 77 and 88). At its thirty-first meeting, the Committee noted that, while there was a substantial toxicological database relating to carotenes and an ADI had been established for synthetic ß-carotene, the same ADI was not applicable to natural carotenes as they did not comply with the specifications for ß-carotene.

At the thirty-fifth meeting, the Committee concluded that there was insufficient evidence to indicate that data relating to one species of *Dunaliella* algae could be applied to others and that the specifications of the test materials were so different from one another that the results of the toxicity tests could not be generalized. There were insufficient data to evaluate any of these materials for the purpose of establishing an ADI. The Committee concluded that carotene isolated from algal sources would be acceptable for food additive use if it was of sufficient purity to meet the specifications for synthetic ß-carotene. Acceptance of algal biomass or crude extracts of carotene from algal sources for use as food additives would be contingent on the provision of evidence of the safety of such materials.

At its thirty-fifth meeting, the Committee considered limited short-term toxicological studies on material stated to have been prepared from three different algal species designated *Dunaliella bardawil, D. salina and D. kona* (Annex 1, reference 88). At its present meeting, the Committee was informed that *Dunaliella bardawil, D. kona and D. salina* were identical, and that, according to current nomenclature, the species used commercially was *Dunaliella salina*. Some of the preparations produced from this species were dehydrated powders prepared by lyophilization or spray-drying and others were vegetable oil extracts.[1]

[1] Although the Committee acccepts that this report does not cover different *Dunaliella* species, the studies are summarized with original names mentioned in the reports or publications submitted.

With respect to the carotene preparations derived from extraction of vegetables, mainly carrots, alfalfa or vegetable oil, the Committee at the thirty-first meeting (Annex 1, reference 77) felt that the need for toxicity tests may be obviated if detailed analytical data were supplied to confirm that natural toxicants occurring at low levels in food/feed stuffsare not concentrated in the extract and that levels of use would not materially exceed the levels of exposure that would result from normal use.

As no toxicological monograph has been prepared previously by the Committee on carotenes from natural sources, data that have been reviewed at previous meetings are incorporated in the present monograph along with the new data that have become available. Because this monograph covers the data on both algal and vegetable carotene preparations, a modified form of the general monograph format has been used, summarizing in order biological data on spray dried concentrated, lyophilized or dehydrated preparations of *Dunaliella*, then data on vegetable oil extracts of *Dunaliella*, then data on carotene extracts from carrots, grass alfalfa and vegetable oil.

ALGAL CAROTENE PREPARATIONS
SPRAY DRIED CONCENTRATED, LYOPHILIZED OR DEHYDRATED PREPARATIONS OF *DUNALIELLA SALINA*
(syn *DUNALIELLA BARDAWIL and DUNALIELLA KONA)*

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion.

Male weanling CD rats were fed *ad libitum* a retinol-deficient diet. After 60 days, the retinol content of the livers was 4-5 µg. Depleted rats were allocated to 9 groups of 6 animals, housed individually, and fed a retinol-deficient diet supplemented as follows: Group 1, retinol at 7.5 mg/kg diet; Groups 2-4, all- *trans* ß-carotene at 12, 29 or 48 mg/kg diet respectively; Groups 5-7, lyophilized *Dunaliella* contributing 29, 58 or 112 mg ß-carotene/kg diet respectively; Group 8, maize oil extract of *Dunaliella* providing 16 mg ß-carotene/kg diet; and Group 9, no supplementation. After seven days repletion, livers were taken for analysis for retinol, retinol isomers and ß-carotene. The liver analyses revealed a comparable content of retinol related to dose of carotene, irrespective of source whether synthetic, algal biomass or algal oil extract. Rats fed the algae or the algal extract-supplemented diets accumulated 9- *cis* retinol in addition to the all- *trans* isomer. Rats fed synthetic ß-carotene, lyophilized algae or algal oil had a liver retinol:ß-carotene ratio of about 3:1. Rats fed algae or algal oil accumulated 9- *cis* ß-carotene and all- *trans* ß-carotene in the liver in a ratio similar to that present in the algae. It was concluded that dried *Dunaliella bardawil* or an oil extract of the alga can serve as a dietary natural ß-carotene source which can satisfy the total requirement of retinol in rats (Ben-Amotz *et al.* 1988). [Note: this study is again cited below in discussion of absorption of oil-extracted algal carotene]

A 45-day-feeding study with 21-day old Sprague-Dawley rats was performed to compare the bioavailability of four sources of ß-carotene; Spray dried *Dunaliella salina* (0.61 % ß-carotene), ß-carotene oil extract of *Dunaliella salina* (24.83 % ß-carotene), oleoresin of carrots (11.5 % ß-carotene) and synthetic ß-carotene (100 %). The diet with *Dunaliella salina* powder (59 g/kg diet) was corrected for the amount of sucrose. The diets were made equal in the percentage of ß-carotene, and the control diet contained 0.036 % ß-carotene.

The rats on the *Dunaliella salina* diets grew more rapidly. Gross macroscopy at autopsy revealed no alterations in the rats.

The bioavailability of *Dunaliella salina* as a source of ß-carotene and oil extract of ß-carotene from *Dunaliella salina* was higher than that of ß-carotene from oleoresin of carrots or

synthetic ß-carotene. These results may be due to the presence of the extra lipids in both the dried *Dunaliella salina* which contains 8.6 % lipid and the oil extract of *Dunaliella salina*. These results lead to the recommendation that dried *Dunaliella salina*, when used as a source of ß-carotene, should be consumed in oil to increase ß-carotene bioavailability. This is because activity of carotene dioxygenase, the enzyme responsible for the conversion of ß-carotene to vitamin A, is increased in the presence of oil (Ghazi *et al.*, 1992).

In one experiment, groups of 1-day-old white Leghorn chicks received a retinol-deficient semi-purified diet or a similar diet supplemented with 8.04 mg retinol/kg diet, or 30 mg synthetic ß-carotene/kg diet or lyophilized *D. bardawil* at 1 g algae/kg diet. The algal powder contained 30 g ß-carotene and 200 g NaCl/kg and provided 30 mg ß-carotene/kg diet. In a second experiment, four similar groups were used except that the lyophilized algal powder was replaced with a similar concentration of drum-dried algae. The drum-dried algal powder contained 34 g ß-carotene, 260 g NaCl, 180 g glycerol, 5.5 g chlorophyll, 210 g protein, 170 g carbohydrate and 120 g lipid/kg (34 mg ß-carotene/kg diet). A third experiment utilized three groups of 15 one-day-old chicks which received retinol-deficient diet alone, or supplemented with lyophilized *D. bardawil* at 0.58 g/kg diet, or with drum dried algae at 1 g/kg diet. In each experiment, the chicks were assessed visually and weighed daily for 5 weeks and at termination serum and liver were analysed for retinol, ß-carotene and lutein.

After an initial lag, the chicks grew equally well on diets containing retinol, ß-carotene or algae in all experiments. Serum and liver concentrations of retinol were normal in all cases except for the chicks receiving retinol-deficient diets without supplements. The serum of chicks fed the algal-supplemented diets contained lutein but no ß-carotene although the ratio of ß-carotene to lutein in the algae exceeded 15:1.

In a separate experiment, two groups of 3 egg-laying hens received a control diet containing 150 g maize meal/kg or the same diet supplemented with 4 g lyophilized *D. bardawil*/kg. The algal preparation contained 50 g ß-carotene and 300 g NaCl/kg (200 mg ß-carotene/kg diet). Eggs from these hens showed an enhanced yolk colour attributable to lutein; no ß-carotene was present in the egg yolk (Ben-Amotz 1986).

2.1.2 Biotransformation

No information available.

2.1.3 Effects on enzymes and other biochemical parameters

No information available.

2.2 Toxicological studies

2.2.1 Acute toxicity studies

2.2.1.1 Mice

Acute toxicity of *Dunaliella bardawil* spray dried powder was established in an LD_{50} test with mice. A single dose of 2.5, 5.0 or 10.0 g *Dunaliella bardawil*/kg diluted with CMC-Na solution was administered by oral intubation. The observation period was 14 days, and mortality, general symptoms, body weights and gross necropsy examination were recorded. The LD_{50} value was greater than 10 g *Dunaliella bardawil*/kg for both male and female mice (Aruga, 1987).

2.2.1.2 Rats

Male Sprague-Dawley rats were given dried *Dunaliella* by gavage at a dose of 5 g/kg bw and observed for the subsequent 14 days. There were neither mortalities nor overt signs of toxicity and all the rats gained weight during the observation period (Lock 1985).

2.2.2 Short-term toxicity studies

2.2.2.1 Mice

In the search for an antioxidative-anticarcinogenic substance, the effects of repeated ingestion (no dose level given) of spray dried *Dunaliella bardawil* on mammary growth and endocrine parameters were examined in mice. In an additional group the mice received also the vitamin A-deficient synthetic standard diet supplemented with synthetic all-trans ß-carotene, whereas the control animals received the normal synthetic diet adequate in vitamin A. The concentration of ß-carotene in both test diets was 0.55 mg/kg. The ingestion of the *Dunaliella bardawil*-containing diet between 20 and 120 days of age showed no deleterious side-effects on mammary gland and uterine growth nor mammatrophic hormone secretion, these results were similar to previously-observed results in aged and mammary tumour-bearing mice. Puberty and body growth were accelerated by *Dunaliella bardawil* compared to the synthetic all-trans ß-carotene (Nagasawa *et al.*, 1989).

2.2.2.2 Rats

Two groups of 10 male and 10 female weanling Sprague-Dawley rats, caged individually, were given powdered diets containing 0 or 10% algal ß-carotene powder for 12 weeks. Body weight gain and food intake were recorded at intervals up to and at termination when the animals were autopsied. Weights of heart, lungs, liver, kidneys, spleen, gonads and adrenals were determined at autopsy and blood was collected for determination of serum glucose, ASAT, ALAT, alkaline phosphatase, uric acid, BUN, triglycerides and cholesterol. One male rat in the treated group died during the study from "non-specific problems" not related to treatment.

Significant differences were observed in neither food intake nor body weight gain between treated and untreated animals of either sex and there were no treatment-related differences in organ weights. Except for one treated male which displayed elevated ASAT and ALAT levels, no significant differences were observed in any of the clinical biochemical parameters between treated and untreated animals of either sex. No histopathological examination was performed (Majnarich 1988).

In a 28-day toxicity study spray dried *Dunaliella bardawil* powder was orally administered to rats (5/group/sex) at dosages of 0.5 and 2.5 g *Dunaliella bardawil*/kg bw/dy. The control animals (only) received 0.5 % aqueous solution of sodium CMC, which was used as the vehicle for the preparation of the test article suspension. Toxicological parameters recorded included food consumption, body weight gain, urinalysis, ophthalmoscopy, haematology, serum biochemistry, organ weights and histopathological examination.

In the males of the 2.5 g *Dunaliella bardawil*/kg bw/dy group a significant increase in the relative weight of the kidneys was noticed. Except slight changes in the thymus and the kidneys in a few animals of the 2.5 g *Dunaliella bardawil*/kg bw/dy group, no histopathological changes were reported. Although the effects were observed mainly in the kidneys, it was suggested by the authors that

the dosage of 2.5 g *Dunaliella bardawil*/kg bw/dy was a NOEL (Furahashi, 1989).

2.2.3 Long-term toxicity/carcinogenicity studies

No information available.

2.2.4 Reproduction studies

The safety of the alga *Dunaliella bardawil* for food use was evaluated in a multigeneration study with rats. Four generations were raised on diets containing 0, 50 and 100 g/kg of dehydrated *D. bardawil*. The caloric value of the diets with the *Dunaliella bardawil* preparations was adapted by lowering the amount of starch. Each experimental group comprised 10 males and 20 females. Starting with an F_0-generation 3 other generations (F_1 - F_3) were raised. The rats of the F_0-generation were kept on the different diets for 1 year and 5 male and 5 female rats were studied for general toxicological effects. No significant differences were observed between the rats consuming algae and the controls, of any generation, in general appearance, behaviour, growth, reproductive performance or gross pathology. The only effect of *D. bardawil* powder observed was a significantly increased relative kidney weights. The blood chemistry and haematology of the first-generation animals, after 1 year on the diets, showed no appreciable differences between the experimental and control animals. The only differences in histopathology observed were a decrease in some chronic inflammations, a slightly higher frequency of metaplasia of the renal pelvis epithelium with ectopic nephrocalcinosis in the renal papillae and an increased frequency of focal bronchopneumonia in rats fed 10 g algae/kg feed when compared with the controls. The latter effect may be attributed to the powdery nature of the algal diet. Although at dose levels effect were recorded on the kidneys and which were not explained, the authors concluded that this multigeneration feeding study may be indicative of the safety of *D. bardawil* for human consumption (Mokady *et al.*, 1989).

2.2.5 Special studies on genotoxicity

A mutagenicity study (Ames test) in *Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 and *Escherichia coli* WP2 uvrA both with and without activation by a liver microsomal S-9 mix was performed at dose levels of 312.5, 625, 1 250, 2 500 and 5 000 µg *Dunaliella bardawil* paste/plate. *Dunaliella bardawil* was not mutagenic in any strain (Aruga, 1988).

2.3 Observations in humans

Nine subjects were maintained on a low-carotene diet for two weeks and serum carotene levels were then determined. For the next ten days the volunteers took a daily dose of the powdered algal preparation providing 75 000 IU ß-carotene (approx 135 mg) in capsule form. Serum carotene was measured on days 7 and 10 of treatment. There was considerable interindividual variation in response to the same dose of carotenes, both in absolute values and in the treatment-dependent increase in serum concentration of carotene. In six of the subjects the serum level of carotene continued to rise between the seventh and tenth day of the study while in three others there was a slight fall in this period. One subject with normal serum carotene levels at the outset showed virtually no response to treatment. No adverse effects due to ingestion of the algal preparation were reported (Cyanotech, 1988).

ALGAL CAROTENE PREPARATIONS

VEGETABLE OIL EXTRACT OF *DUNALIELLA SALINA*
(syn *DUNALIELLA BARDAWIL and DUNALIELLA KONA)*

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution and excretion.

In an experiment fully described in section 2.1.1 describing results with spray dried concentrated, lyophilized or dehydrated preparations of *Dunaliella*, male weanling CD rats were fed a retinol-deficient diet *ad libitum*. After depletion, rats were allocated to groups and fed a retinol-deficient diet supplemented with one of: retinol; all- *trans* ß-carotene; lyophilized *Dunaliella*; maize oil extract of *Dunaliella*; and no supplementation. After seven days repletion, livers were taken for analysis of retinol, retinol isomers and ß-carotene. The liver analysis revealed a comparable content of retinol related to dose of carotene, irrespective of source i.e. synthetic, algal biomass or algal oil extract. It was concluded that dried *Dunaliella bardawil* or an oil extract of the alga can serve as a dietary natural ß-carotene source which can satisfy the total requirement of retinol in rats (Ben-Amotz *et al.* 1988).

2.1.2 Biotransformation

No information available.

2.1.3 Effects on enzymes and other biochemical parameters

No information available.

2.2 Toxicological studies

2.2.1 Acute toxicity studies

No information available.

2.2.2 Short-term toxicity studies

No information available.

2.2.3 Long-term toxicity/carcinogenicity studies

No information available.

2.2.4 Reproduction studies

No information available.

2.2.5 Special studies on genotoxicity

The commercial, carotene-rich corn oil extract of *Dunaliella salina* was inactive in an *in vitro* primary hepatocyte unscheduled DNA synthesis assay (Cifone 1987).

The extract was negative in an assay of forward mutation at the HGPRT locus in cultured Chinese hamster ovary cells, with or without metabolic activation with rat liver S9 fraction. A dose-related cytotoxicity was noted at concentrations above 2.0 µl/ml without S9 and above 10.0 µl/ml in the presence of S9 (Young 1987).

The material was not mutagenic in the *Salmonella*/microsome assay (Ames test) with *Salmonella typhimurium* strains TA-1535, TA-1537, TA-1538, TA-98 and TA-100 with or without metabolic

activation (Jagannath 1987).

In an *in vivo* mouse micronucleus assay using adult ICR mice, the commercial carotene extract did not induce a significant increase in micronuclei in bone marrow polychromatic erythrocytes (Ivett 1987).

2.3 Observations in humans

After a depletion period of 10 days on a low-carotene diet, 12 male and 20 female healthy adults were randomly assigned to one of five treatment groups. Two groups received capsules of carotene obtained by vegetable oil extraction of *Dunaliella salina* providing ß-carotene at levels of 8 or 24 mg and alpha-carotene at levels of 1.1 or 3.2 mg respectively. Two further groups received carrots (69.1 or 207.3 g respectively) that provided a similar amount of ß-carotene to the *Dunaliella salina* extract groups; the corresponding amounts of alpha-carotene were 6.3 and 18.9 mg respectively. A fifth group received placebo capsules. The subjects received the treatment for seven days and then underwent another depletion phase of 7 days.

Treatment with carotene capsules or carrots led to an expected increase in serum alpha- and ß-carotenes, with the higher dose treatments being less efficient per mg carotene consumed. The encapsulated algal carotenes were more efficient at raising serum values per mg fed, consistent with other reports that carotenes are better absorbed from oily solution than a vegetable matrix (Jensen *et al.* 1985).

In a study on the bioavailability of cis- and trans-ß-carotenes, 16 healthy adults, who had been on a low-carotene diet for ten days, were fed either ß-carotene extracted from *Dunaliella salina* alga, containing approximately equal amounts of all-trans-ß-carotene and 9-mono-cis-ß-carotene, or ß-carotene in the form of fresh carrots containing predominantly trans-ß-carotene, or avocado

oil-placebo capsules. Subjects were randomly divided into three groups: they consumed daily in a single dose either 3 ß-carotene capsules (24 mg ß-carotene), 207.3 g carrots (24 mg ß-carotene); or 3 ß-carotene free placebo capsules for seven days. HPLC determinations of serum trans-cis ß-carotene ratios showed trans ß-carotene to be the predominate serum isomer before and during all treatments. Serum trans-ß-carotene concentrations were significantly increased in the ß-carotene capsules and carrot groups. Cis-ß-carotene concentrations were increased in the carrot and placebo groups. However, the serum isomer increments for those taking ß-carotene capsules and carrots strongly favoured trans-ß-carotene over cis-ß-carotene. These data demonstrate a predominant absorption of intact trans-ß-carotene over intact cis-ß-carotene into human serum even when approximately equivalent amounts of these isomers were ingested. This selective absorption of intact ß-carotene isomers might be a factor in their biopotency in humans (Jensen *et al.*, 1987).

CAROTENE EXTRACTS FROM VEGETABLES
(Carrots, alfalfa and vegetable oil)

2. BIOLOGICAL DATA

2.1 Biochemical aspects

No information available.

2.2 Toxicological studies

No information available.

2.3 Observations in humans

The acute effects of consuming alpha- and ß-carotene from carrots on serum alpha-carotene and ß-carotene levels were investigated in 17 adult subjects (18-58 years of age). After a 10-day low-carotene diet, the subjects were randomized into three groups based on day 6 ß-carotene levels. On day 11, fasting baseline blood was drawn. Either 3 carrots, 1 carrot or 3 placebo capsules were then consumed following a low-carotene breakfast. Blood was drawn 1, 2, 3, 4, 5, 7 and 24 hours post-treatment and alpha- and ß-carotene levels were determined by HPLC. Treatment of 3 carrots yielded significantly greater peak alpha- and ß-carotene levels in serum at 5 hours post-treatment than did treatments with 1 carrot or 3 placebos. These results suggest the best condition for drawing blood samples to assess the serum carotene status of adults is at fasting state and that significant alterations in serum can occur within 5 hours of a carotene rich meal (Jensen *et al.*, 1986).

3. COMMENTS

Few new toxicological data have become available since the previous review by the Committee (Annex 1, reference 88). There were no data from long-term toxicity or teratogenicity studies, although a multigeneration study on dehydrated *Dunaliella bardawil* (= *salina*) in rats did not reveal any adverse effects on reproductive performance or gross fetal morphology. However, a NOEL was not identified in this study, as animals of the F_0-generation maintained on diets containing 5% and 10% algal carotene for one year showed renal pathological changes. In addition, there was focal bronchopneumonia at the higher level. Although the renal changes (metaplastic changes in the pelvic epithelium and nephrocalcinosis) might have been due to nutritional imbalance, this was not clearly established. The focal bronchopneumonia observed in the lung at the higher dose level may have been associated with inhalation of powdered diets, although both low-dose and control rats also received powdered diets without showing similar effects. The Committee considered that the available short-term toxicity studies inadequate for establishing an ADI because of the small numbers of animals tested, lack of or inadequate histopathological examination, or inadequate reporting.

There were virtually no systematic toxicological studies available on the oil-extract of alga. Available data on the dried material could not be extrapolated to the oil extract since the specifications are quite different and lipophilic materials may have been concentrated during the oil extraction process.

4. EVALUATION

The Committee considered the data inadequate to establish an ADI for the dehydrated algal carotene preparations or for the vegetable oil extracts of *Dunaliella salina*. There is no history of use of *Dunaliella* algae as food.

No relevant toxicological data on vegetable extracts were available. However, the Committee concluded that there was no objection to the use of vegetable extracts as colouring agents, provided that the level of use did not exceed the level normally present in vegetables. Implicit in this conclusion is that the extracts should not be made toxic by virtue of the concentration of toxic compounds (including toxicants naturally occurring in the vegetables) nor by the generation of reaction products or residues of a nature or in such amounts as to be toxicologically significant

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See Also:
Toxicological Abbreviations

III. CHEMISTRY, HISTORY, TAXONOMY MICROBIOLOGY AND MANUFACTURE OF *Dunaliella bardawil* Powder

A. Chemistry, History, Taxonomy and Classification

The halophilic, photosynthetic green algae, *Dunaliella bardawil,* has been known and studied for 100 years (Oren, 2005). This genus includes several ill-defined unicellular species that live in a wide range of marine habitats from seawater to hyper-saline waters (Brock, 1975; Polle, Tran, and Ben-Amotz, 2009; Zamir, 1992; Oren, 2005; and Butcher, 1959). Usually algae of the genus *Dunaliella* are consistently found in sea water and evaporation salt ponds which contain greater than 2 M salt and greater than 1 M magnesium (Ben-Amotz, 2009 and Brock, 1975). This organism is one of the 'most halotolerant eukaryotic' organism known, showing a remarkable degree of adaptation to a variety of salt concentrations, from as low as 0.1 M to salt saturation of >5 M (González, Gómez, and Polle, 2009; Borowitzka 1981, and Borowitzka, 1986). There have been many decades of research and development of aquaculture systems, principally in Australia and Israel, to commercialize *Dunaliella bardawil,* rich in carotenoids for use as an animal and human food source (Rao, 2009).

The subject powder consists of the dehydrated biomass of the alga *Dunaliella bardawil* and, therefore, is a mixture which largely consists of protein, carbohydrate, fat, minerals and carotenoids. Further description of the isolation and cultivation of the cells as well as their unique characteristics is provided below. The parameters on growth conditions for the algae are explained in the manufacturing procedure later in this Section. More details of the composition are given in the product specifications that follow.

The microalgae taxonomy and phylogeny are the following:

Kingdom:	Plantae
Phylum:	Chlorophtya
Class:	Chlorophyceae
Order:	Chlamydomonadales*
Family:	Dunaliellaceae
Genus:	*Dunaliella*
Species/strain	*D.bardawil*

* The National Center for Biotechnology Information has revised the name of the Order for this alga in its database.

V. SAFETY DATA FOR *DUNALIELLA bardawil* POWDER

A. *Dunaliella bardawil* Powder Safety Considerations

The safety of Nikken's *Dunaliella bardawil* powder (hereinafter in this section referred to under each subsection initially as *Dunaliella bardawil* powder and subsequently then as "the powder "or" the dried algal powder" has been demonstrated by a considerable number of toxicology and clinical studies. Many of these studies were of complex design with several test materials although most included at least one group of animals given a crude dried algal powder. Some of the studies were focused on the efficacy of the major component, β-carotene, and many studies used several test materials that contained varying levels of β-carotene. In addition, many of these studies were not available for review by JECFA in 1993 (WHO, 1993), and this will be discussed more fully subsequently. Those studies available to the Expert Panel that were not considered by JECFA are highlighted in the discussions to follow.

One issue that arose in the interpretation of the studies is the amount of salt present in the test sample that was carried over from the brine pits where the algae were grown. It is common practice now to wash *Dunaliella bardawil* algae before drying to remove some of the excess salt. This was not the case with early manufacturing samples with which at least one of the safety studies were conducted. It is important to note that the Nikken product contains 2-3% salt, while the test sample in the Mokady study discussed below contained 24.7% salt.

1. Acute Toxicity Studies for *Dunaliella bardawil* Powder

Two unpublished acute studies addressing the toxicity of *Dunaliella bardawil* powder given as single doses to mice or rats have been conducted. Both studies were reviewed by JECFA. No lethality was seen within 14 days after administration, and no clinical signs of toxicity were found in either study.

The powder was nontoxic and not lethal when administered by oral gavage to male and female Crj:CD-1 (ICR) Charles River mice (10/sex/group) at 2.5 g/kg, 5.0 g/kg and 10 g/kg (Aruga, 1987) [Attachment 9]. There were no test article-related changes in body weight, and no visible abnormalities were observed in mice at the post-mortem examination. The study was performed in compliance with Japanese Good Laboratory Practice Standard for Safety Studies on Drugs.

Male Sprague-Dawley rats were administered the powder by oral gavage at a dose of 5 g/kg and were observed for the subsequent 14 days (Lock, 1985 copy unavailable, summary available in WHO, 1993). There were neither mortalities nor overt signs of toxicity, and all the rats gained weight during the observation period.

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