

RBM #9505101. Yu P, Maraschin R: 14 -day oral toxicity study in rats. *Haematococcus pluvialis*, unicellular green algae. Istituto Ricerche Biomediche. pp 1-31, Sept 1995.



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**CORRIGE:**

Sampling  
time (hour) ⇒

0                    1                    2                    4                    8                    12

Animal no.  
and sex ↓

25M 26M	21M 22M	23M 24M	25M 26M	21M 22M	23M 24M
31M 32M	27M 28M	29M 30M	31M 32M	27M 28M	29M 30M

*Ivrea, May 25, 1995*

*Mr. Enrico Gillio Tos*

*RBM Study Director*

## ALLOCATION TO GROUPS AND IDENTIFICATION SYSTEM

Before commencement of the treatment all the animals were weighed: animals at the extremes of the body weight range were discarded.

The required number of rats (12M + 12F) was allocated to the dose groups by means of a computerized stratified sequenced randomization program.

Each rat was numbered and individually identified by an ear-tag.

Numbering of the animals went from 8394 to 8417.

Each single cage bore a tag on which the experiment number, dosage group, sex, progressive cage and animal numbers were indelibly marked.

The color of the tag indicated the dose group to which the cage belonged.

All experimental materials (cages, beakers, trays etc.) belonging to each group were color-tagged.

The animals and the cages were numbered and color-tagged as follows:

Group 1 (white)	0 g/kg/day (Control Article)		
Males no.s	8394 - 8399	(cages no.s	1 - 3)
Females no.s	8400 - 8405	(cages no.s	4 - 6)
Group 2 (red)	6 g/kg/day of Haematococcus pluvialis, unicellular green algae		
Males no.s	8406 - 8411	(cages no.s	7 - 9)
Females no.s	8412 - 8417	(cages no.s	10 - 12)

The animals of each group were housed in 3 cages / sex of 2 animals / cage

## TEST ARTICLE FORMULATE PREPARATION

Every day, an exact amount of test article was weighed into a mortar, ground with some drops of vehicle, transferred into a suitable graduated container and made up to final volume with vehicle in order to obtain the final concentration of 300 mg/ml

The suspension was kept magnetically stirred until the end of the daily administration.

The administration was performed within 4 hours of the preparation of the test article suspension.

## METHOD OF ADMINISTRATION

Oral (by gavage).

The volumes administered were adjusted on the basis of the most recent individual body weight recorded.

Control animals received 20 ml/kg/day of the vehicle.

## TYPE AND FREQUENCY OF OBSERVATIONS, ANALYSES AND MEASUREMENTS

### CLINICAL OBSERVATIONS

#### MORTALITY

Throughout the study, inspections for mortality were made twice a day (early in the morning and late in the afternoon).

Animals found dead before the end of the trial were subjected to autopsy and the organs listed under the "Gross Pathology Examination" paragraph were removed and examined.

#### CLINICAL SIGNS

Physical appearance, behaviour and clinical signs of the rats were observed daily.

Any deviation from normality was recorded.

#### BODY WEIGHT

Each animal was weighed prior to the beginning of the treatment period (on day 0, the day before the first administration) and then at weekly intervals throughout the study period.

At the beginning of the study, the body weight variation of the test animals did not exceed  $\pm 20\%$  of the mean weight.

## FOOD AND WATER CONSUMPTION

Food consumption was recorded, for each cage, at weekly intervals throughout the study period.

Consumption was calculated as the difference between the known offered amount per cage and the remainder recorded after 7 days. Individual food intake was then calculated, in g/animal/day, as reported in the tables, for each 7-day period.

Water consumption was not measured.

## LABORATORY INVESTIGATIONS

At the end of the 14-day treatment period (week 3; day 15), the hematological examinations, blood chemistry tests and urinalyses listed below were performed on all the animals.

In order to collect urine samples, on the day before the scheduled analysis the animals received 10 ml/kg of tap water (by gavage), as water load; subsequently they were kept in metabolism cages for about 16 hours, without food or water.

On the scheduled analysis day, blood was sampled from one of the sublingual veins, while the fasted animals were slightly anesthetized with ether.

The following parameters were determined:

## HEMATOLOGY

Parameters	Methods	Units of measure
Erythrocytes	Using the "Cellanalyzer 480" cell counter (DELCON)	$\times 10^6$ cells/mm <sup>3</sup>
Hemoglobin	Colorimetric method, using the "Cellanalyzer 480" cell counter (DELCON)	g/100 ml
Leukocytes (total WBC count)	Using the "Cellanalyzer 480" cell counter (DELCON)	$\times 10^3$ cells/mm <sup>3</sup>
WBC differential count	Staining of fresh sample with modified May-Gruenwald and Giemsa (Merck) solution. Microscopic count	%

Parameters	Methods	Units of measure
Mean Corpuscular Volume (MCV)	Using the "Cellanalyzer 480" cell counter (DELCON)	$\mu^3$
Hematocrit	Calculated with the following formula: $Hematocrit = MCV \times \text{Number of Erythrocytes}$	%
Mean Corpuscular Hemoglobin Concentration (MCHC)	Calculated with the following formula: $MCHC = \frac{Hemoglobin(g/100 ml) \times 100}{Hematocrit}$	%
Mean Corpuscular Hemoglobin (MCH)	Calculated with the following formula: $MCH = \frac{Hemoglobin(g/100 ml) \times 10}{Erythrocytes(x 10^6 / mm^3)}$	pg
Platelets	Using the "Cellanalyzer 480" cell counter (DELCON)	$\times 10^3$ cells/mm <sup>3</sup>
Prothrombin time	Reagent: Calcic thromboplastin (Boehringer Mannheim GmbH Diagnostica) at the KC10A (Mascia Brunelli) coagulometer, on citrated plasma	sec.

### BLOOD CHEMISTRY

Parameters	Methods	Units of measure
Glucose	Enzymatic method on serum GLUCOSE - UV Test (Abbott Lab. Diagn. Div.) at the SPECTRUM-ABBOTT	mg/100 ml
Urea	Enzymatic method on serum. UREA-UV Test (Abbott Lab. Diagn. Div.) at the SPECTRUM-ABBOTT	mg/100 ml
Creatinine	Kinetic-colorimetric method on serum. CREATININE Test (Abbott Lab. Diagn. Div.) at the SPECTRUM-ABBOTT	mg/100 ml
Total bilirubin	Colorimetric method on serum. A-Gent BILIRUBIN Test (Abbott Lab. Diagn. Div.) at the SPECTRUM-ABBOTT	mg/100 ml

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Parameters	Methods	Units of measure
Alkaline phosphatase	Kinetic-colorimetric method on serum at 37°C. A-Gent ALKALINE PHOSPHATASE Test (Abbott Lab. Diagn. Div.) at the SPECTRUM-ABBOTT	IU/l
Serum glutamic oxaloacetic transaminase (SGOT or AST)	Kinetic method on serum at 37°C. AST Test (Abbott Lab. Diagn. Div.) at the SPECTRUM-ABBOTT	IU/l
Serum glutamic pyruvic transaminase (SGPT or ALT)	Kinetic method on serum at 37°C. ALT Test (Abbott Lab. Diagn. Div.) at the SPECTRUM-ABBOTT	IU/l
Total Cholesterol	Enzymatic-colorimetric method on serum. CHOD-PAP - Monotest (Boehringer Mannheim GmbH-Diagnostica) at the SPECTRUM-ABBOTT	mg/100 ml
Triglycerides	Enzymatic-colorimetric method. Triglycerides GPO-PAP (Boehringer Mannheim GmbH-Diagnostica) at the SPECTRUM-ABBOTT	mg/100 ml
Total protein	Colorimetric method on serum. Test Combination (Boehringer Mannheim GmbH-Diagnostica) at the SPECTRUM-ABBOTT	g/100 ml
Serum protein electrophoresis	The Sepratek (Gelman) micro- method is used to separate the albumin from the alpha 1, alpha 2, beta and gamma globulin fractions. Strips, stained with Ponceau S, are read by the CLINISCAN II (Helena Instruments) apparatus	%
A/G ratio	Calculated automatically by the computer	
Sodium	Ion-selective electrode determination at the SPECTRUM-ABBOTT	mEq/l
Potassium	Ion-selective electrode determination at the SPECTRUM-ABBOTT	mEq/l
Calcium	Colorimetric method on serum. A-Gent CALCIUM Test (Abbott Lab. - Diagn. Div.) at the SPECTRUM-ABBOTT	mg/100 ml

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Parameters	Methods	Units of measure
Chloride	Ion-selective electrode determination at the SPECTRUM-ABBOTT	mEq/l
Inorganic phosphorus	UV method on serum. PHOSPHORUS Test (Abbott Lab - Diagn. Div.) at the SPECTRUM-ABBOTT	mg/100 ml

## URINALYSIS

**Diuresis** - Volume of urine excreted in 16 hours (ml/16h)

**Specific gravity** - Determined with the RD-10 Densitometer (Boehringer Mannheim GmbH)

**Semiquantitative determinations** - Fresh, non-centrifuged urine was analyzed for leukocytes, nitrites, pH, protein, glucose, ketone bodies, urobilinogen, bilirubin and blood (Combur 9 Test-RL-Boehringer Mannheim GmbH) at the "Urotron RL 9 System" apparatus (Clin. Int. GmbH). The read values are automatically transformed in scores by the instrument; the correspondence between score and nominal values for each parameter is detailed below:

Score	0	1	2	3	4	5	6
Leukocytes (Leuk./ $\mu$ l)	neg.	25-100	100-500	>500	/	/	/
Nitrites	neg.	pos.	/	/	/	/	/
Protein (mg/dl)	neg.	15-30	30-60	60-100	100-200	200-500	>500
Glucose (mg/dl)	norm.	50-100	100-200	200-300	>300	/	/
Ketone bodies (mg/dl)	neg.	10-50	50-150	>150	/	/	/
Urobilinogen (mg/dl)	norm.	1-4	4-8	8-16	>16	/	/
Bilirubin (mg/dl)	neg.	0.5-1.5	1.5-3	3-6	6-12	>12	/
Blood (Ery./ $\mu$ l)	neg.	10-50	50-150	150-250	>250	/	/

pH : from 5 to 9, with a differential of one unit.

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**Microscopic examination** - The sediment from each sample of urine was examined microscopically for the presence of epithelial cells, leukocytes, erythrocytes, crystals, casts, bacteria and other abnormal components.

The presence of the various constituents of the sediment was quantified as follows:

0	Absent
1	Occasional in some fields
2	Occasional in all fields
3	Many in all fields

## POST-MORTEM EXAMINATIONS

### GROSS PATHOLOGY

At the end of the treatment period the body weight of each animal that had been fasted overnight (about 16 hours) was recorded before the animal was killed by excision of the femoral arteries after having been completely anesthetized with an i.p. injection of an overdose of sodium pentobarbital at the dose of 50 mg/kg. Each animal was subjected to a detailed gross necropsy.

The following organs and tissues were removed, and those with an asterisk \* were also trimmed and weighed. Individual organ weight/fasted body weight ratios were calculated.

skin and mammary gland  
urinary bladder  
prostate  
testes\*  
epididymides  
seminal vesicles  
uterus  
ovaries\*  
spleen\*  
stomach  
intestine, duodenum, ileum, cecum, colon, rectum  
mesenteric lymph nodes  
pancreas  
liver\*

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**kidneys\*****adrenals\***submandibular salivary glands and lymph nodes  
sternum with bone marrow**heart\***

thymus

lungs

aorta

trachea

esophagus

thyroids and parathyroids

eyes

Harder's lacrimal glands

tongue

**brain\*****pituitary\***spinal cord: thoracic, cervical and lumbar  
vertebrae

gross lesions

Samples of brain, liver, fat and muscle were taken from 2 rats/sex/group (those with the lowers identification numbers), were kept frozen at -20°C and subsequently delivered to the Sponsor on November 13, 1995 for assay of content of astaxanthine (as indicated by the Sponsor).

**HISTOLOGIC EXAMINATION**

All or a part of the above organs were fixed in 10% buffered neutral formalin except the eyes that were fixed in Davidson's fluid.

As the post-mortem examination did not show target organs, no histologic examination was performed.

## DATA PROCESSING AND EVALUATION

All raw data were recorded on appropriate forms bound in numbered registers, stored and processed by a computer system.

All units of measure of the input data were selected so that the third decimal place would not be determinant. The computer rounds off figures at the second decimal place (except for the S.D.).

By RBM - Internal definition, day 0 is the day immediately preceding the start of treatment (day 1). The days of the experiment, both for pre-trial and experimental phases, are numbered according to this definition.

In order to gather together the observations carried out on different calendar dated a "nominal day", associated to each single observation, has been introduced.

This day, chosen by the Study Director, coincides with the actual treatment day (defined as the difference between the date of the event and the date relative to "day 0", already previously defined) only for body weight and food consumption measurements.

The term "week", which appears in the tables is calculated automatically on the basis of the "nominal day", according to the following formula:

week = integer part  $[(\text{nom. day} + 6)/7]$  if nom. day greater or equal to 0

week = integer part  $[(\text{nom. day} - 6)/7]$  if nom. day less than 0

The term "day", which appears in the tables and appendices, is the actual day.

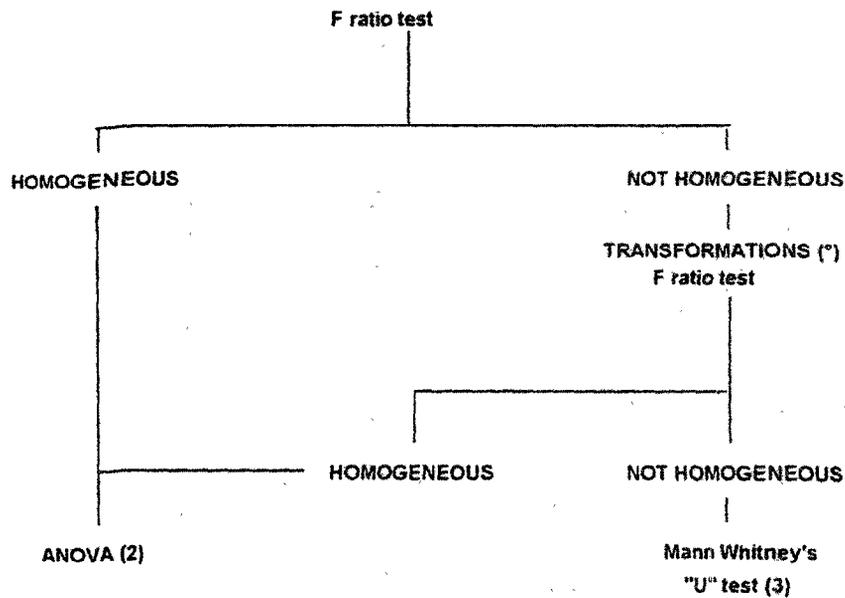
Recordings were expressed both in absolute figures and as mean and standard deviation (M.  $\pm$  S.D.).

The parameters statistically examined were:

- body weight
- body weight gain

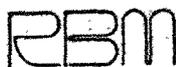
- food intake
- hematology parameters
- blood chemistry parameters
- urinalysis (except the semi-quantitative analysis and the microscopic examination of the sediment of the urine)
- organ weights (absolute and relative to body weight)

All the above data were compared according to this decision tree:



(°) = The transformations applied were inverse, logarithm decimal, square and square root.

If one transformation was successfully applied, ANOVA and Dunnett's test (when necessary) were applied on transformed data.



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In the tables, under the ANOVA heading, both the kind of analysis of variance performed (parametric and nonparametric) and the relative significance levels, indicated below, were reported.

Levels of significance were indicated by asterisks:

*	P	< 0.05
**	P	< 0.01
***	P	< 0.001

The Head of EDP Unit guarantees that whatever emerged during the statistical analysis of the data is faithfully reproduced in the tables of group data and the results of these analysis, as interpreted by the individual scientists, are included in this report.

## RECORD FILING

The protocol, a reserve sample of the batch of the test article used, the raw data bound in registers numbered 950501/0, 1, 2, 3 and 4, the final report, the organ preserved in formalin, and all other documents pertinent to the conduct of this study, including records and reports of maintenance, cleaning, calibration and inspection of equipment, are filed at RBM premises.

## PROCEDURAL DETAILS

Procedures followed during the study were those documented in the RBM Standard Operating Procedures collection.

Protection of animals used in the experiment is in accordance with Directive 86/609/EEC, enforced by the Italian D. L. No. 116 of January 27, 1992.

Physical facilities and equipment for accommodation and care of animals are in accordance with the provisions of EEC Council Directive 86/609.

The Institute is fully authorized by Competent Veterinary Health Authorities.

## REFERENCES:

- 1) **SNEDECOR G.W.**: Fifth Ed., Chapter 4 - ISCP-AMES IOWA, 1959.
- 2) **ARMITAGE P.**: Statistical Methods in Medical Research, Blackwell Scientific Publ., 1971.
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General reference sources for information on laboratory investigations in experimental animals:

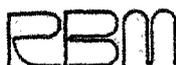
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- **BENIRSCHKE K., GARNER F.M., and JONES T.C.**: Clinical Biochemistry, p. 1750, in: Pathology of Laboratory Animals, Vol II, Benirschke K., Garner F.M., and Jones T.C. (ed.) Springer - Verlag, New York, 1978.

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## RESULTS



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## CLINICAL OBSERVATIONS (*Dr. Ping Yu*)

### MORTALITY

Two treated animals, male no. 8408 and female no. 8412 died respectively on day 11 and on day 15 of the study due to incidental causes.

For both animals the cause of death was considered erroneous gavage of the compound into the trachea, even if the female died just after the ether anesthesia for blood sampling procedures performed on the day of the final killing.

Lungs showed moderate darkness (in the male) or adhesions and increased firmness (in the female).

In addition reddish fluid (similar to the administered test compound) was found in the thoracic cavity of both animals, suggesting a rupture of the esophagus and/or trachea, during gavage procedures.

No compound-related modifications were found in either rat.

### CLINICAL SIGNS

No clinical abnormalities were observed in any animal.

### BODY WEIGHT (*TABLES 1 - 4; FIGURES 1 AND 2*)

No effects on body weight were noted in male and female rats administered *Haematococcus Pluvialis*, unicellular green algae at the dose of 6 g/kg/day for two consecutive weeks. Mean body weight data from treated animals appeared comparable to those of the controls in both sexes.

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Figure no. 1  
 RBM exp. 950501  
 Body weight Males (Rats)

Gr# 1 —————  
 Gr# 2 - - - - -

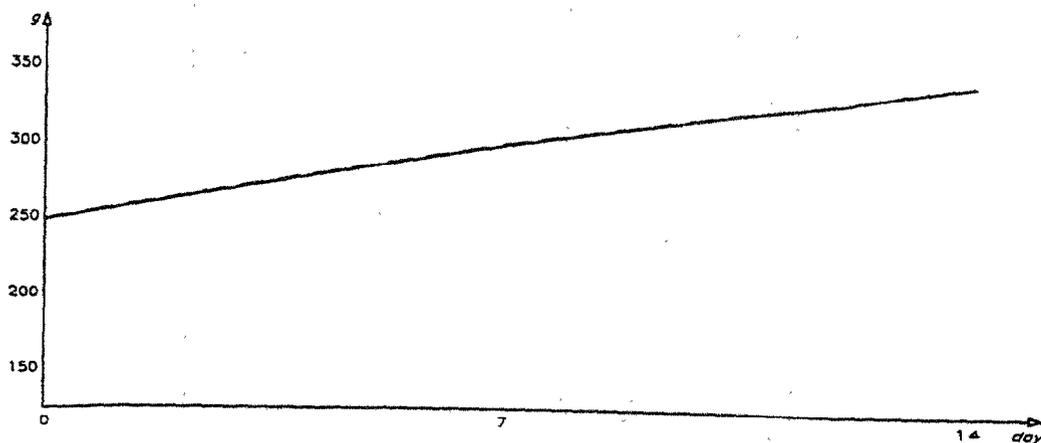
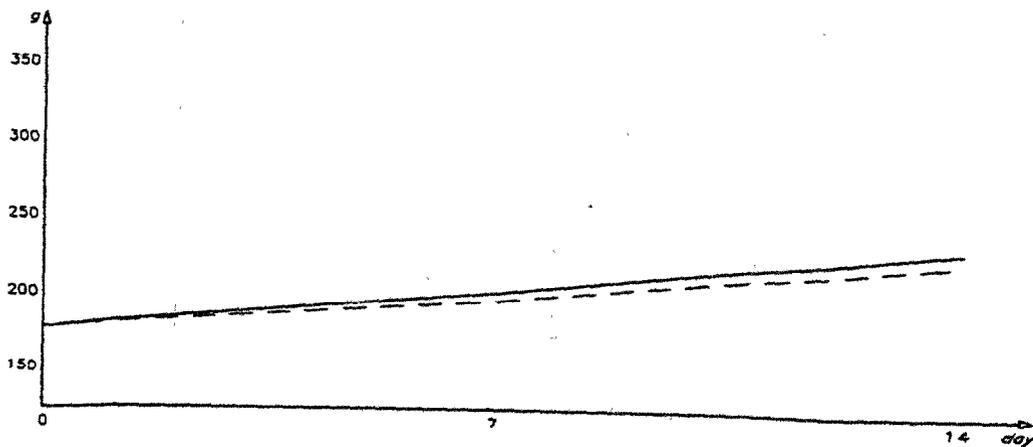


Figure no. 2  
 RBM exp. 950501  
 Body weight Females (Rats)

Gr# 1 —————  
 Gr# 2 - - - - -



**FOOD CONSUMPTION (TABLES 5 AND 6; APPENDIX 3)**

No variations in the amount of food consumed by the animals were found comparing data obtained in the treated group of both sexes with those of the respective control group.

**LABORATORY INVESTIGATIONS (Dr. Germano Oberto)****HEMATOLOGY (TABLES 7 AND 8; APPENDIX 4)**

Hematological profiles of both males and females, determined after 2 weeks of dosing did not appear adversely affected by the test article administration.

At the statistical analysis of data the only change noted was a negligible increase in mean corpuscular volume (MCV) compared to the control value, which was confined to the male group. No statistically significant variations were seen in the female group.

**BLOOD CHEMISTRY (TABLES 9 AND 10; APPENDIX 5)**

Blood chemistry tests performed at the end of the study period did not reveal treatment-related changes in either sex.

Statistical assessment of data showed a slight decrease in GOT serum activity in the treated male group, when compared to the control value; this change was considered incidental and devoid of toxicological relevance.

No variations of statistical relevance were highlighted in the female group.

### URINALYSIS (TABLES 11 AND 12; APPENDIX 6)

No changes in urine parameters were considered to have been induced by treatment in either males or females.

The slight decrease in the specific gravity of urine which was highlighted by the statistical analysis in the treated male group, compared to controls, was considered incidental, as it was related to the slightly increased volume of urine excreted and as all individual data fell within the norm.

From examination of individual data, individual animals (i.e., male no. 8396 of control group and female no. 8412 of the treated group) showed a particularly high presence in the urine of bilirubin/urobilinogen (the male) or of glucose (the female) at the semi-quantitative test.

These findings were considered incidental, as they were confined to individual rats, without evidence of other clinical or laboratory alterations.

### POST-MORTEM EXAMINATIONS (Dr. Michela Carbonatto)

#### ORGAN WEIGHT AND GROSS PATHOLOGY (TABLES 13-18; APPENDICES 7-9)

No compound-related changes were seen.

All organ weight data were comparable among control and treated groups and no modifications of note were seen at the gross pathology examination.

The only statistically significant change was a slight decrease of the absolute mean weight of adrenals in treated females. This was considered incidental as the individual values were in the range of control rats of this or other studies performed in our laboratory, with one control value (animal no. 1404, 80 mg) slightly exceeding this range.

No statistical differences were found in males between treated and control groups.



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## SUMMARY AND CONCLUSIONS

In this study the effects of repeated administration of the test article **Haematococcus pluvialis, unicellular green algae** on Sprague Dawley CrI:CD(SD)BR rats were evaluated.

**Haematococcus pluvialis, unicellular green algae** was administered by oral route (gavage) as a suspension in 20% intralipid solution once a day for 14 consecutive days at the maximum administrable dose, 6 g/kg/day (group 2). The administration volume was 20 ml/kg/day, the test substance concentration in the vehicle being 300 mg/ml.

Control animals (group 1) received 20 ml/kg/day of 20% intralipid solution (control article).

Each experimental group consisted of 6 males and 6 females.

Throughout the study routine clinical observations (clinical signs, body weight and food intake) and laboratory investigations (hematology, blood chemistry and urinalysis) were carried out. At the end of the 14-day dosing period, all animals were killed for pathology investigations.

No treatment-related deaths occurred during the course of the study. Routine clinical observations and laboratory investigations did not show any adverse changes in the test article-treated animals of either sex.

At the post-mortem examination (organ weight and gross pathology) no compound-related changes were seen.

In conclusion, **Haematococcus pluvialis, unicellular green algae**, when administered to Sprague Dawley rats by oral route for 14 days at the maximum administrable dose, 6 g/kg/day, proved to be well tolerated as no untoward changes were found in any of the treated animals.

Dr. Ping Yu  
RBM Study Director

March 25, 1996

Dr. Roberto Maraschin  
Scientific Director Recognized by  
the Italian Health Authorities as  
Responsible for General Toxicology  
Experimentation



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RBM EXP No. 950501

*Asst file 207 1.3.4.*

**Haematococcus pluvialis, unicellular green algae**

**"14-DAY ORAL TOXICITY STUDY  
IN RATS"**

RBM EXP. No. 950501

*Issued on March 25, 1996*

**SPONSOR**

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**Istituto di Ricerche Biomediche**  
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Italy



RBM EXP No 950501

EXP. No 950501

## TITLE OF THE STUDY

"14-day toxicity study in Sprague Dawley Cri:CD (SD) BR rats treated with the test article **Haematococcus pluvialis, unicellular green algae** administered by oral route at the doses of **0 and 6 g/kg/day**".

## PURPOSE OF THE STUDY

The purpose of the study was to evaluate the toxicity of the test article **Haematococcus pluvialis, unicellular green algae** administered by oral route to the rat.

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This report consists of 90 pages.

Ivrea,

Dr. Ping Yu *Yu*  
 RBM Study Director *March 25, 1996*

## FOREWORD

On behalf of **KINETICON AB Kungshangsvagen, 31 - S - 75323 UPPSALA - Sweden**, Istituto di Ricerche Biomediche "Antoine Marxer" RBM S.p.A., authorized by the Italian Health Authorities (1-2) to conduct safety studies, has performed a toxicity study by oral route of 14 days duration in Sprague Dawley Crl:CD(SD) BR rats (RBM - Experiment no. 950501), with the test article

### Haematococcus pluvialis, unicellular green algae

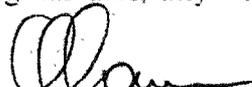
A sample of the substance used, along with pertinent documentation, is held in sufficient quantity in the RBM archives and is at the disposal of the Ministero della Sanità.

The undersigned declare that the experiment was conducted using the same batch of substance as that of the sample held on file.

For verification by the Ministero della Sanità, the undersigned moreover guarantee the identification and classification of all those materials, documents and recordings used in conducting the experiment, held on file for a period of at least 10 years from the date of this report. Following this time, they will be placed at the disposal of the Sponsor.

  
Dr. Roberto Maraschin

Scientific Director Recognized by  
the Italian Health Authorities as  
Responsible for General Toxicology  
Experimentation

  
Dr. Angelo Conz

General Manager of the Istituto  
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Marxer", RBM S.p.A.

Ivrea, March 25, 1996

- (1): **Pharmaceuticals:**  
Authorization dated March 12, 1976 in accordance with "Circolare 73", May 16, 1974
- (2): **Chemicals:**  
Authorization in accordance with DPR 927/81 (D.M. dated January 7, 1988 published in G.U. No. 12, dated January 16, 1988).

**QUALITY ASSURANCE STATEMENT**

RBM Experiment number: 950501

Study title: "14-day toxicity study in Sprague Dawley CrI:CD (SD) BR rats treated with the test article *Haematococcus pluvialis*, unicellular green algae administered by oral route at the doses of 0 and 6 g/kg/day".

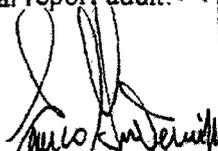
In compliance with the Principles of Good Laboratory Practice this study has been inspected by the Quality Assurance Unit.

Inspections were made by the Q.A.U. of the various phases of the study described in this report. The dates on which the inspections were made and the dates on which the findings were reported to the Study Director and to the facility management are given below:

Dates of inspection/audit	Dates of report to Study Director and Management
October 12, 1995	October 12, 1995
October 13, 18 and 19, 1995	October 23, 1995
November 2, 1995	November 3, 1995
December 15-19, 1995	December 19, 1995

This report has been audited by the Q.A.U. and was found to be an accurate description of such methods and procedures as were used during the conduct of the study and an accurate reflection of the raw data.

Date of final report audit: April 3, 1996

  
 Enrico Invernizzi  
 Head of Quality Assurance Unit

Date: April 3, 1996

RBM EXP. No. 950501

**RBM MANAGEMENT DECLARATION OF GLP COMPLIANCE**

Study No 950501 entitled :

"14-day toxicity study in Sprague Dawley Crl:CD (SD) BR rats treated with the test article *Haematococcus pluvialis*, unicellular green algae administered by oral route at the doses of 0 and 6 g/kg/day"

was performed in compliance with the OECD-GLP in the testing of chemicals, [C(81) 30 (final)], regulations also enforced by the Italian Health Authority [(D.M. dated June 26, 1986 as published in G.U. No. 198, dated August 27, 1986 and D.L. January 27, 1992, No. 120 as published in G.U. (Supplement) No. 40, February 18, 1992)].

  
Dr. Ping Yu  
RBM Study Director

  
Dr. Angelo Conz  
General Manager of the Istituto  
di Ricerche Biomediche "Antoine  
Marxer", RBM S.p.A.

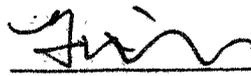
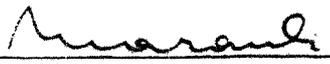
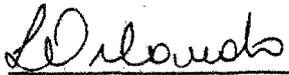
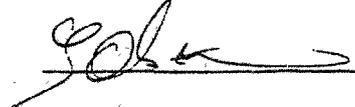
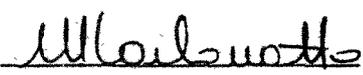
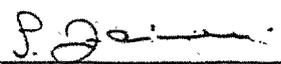
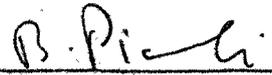
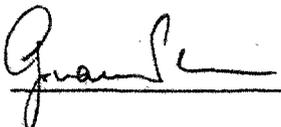
Ivrea, Aprile 4, 1996

RBM EXP No. 950501

## SCIENTISTS INVOLVED IN STUDY

STUDY No. 950501

Haematococcus pluvialis, unicellular green algae 14-day oral toxicity study in rats

RBM Study Director	Dr. Ping Yu	
Scientific Director Toxicology	Dr. Roberto Maraschin	
Head of General Toxicology Unit	Dr. Luciana Orlando	
Laboratory investigations	Dr. Germano Oberto	
Head of Pathology Unit	Dr. Sergio Peano	
Post-mortem examinations	Dr. Michela Carbonatto	
Head of Pharmacy Unit	Mr. Pietro Zaninelli	
Formulate Preparation	Dr. Bruna Piccioli	
Head of EDP Unit	Dr. Giovanni Peretti	

**RBM**

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RBM EXP No. 950501

## **MATERIALS AND METHODS**

## EXPERIMENTAL DESIGN

RBM Experiment No.: **950501**  
 Test article: **Haematococcus pluvialis, unicellular green algae**  
 Vehicle and control article: **20% intralipid solution, manufactured by Pharmacia AB**

Sponsor: **KINETICON AB**  
**Kungsangsvagen 31**  
**S-75323 UPPSALA**  
**Sweden**

### Dose levels (\*)

Group 1: **0 g/kg/day (Control article)**  
 Group 2: **6 g/kg/day of Haematococcus pluvialis, unicellular green algae**

(\*) The dose level administered in this study was selected by the Sponsor.  
 6 g/kg/day corresponds to the maximum administrable dose.

Administration route: **oral (by gavage)**

Administration volume: **20 ml/kg b.w.**

Concentration of Haematococcus pluvialis, unicellular green algae in the vehicle: **300 mg/ml at the dose of 6 g/kg/day**

Reason for selection of the administration route: **the compound is used for animal feed**

Dosing regimen: **once a day, 7 days a week.**

Total duration of dosing: **14 consecutive days**

Species, strain and substrain of the test system: **Sprague Dawley CrI:CD (SD) BR rat.**

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Justification for selection of the test system:

the Sprague Dawley rat was chosen as rodent species, since it is widely accepted by Health Authorities as an appropriate experimental model with documented susceptibility to a wide range of toxic substances

Number and sex of animals:

12M+12F (6 animals/sex/group)

Beginning of treatment:

- Males and Females: October 19, 1995

Killing of the animals

- at the end of treatment:

- Males and Females: November 2, 1995

### TEST ARTICLE CHARACTERIZATION

Identification:

**Haematococcus pluvialis, unicellular green algae**

Batch:

950918

Characteristics:

red-brown powder

Manufacturing date:

August 17, 1995

Expiry date:

February 17, 1996

Storage:

-20°C in the dark

### VEHICLE AND CONTROL ARTICLE CHARACTERIZATION

Identification:

Intralipid 20% (Trade name)

Characteristics:

sterile, pyrogen free emulsion

Composition (as reported on the label):

Soybean lipids	200 g
Phospholipids from yolk	12 g
Glycerol	22.5 g
Water for injection	to reach 1000 ml

Batch:

66727-51

Manufacturing date:

February, 1995

Expiry date:

July, 1996

Producer:

Pharmacia AB - Sweden

## ANIMAL HUSBANDRY

### SUPPLY, ACCEPTANCE OF THE ANIMALS AND SELECTION FOR THE EXPERIMENT

The 24 (12M + 12F) Sprague Dawley CrI:CD (SD) BR rats, selected for this study from a larger group (14M + 14F) than that required, were purchased from Charles River Italia S.p.A., Via Indipendenza 11 - 22050 CALCO (Como) - (received on September 29, 1995 - shipping slip no. 07276).

When received, the rats were about 4 weeks old; the males weighed about 75-85 g and the females about 60-70 g.

On arrival at RBM, all animals were clinically observed and weighed: their weight conformed to that required. During the acclimatization period of about 3 weeks the rats were housed in a quarantine room (B09A) and their health status was assessed by daily clinical observations.

Before dosing commenced, all the animals received were weighed. The body weight increase proved to be within the limits of normal variability for this strain.

### ACCOMMODATION OF THE ANIMALS

After the acclimatization period, the rats were housed in room B20A, of a limited access, barriered rodent facility.

Animal room controls were set to maintain temperature and relative humidity at  $22^{\circ}\text{C} \pm 2$  and  $55\% \pm 15$ , respectively. There were approximately 20 air changes per hour (filtered on HEPA 99.97%). The rooms were illuminated by artificial lighting with a 12-hour circadian cycle (7 a.m. - 7 p.m.).

An automatic standby power is brought into operation should the main supply fail.

For the entire duration of the study the rats were kept in wire cages measuring 40.5x38.5x18h cm, with stainless steel feeders. The waste that dropped through the wire bottom onto a removable paper was periodically disposed of.

The distribution of the cages in the animal room was designed to minimize possible environmental effects on the test animals.

The position of the cages in the racks was alternated by groups horizontally and vertically.

The diagram of the cage location in the animal room is kept in the study file.

## DIET AND WATER SUPPLY

The rats were fed a diet coded "4 RF 21 GLP Top Certificate" produced by the Charles River Italia's feed licensee Mucedola S.r.l., Settimo Milanese.

On the label, the contents declared by the producer, were:

Moisture	12.00 %
Crude protein	18.50 %
Crude fat	3.00 %
Crude fiber	6.00 %
Ash	7.00 %

The Producer supplemented the diet with vitamins and trace elements.

According to the analytical certificates provided by the Supplier, the contents of the batches of diet used in this study were within  $\pm 5\%$  of the declared values and the presence and the levels of contaminants were within the limits proposed by EPA-TSC A (44FR : 44053-44093, July 26, 1979).

Animal feed, in compliance with RBM SOPs, is analyzed twice a year for bacterial contamination.

The diet was available "ad libitum" to the animals.

Filtered water was distributed by means of an automatic watering valve system

The drinking water offered to the animals came from the municipal water main.

The water is periodically analyzed for microbiological count, for the presence of heavy metals, other contaminants (e.g. solvents, pesticides) and other physical and chemical properties

The acceptance limits for the quality of drinking water are those defined in EEC Directive 80/778.

Contaminants that might interfere with the objectives of the study are not expected to be present either in the diet or in the water.

The analytical certificates of the animals' feed and water are filed at RBM premises.