RHODOSPIRILLUM RUBRUM FOOD ACCEPTABILITY

STUDY FOR MELISSA

Technical note on Work package 30.2

Confidential

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INTRODUCTION

MELISSA, a model of a future biological life support system for manned missions to space, contains four compartments: a liquefying compartment, a phototrophic compartment, a nitrifying compartment, and a photosynthetic compartment. A previous food acceptability study was conducted to determine the suitability of *Spirulina*, growing in the photosynthetic compartment, as a component of the consumer diet in the MELISSA ecosystem model (Tranquille and Emeis, 1991 [1]; Tranquille et al, 1994 [2]). This report centres on *Rhodospirillum rubrum (R.rubrum)*, growing in the second (phototrophic) compartment of MELISSA.

Aim of the Study

The aim of the present food acceptability study was to test, in rats, the suitability of *R.rubrum* as a component (at 10 % w/w) of the consumer diet in the MELISSA model over a medium-term period.

Study Design

The study involved two groups of eight rats each, and extended over a period of eight weeks. The groups were fed either a normal semi-synthetic rat diet (control group), or were fed this normal rat diet with 10% (w/w) freeze-dried *R.rubrum* added (experimental group). A series of physiological, biochemical and metabolic variables were measured during and at the end of the study to assess the effect of *R.rubrum* on the general health of the rats.

As *R.rubrum* contains a high content of polyhydroxybutyric acid (PHB), an initial acceptability test had been conducted with a small number of rats to see if they would accept and endure PHB in their diet. These data were presented in Technical Note 30.1 [3]. The conclusion in that note was that adding 1% (w/w) PHB to a normal rat diet is not harmful in the short term (6 weeks).

MATERIALS

Rats

Sixteen young male Wistar rats, weighing approximately 120 grams, were obtained from Iffa-Credo (Someren, the Netherlands). The animals were housed individually under normal animal house conditions - 12 hours of light and 12 hours of darkness. The temperature was set at 21-22°C, and humidity was 60-70 %. The animals were assessed 2-3 times a week: they were weighed, their food and water intake were determined, and they were given fresh food and water *ad libitum*.

Dietary materials

The *R.rubrum (ATCC 25903)* was obtained as a freeze-dried powder through ESA/ESTEC. It had been produced by CBB Developpement, Rennes, France, and Intertechner, Cherbourg,

France.. The preparation of the batch of *R.rubrum* used in the present study is described in detail in Technical Note 29.2 [4]. The composition of the freeze-dried material was [4]:

Protein	49.2 ± 5.7 % of dry weight
Polyhydroxybutyric acid	16.6 ± 3.9 %
Glycogen	10.5 ± 0.2 %
DNA	0.47 ± 0.06 %
RNA	3.5 ± 0.5 %

The normal control rat diet was a semi-synthetic rat chow flour containing all essential nutrients, as defined by "Nutrient Requirements of Laboratory Animals" [5].
It was formulated and supplied by Hope Farms BV (Woerden, the Netherlands), and contained (gram per kg): standard vitamin mix 2.5; standard trace element mix 2.5; $CaHPO_4.2H_2O$ 13; $CaCO_3$ 10; KH_2PO_4 7; KCl 7; NaCl 3; $MgSO_4.7H_2O$ 4; MgO 2; methionine 2; choline chloride (50%) 4; corn starch 100; cellulose 50; sunflower oil 50, casein 200, glucose.H_2O 543.

Method of diet formulation
Both the control diet and the *R.rubrum* diet were formulated as follows: water (600 ml) and agar-agar (12 g) were heated until the agar dissolved, and then cooled to room temperature while stirring.
For the control diet, 600 ml of the 2% agar-agar was carefully added to 1400 g of the control diet flour.
For the test diet, 600 ml of the 2% agar-agar was added to 1260 g of the control diet flour and 140 gram (10%) freeze-dried *R.rubrum*.
The flour and agar mixtures were kneaded into a firm dough, which was shaped into portions of approximately 400 g, and kept frozen at -20°C until used.

METHODS

Experimental
The rats were randomly divided into two groups of eight rats, and fed either the control diet or the experimental diet for eight weeks *ad libitum*. Tap water was supplied *ad libitum*. Food and water intake, and body weight were assessed 2-3 times per week. After eight weeks, blood, urine and tissues were collected. From blood, platelet-poor citrated plasma (one part of 3.8 % trisodiumcitrate to nine parts of blood) and serum were prepared by centrifugation for 10 min at 2000 x g. Plasma, serum and urine were stored at -70°C until assay.
Tissue weights were determined on a Mettler analytical balance. Haematocrit was determined on a Hawksley microhaematocrit centrifuge.

Analysis of plasma, serum and urine variables
A series of clinical-chemical tests was carried out to check the animals' health on the two different diets. The variables measured were:

Parameter	*Assayed on*	*Procedure*
Haematocrit	blood	Ht centrifuge
Haemoglobulin	blood	spectrophotometry
Total cholesterol	serum	enzymatically
Total triglycerides	serum	enzymatically
Glucose	plasma, urine	Reflotron
Urea	plasma	Reflotron
Uric acid	plasma, urine	Reflotron
Allantoin	urine	spectrophotometry
Urinary protein	urine	spectrophotometry
Creatinine	plasma	Reflotron
tPA antigen	plasma	ELISA
von Willebrand factor	plasma	ELISA
GOT	plasma	Reflotron
GPT	plasma	Reflotron

Haemoglobin was measured spectrophotometrically using Drabkin's [6] procedure. Total cholesterol and total triglycerides were measured enzymatically, using test kits from Boehringer-Mannheim (Mannheim, Germany), according to the manufacturer's instructions. Allantoin was determined spectrophotometrically by the procedure of Borchers [7]. Protein was measured using Lowry's method [8]. tPA antigen and von Willebrand factor antigen were measured by enzyme-linked mmunosorbent assays (ELISA) [9,10]. Glucose, urea, uric acid, GOT and GPT were assayed using Reflotron (Boehringer-Mannheim) dry-chemistry, according to the manufacturer's procedures.

Data presentation and statistics
Data will be presented as mean $\pm$ standard deviation (n = 8), or as median (95 % confidence interval).
To determine whether a difference between the experimental group and the control group was significant, the parametric Student's t-test or the non-parametric Mann-Whitney U-test was used. Differences were considered significant if P (two-sided) was < 0.05.

RESULTS

Growth; food and water consumption
Figs 1 and 2 show graphically the growth of individual rats on the control diet (Fig 1) and on the test diet (Fig 2) during the eight weeks of the experiment. Fig 3 shows the averaged data for the two groups.
The growth curves all followed a similar pattern. The rats on the *R.rubrum* test diet grew

slower during the first week on the diet, compared to the control rats. Table 1 shows the body weight changes of the rats in the first eight days, and for the remainder of the experimental period. The growth rates of the two groups of rats were no longer significantly different after day 8 (Table 1).
The rats on the *R.rubrum* test diet consumed less food (on average 31 gram/rat.day) than the rats on control diet (on average 37 gram/rat.day), especially during the first week of the experiment (*R.rubrum* diet: 23 gram/rat.day; control diet 33 gram/rat.day). In this respect it should be noted that it is quite common that rats need a few day to adjust to a new diet.
The *R.rubrum* test rats drank more water than the control rats. After taking into account the water content of the food, the water consumption of *R.rubrum*-consuming rats averaged 43 ml/rat.day, and of control rats 40.5 ml/rat.day. The data are summarized in Table 2.

General condition of the rats
During the experimental study the general health of the rats was assessed regularly, and all sixteen rats taking part in the experiment appeared well and healthy. At the conclusion of the experiment, all animals had healthy teeth, skin and bowels. The major organs were macroscopically inspected and found to be normal. Table 3 shows the average weights of five tissues of the rats on the control diet and the *R.rubrum* test diet. All weights fell within the normal range. However, a significant difference between the control and *R.rubrum* groups was observed in heart weight, the hearts of rats on the *R.rubrum* test diet being significantly larger than those of rats on the control diet (Table 3)

Blood and urine variables
A series of biochemical and metabolic parameters were determined in blood and urine collected at the end of the 8 week experimental period (Tables 4 and 5).
Haematocrit and haemoglobin levels were normal in both groups of rats.
Kidney function in the test rats remained normal, as evidenced by normal the urinary protein content, and normal creatinine, urea, and uric acid levels. Glucose metabolism was also found to be normal in both the control and test rats. So was liver function, as determined by measurements of the liver enzymes GOT and GPT.
Endothelial cell function was not impaired, despite a small, borderline significant, decrease in plasma von Willebrand factor levels. Plasma tPA levels were, however, not influenced (Table 4).
The presence of relatively large amounts of DNA in the test diet animals was not refelected in a significant increase in the urinary levels of allantoin, the end product of purine metabolism in rats. The urinary allantoin concentration was slightly, but not significantly, increased in the group consuming the *R.rubrum*-diet (Table 5).
A highly significant difference between the control group and the *R.rubrum* test group was found for total serum cholesterol and triglycerides concentrations (Table 4). Subsequent analysis by Fast Protein Liquid Chromotography showed that the difference in cholesterol and triglyceride values was due to a severe decrease of very low-density lipoproteins (VLDL; data courtesy Dr. J.M.G. Princen, Division of Cardiovascular and Connective Tissue Research TNO-PG). This indicates that the presence of *R.rubrum* in the diet had a very

significant effect on the production and/or clearance of VLDL in the rat.
No change in high-density lipoprotein level was seen (please note that rat serum contains virtually no low-density lipoproteins).

GENERAL CONCLUSIONS

From the observations presented in this report several conclusions can be drawn. The main purpose of Work Package 30.2 was to investigate whether the rats could tolerate 10% *R.rubrum* in their normal diet, and whether there was any indication of a toxic effect.
After an initial period of slow growth, most likely due to adaptation to the dietary shift, the rats on the *R.rubrum* test diet grew normally for the remainder of the experimental period. No differences as the result of the two dietary regimens were seen in lung, liver, kidneys and adrenal weight or general function. No effects on general health were noted. No evidence of generalized toxic effect was noted.
However, significant changes were seen in the *R.rubrum* group, compared to the control group, in the following:

1. an increase in heart weight;
2. a (borderline significant) decrease in plasma level of von Willebrand factor
3. a very significant decrease of serum total cholesterol and triglyceride levels.

This latter difference may indicate a profound effect of *R.rubrum* on VLDL metabolism, while high-density lipoprotein metabolism remained unaffected. The increase in heart size might be due to a rise in peripheral vascular resistance, but there is no independent evidence to support this conclusion. The decrease in the von Willebrand factor levels of the *R.rubrum* test rats, though not statistically significant, might also point towards changes in the vascular system of the test rats. The above results, observed after *R.rubrum* had been incorporated into a normal rat diet to a concentration of 10% for only eight weeks, might indicate that the dietary addition of *R.rubrum* might in the long run affect the general condition of rats.

REFERENCES

[1] Tranquille N, Emeis JJ. Food acceptability studies for Melissa. Final report on ESA contract #113088; 1992

[2] Tranquille N, Emeis JJ, de Chambure D, Binot R, Tamponnet C. Spirulina acceptability trials in rats. A study for the "Melissa" life-support system. Adv Space Res 1994; 14:167

[3] Tranquille N, Emeis JJ. Rhodospirillum rubrum food acceptability study for MELISSA. Technical note 30.1; 1996

[4] Grizeau D, Dupre C, Guary J-C. Production of freeze-dried biomass of Rhodospirillum rubrum: furniture and analytical results. Technical note 29.2; 1966

[5] Nutrient Requirements of Laboratory Animals. National Academy of Sciences publication #10, 3rd revised edition. Washington DC, 1978

[6] Drabkin DL, Austin JH. Spectrophotometric studies. V. A technique for the analysis of undiluted blood and concentrated haemoglobin solutions. J Biol Chem 1935/1936; 112:105

[7] Borchers R. Allantoin determination. Anal Biochem 1977; 79:612

[8] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193:265

[9] Tranquille N, Emeis JJ. The simultaneous acute release of tissue-type plasminogen activator and von Willebrand factor in the perfused rat hindleg region. Thromb Haemostas 1990; 63:454

[10] Emeis JJ, Hoekzema R, de Vos AF. Inhibiting interleukin-1 and tumour necrosis factor-α does not reduce the induction of plasminogen activator inhibitor type-1 by endotoxin in rats in vivo. Blood 1995; 85:115

TABLE 1
BODY WEIGHT CHANGES (GRAMS)

Day #	Control group		R.rubrum group		Student t	p value
	body weight	weight change	body weight	weight change		
0	126 ± 7*		120 ± 5		2.024	0.062
8	192 ± 9		156 ± 10		7.891	< **0.0001**
Δ 0 → 8		67 ± 8		36 ± 11	6.405	< **0.0001**
32	311 ± 25		264 ± 11		4.852	**0.0003**
Δ 8 → 32		117 ± 18		108 ± 11	1.249	0.232
55	380 ± 33		342 ± 27		3.179	**0.007**
Δ 32 → 55		73 ± 17		78 ± 19	0.552	0.590

* mean ± SD

TABLE 2
FOOD AND WATER INTAKE

	Control group	R.rubrum group	Student t	p value
Water intake (ml/rat.day)	25 ± 6*	29 ± 5	2.044	**0.048**
Food intake (gram/rat.	37 ± 7	31 ± 7	2.371	**0.023**
Water content (%)	42	45		
Total water intake (ml/rat.day)	40.5	43		
Dry food intake (gram/rat.day)	21.5	17		

* Mean ± SD

TABLE 3
RELATIVE TISSUE WEIGHTS (mg / gram body weight)

Tissue	Control group	R.rubrum group	Student t	p value
heart	2.97 ± 0.14*	3.21 ± 0.17	3.095	**0.008**
lungs	3.65 ± 0.30	3.62 ± 0.38	0.159	0.876
kidneys	6.78 ± 0.42	7.11 ± 0.44	1.519	0.151
liver	38.1 ± 1.51	37.2 ± 2.92	0.782	0.447
adrenals	0.13 ± 0.01	0.13 ± 0.02	0.532	0.603

* Mean ± SD
** Taken from:

TABLE 4
CLINICAL CHEMISTRY OF SERUM / PLASMA

Parameter	units	Control group	R.rubrum group	Student t	p value
glucose	mmol/liter	9.2 ± 0.6*	9.6 ± 0.8	1.255	0.230
total cholesterol	mmol/liter	1.6 ± 0.1	1.2 ± 0.1	5.834	**< 0.0001**
total triglycerides	mmol/liter	1.4 ± 0.6	0.5 ± 0.1	4.475	**0.0005**
urea	mmol/liter	6.2 ± 0.6	6.6 ± 0.4	1.387	0.187
uric acid	μmol/ liter	< 120	< 120		n.s.
creatinine	μmol/ liter	< 44	< 44		n.s.
GOT	units/liter	63 ± 5	69 ± 10	1.364	0.194
GPT	units/liter	29 ± 2	27 ± 3	1.747	0.102
tPA antigen	ng/ml	2.6 ± 0.8	3.2 ± 0.7	1.301	0.216
von Wille-brand factor	units/ml	80 ± 13	64 ± 17	2.109	0.055

* Mean ± SD

TABLE 5
CLINICAL CHEMISTRY OF BLOOD / URINE

		Control group	R.rubrum group	Student t or Mann-Whitney U	p value
Blood					
hematocrit	%	42 ± 2*	42 ± 2	t = 0.867	0.401
haemoglobulin	%	100 ± 5	98 ± 4	t = 0.971	0.348
Urine					
glucose	mmol / L	< 0.5	< 0.5		n.s.
protein	mg / ml	0.98 ± 0.24	1.11 ± 0.11	t = 1.352	0.198
uric acid	mmol / L	1.25 (0.94-1.74)**	1.20 (0.94-1.94)	U = 30.5	0.878
allantoin	% control	77 (63-137)	114 (86-159)	U = 16.5	0.318

* Mean ± SD
** Median (95% confidence interval)

FIGURE 1

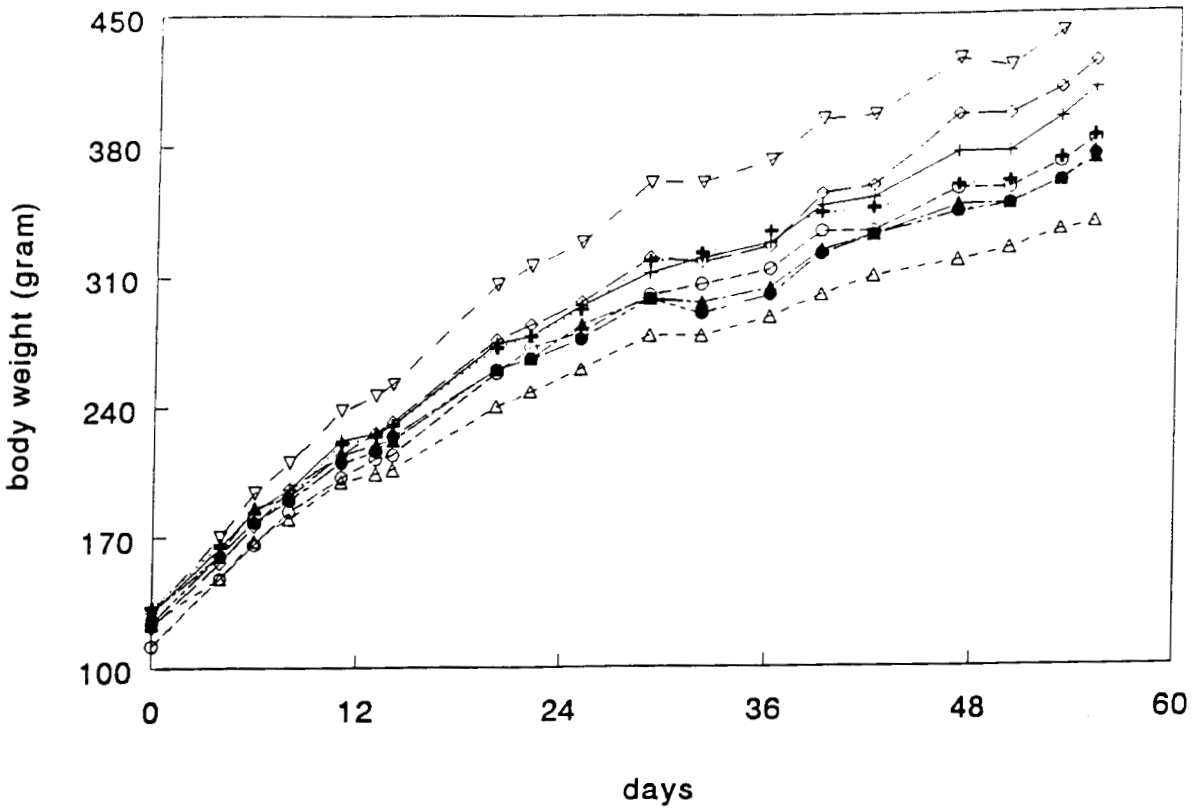

Figure 1.
Body weight changes of the eight individual rats fed the control diet over the 56-day experimental period

FIGURE 2

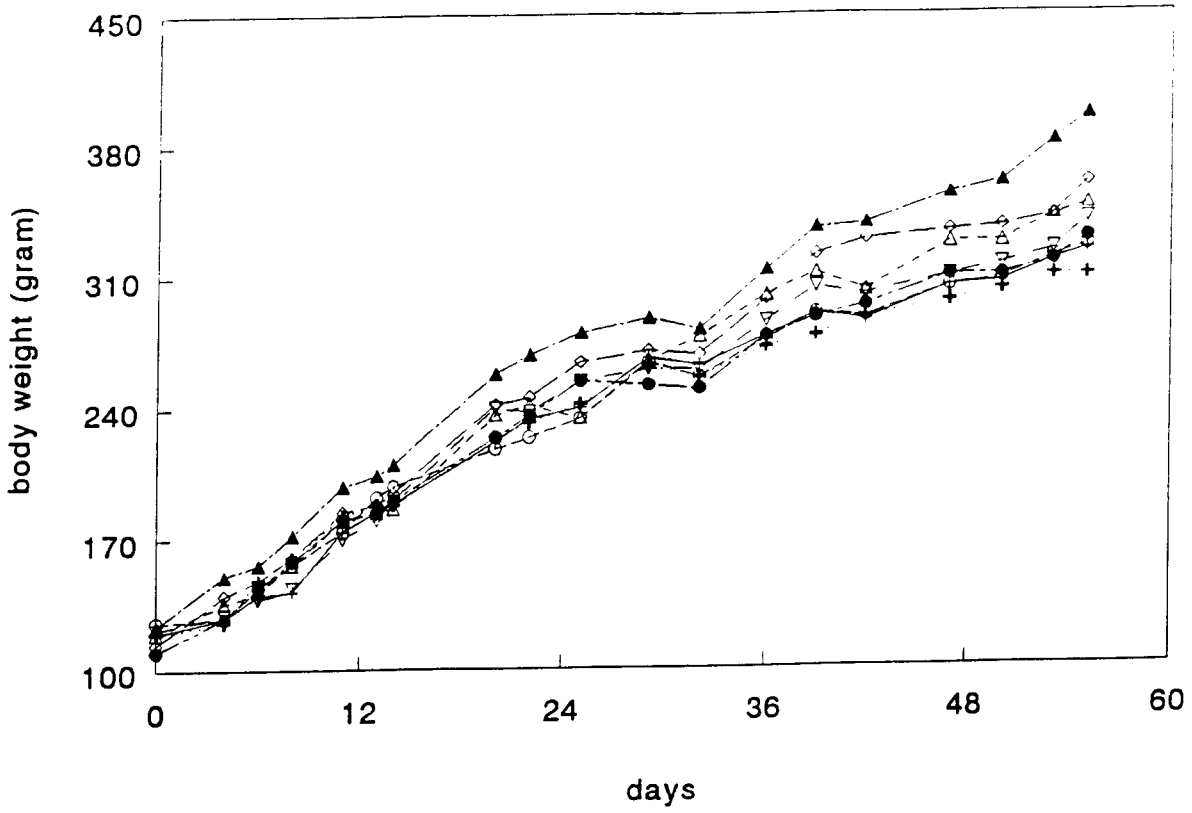

Figure 2.
Body weight changes of the eight individual rats fed the *R.rubrum*-containing diet over the 56-day experimental period

FIGURE 3

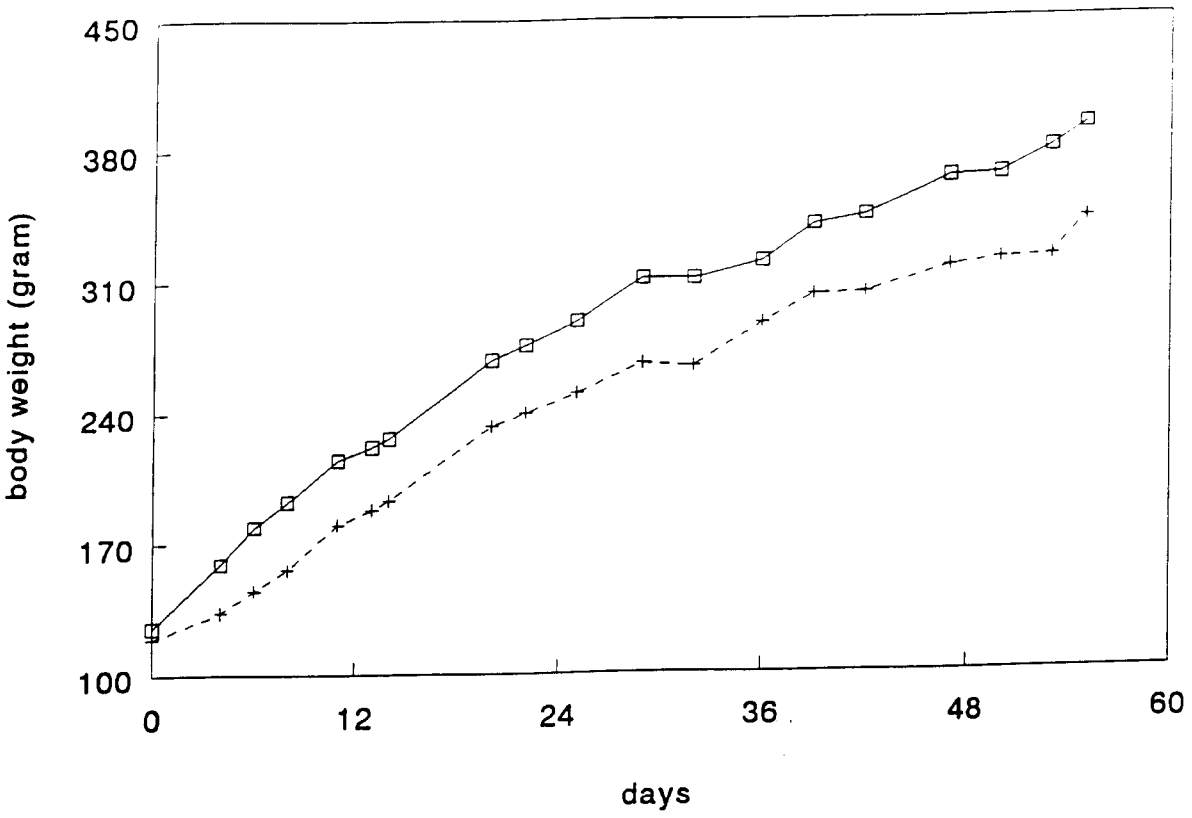

Figure 3.
Mean changes in body weight of the control group (□) and the *R.rubrum* group (+) over the 56-day experimental period