”BABEŞ-BOLYAI” UNIVERSITY
Faculty of Biology and Geology

STUDY OF PHYSIOLOGICAL EFFECTS OF ARTHROSPIRA (SPIRULINA) BIOMASS ON ANIMAL EXPERIMENTAL MODELS

Ph. D Thesis Summary

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CONTENTS

KEY-WORDS: *Arthrospira (Spirulina) platensis*, *Mus musculus* mice, *Wistar* rats, hepatoprotective, antioxidant, hematopoiesis, immunostimulating, thyroid, alcoholic liver

LIST OF ABBREVIATIONS

ALAT – alanine aminotransferase

ALD – alcoholic liver disease

ASAT – aspartate aminotransferase

CCl_4 – carbon tetrachloride

DIT – diiodine thyrosine

Epo – erythropoietin

GER – granular endoplasmic reticulum

HDL – high density lipoproteins

LDL – low density lipoproteins

MEV - mean erythrocyte volume

MIT – monoiodine thyrosine

NADH – reduced nicotinamide adenine dinucleotide

PHA – phytohemagglutinine

RBC – red blood cell

ROS – reactive oxygen species

SER – smooth endoplasmic reticulum

SOD – superoxide dismutase

TAG – triacyl glicerol

VLDL – very low density lipoprotein

1. Introduction

Cyanobacteria of the genus *Spirulina* have long been utilised as food additives in human and animal nutrition, due to their high content of proteins, vitamins, essential amino acids, minerals, essential fatty acids (such as γ-linolenic acid, a precursor of eicosanoids) and as antioxidant agents of the carotenoid type (Belay *et al*., 1996).

Starting from the fact that the biological activity of cyanobacteria depends in a great measure on the environmental conditions, the purpose of this thesis was to investigate, from the toxicological viewpoint, a local, wild strain of *Arthrospira (Spirulina),* but especially to evaluate the potential beneficial effects, at the physiologic level, on mammalian organism (mouse, rat).

For this purpose, several blood (hematologic, biochemical and enzymatic) and hepatic parameters were determined, under various conditions. In these experiments, in which we monitored/evidenced several actions/effects of *Arthrospira (Spirulina)* (hepatoprotective, hematopoietic, thyroid function stimulating, immunostimulating), we used not only the classical clinical methods but also electron microscopy and spectrophotometric measurements of certain bioenergetic parameters (membrane potential, calcium fluxes and mitochondrial swelling).

2. Biological actions of *Arthrospira (Spirulina) platensis*

One of the major preoccupations of the modern biomedical sciences is to find and promote new prime materials necessary for producing better, more effective, medical preparations. În the last decades, a special attention was given to cyanobacteria. Their study receives a greater and greater importance in obtaining and selecting new materials with nutritional and/or technical roles (Hills, 1985, Belay *et al*., 1993, cit. by Rudic *et al*., 1995). In this respect, the cyanobacterium *Arthrospira (Spirulina) platensis* is in the center of the attention, due to the exceptional value of its biochemical compounds which overpasses that of the all plant species studied (Hendrickson, 1989).

Toxicological and nutritional evaluations confirm that *Arthrospira (Spirulina)* biomass can be used as a nutritional supplement. The positive physiological effects of cyanobacteria, in general, is attributted to their high content of vitamins, minerals and essential fatty acids, improving the **immune response**, **fertility, body weight** and **external aspect** (Certik and Shimizu, 1999).

The antioxidant properties of *Arthrospira (Spirulina)* and its extracts have attracted the attention of the specialists. In one of their studies, Manoj *et al*. (1992) show that the alcoholic extract of *Arthrospira (Spirulina)* inhibits lipid peroxidation at a greater extent (65%) than the chemical antioxidants, such as α-tocopherol (35%) and β-caroten (45%). The aqueous extract of *Spirulina* also had a greater antioxidant effect (65%) than galic acid (54%) and than chlorogenic acid. An interesting aspect of their conclusions was that the aqueous extract had a significant antioxidant effect even after it was ridden of polyphenols.

Antioxidant and **anti-inflammatory effects** of *Arthrospira (Spirulina)* were also studied by Dartsch (2008), who used 4 basic preparations of *Spirulina platensis:* (1) BioSpirulina, (2) SpiruComplex, a preparation with Selenium (0.006%), Chromium (0,003%) and Zinc (0.115%), (3) SpiruZink, a preparation with natural Zinc (0.25%), (4) Zinkspirulina + Acerola (sour cherry of Barbados or tropical sour cherry) a preparation with Zinc (0.125%) and Acerola powder (it contains 5.5% ascorbic acid). All *Spirulina* preparations were able to inactivate the oxygen free radicals generated by Potassium superoxide. The most powerful inactivator was ZinkSpirulina + Acerola, with a significant inhibition at 100 μg/ml.

Several experiments demonstrate the **hepatoprotective effect** of *Arthrospira (Spirulina)*. Vadiraja *et al*. (1998) studied the effect of phycocyanin *c* from *Spirulina* în the presence of carbon tetrachloride (CCl_4), which induces hepatotoxicity in rats. A single dose of 200 mg/kg body weight was administered intraperitoneally to rats, at one and three hours after the administration of CCl_4 (0.6 ml/kg). In both cases, phycocyanin reduced significantly the hepatotoxicity induced by this substance, known as a free-radical generator.

Laboratory studies performed on mice, hamsters, chickens, cats and fish showed that *Arthrospira (Spirulina)* **stimulates immunity** and the **synthesis of blood cells** (Kozlenko and Henson, 1997). The investigators showed that these cyanobacteria stimulate the multiplication and activation of the macrophages, preparing them for the distruction of microorganisms. *Spirulina* accelerates antibody production, ensuring the best protection against the invading microorganisms. The increase in the level of IgA in saliva is correlated to the dietary supplementation with *Arthrospira (Spirulina).* This cyanobacterium has a special role in re-establishing cell and key organ functions (liver, thymus, spleen, lymph ganglia, tonsils, bone marrow), improving their capacity to function properly despite the aggresions exerted by the environmental toxins and infectious agents. Rasool and Sabina (2009) investigated the **immunomodulating effect** of *Arthrospira (Spirulina) fusiformis* in mice. It is known that the T lymphocytes respond to the stimulation of mitogens (unspecific polyclonal activators), such as phytohemagglutinin (PHA) and concanavalin A, by entering cell division. Consequently, the mitogen acts first by binding to the cell surface receptors, which, in their turn, initiate a cascade of biochemical reactions that lead to cell proliferation. In the above mentioned study, the authors noticed that *Spirulina* inhibited cell proliferation stimulated by PHA, meaning that *Spirulina* can inhibit the cell-mediated immune response.

Many of the recent studies have shown the capacity of *Arthrospira (Spirulina)* to **inhibit viral replication**. În 1996, the laboratory of viral pathology of the the Dana-Farber Oncological Institute (attached to the Medical Faculty of Harvard University), following a study on the aqueous extract of *Spirulina*, have established that the extract inhibits HIV-1 replication in T lymphocytes of human origin, peripherral mononuclears and Langerhans cells.

One of the first studies on **seric cholesterol lowering** by *Arthrospira (Spirulina)* was made on rats, by Devi and Venkataraman, 1983 (cit. by Belay, 2002). Since then, more studies have confirmed these results on several animals and man. Bertolin *et al*. (2009) performed a study on the lipid profile in rats with hypercholesterolemia, induced for 60 days. The control group did not show significant differences of the total cholesterol, LDL, VLDL and TAG, while the level of HDL decreased significantly during the treatament. The group given a hipercholesterolemic diet and supplemented with *Spirulina* also presented hipercholesterolemia, a fact suggesting that the association of this cyanobacterium with the hipercholesterolemia does not change the level of lipids.

However, the administration of *Spirulina* biomass in a therapeutic modality (cyanobacteria were administered 30 days after the induction of hipercholesterolemia) led to a significant reduction of the level of total cholesterol, in comparison with the initial values induced by the hyperlipidemic diet.

The results of the above-mentioned studies create the premises of a potential utilisation of *Spirulina,* along with other more conventional approaches, in a strategy of food supplementation focused on the prevention and reduction of major health problems such as **cancer, cardiovascular diseases** (Riss *et al*., 2007), **degenerative diseases of the central nervous system,** such as **Alzheimer** and **Parkinson** (Stromberg *et al*., 2005), **intoxications with heavy metals** (Sharma *et al*., 2002), **inflammatory processes** that involve the participation of NADPH oxidase (Riss *et al*., 2007), in general associated with alterations induced by free radicals.

3. The purpose of our experimental research

Through the experimental research pesented in this thesis we intended to study several effects produced by the administration of a locally isolated strain of *Arthrospia (Spirulina) platensis* to mice and rats, under normal and/or induced pathological conditions The problems proposed for resolution refer to: the antioxidant hepatoprotective effects of *Spirulina* on experimental models with ethanol-intoxicated rats as compared to physiologically normal ones; hematopoietic effects on experimental models with anemiated and physiologically normal animals; the role of *Spirulina* powder in stimulating the thyroid function in mice and rats; the immunostimulatory action of the cyanobacterium (*Spirulina*) on experimental models with immunosuppressed animals.

Although the antioxidant properties of *Spirulina* are known, its profilactic and curative values in the pathology of alcoholic liver have not been taken into consideration. Our *in vivo* experimental model involves the intoxication with ethanol and the parallel administration of *Spirulina*, as food supplement.

The use of electron microscopy in surprising the finest changes at the tissue/cell levels was complemented with studies on mitochondrial preparations intended to quantify certain bioenergetic (biochemical/biophysical) parameters of utmost importance in the survival of the cells (mitochondrial membrane potential, calcium fluxes and matrix swelling).

Following the investigations performed in the present study, we consider that there is enough experimental evidence/proof for potential hepatoprotective, hematopoietic, immunostimulating, hypoglycemic and hypocholesterolemic effects of *Spirulina* powder employed by us.

4. Material and methods

4.1. Biological material

***Spirulina* powder**

Arthrospira (Spirulina) is normally grown under controlled conditions of temperature, salinity, oxygen concentration and does not develop spontaneously in temperate climates. Nevertheless, there exist natural environments in our country where these cyanobacteria grow spontaneously, producing large quantities of biomass. The thermo-mineral waters from western Romania offer the appropriate temperature conditions, along with the carbon source and minerals necessary for a large scale cultivation of *Spirulina*, at a cost which is 80% lower than the one used in the classical system (Dragoş, 2000).

In our experiments, we used a local, wild strain of *Arthrospira (Spirulina).* Washed, dried and milled cyanobacteria, as powder, was obtained from S.C. Eden-Vet S.R.L. Oradea (Romania), being produced in collaboration with the Biological Research Institute of Cluj-Napoca. *Spirulina* concentration and its way of administration are presented within the experimental protocols.

Experimental animals

The experiments were performed on female, adult Wistar rats weighing initially 150±20 g and on mice (*Mus musculus* var. *albicans)*, with a mean weight of 20 g each. The animals were maintained during the entire experiment in our zoobase (Department of Molecular Biology and Biotechnology, Faculty of Biology and Geology). The zoo-hygienic conditions were appropriate for the species and age of the animals: constant temperature of 18^{o}C, constant humidity, illumination regimen of 12 hours/day, free access to food and water. The food consisted of a complex mixture (Larsen diet, with or without *Spirulina* powder). Animals were gently handled, to avoid producing unnecessary stress or pain and sacrification was done under anesthesia.

4.2. Testing methods and techniques

Methods and techniques used in measurements of hematologic and hepatic tissue parameters. All the parameters measured were schematically presented in Table 4.1. **They are grouped into** 3 categories: morphological – hematologic (morpho-hematologic), biochemical – hematologic (biochemo-hematologic) and hepatic tissue (hepatologic) parameters.

Monitoring the **morpho-hematologic parameters from the periferral blood** of the animals was necessary for establishing whether the cyanobacteria tested possess hematopoietic and/or immunostimulating properties. The number of red blood cells (RBC count), hemoglobin concentration and hematocrit give indications about the way in which the iron metabolism is affected (inhibited or stimulated), considering the fact that *Spirulina* contains appreciable amounts of iron. The experiments also included the investigation of certain **biochemical parameters of blood and hepatic tissue**. To establish the hypoglycemic and hypocholesterolemic effects, glycemia and cholesterolemia were monitored.

Carbohydrate metabolism was assessed by quantitative measurements of glycogen and tissue glucose, as well as enzyme activities of ASAT, ALAT and LDH in the serum, since their presence here gives information about membrane permeabilisation.

Table 4.1. Hematologic and hepatologic parameters

Hematologic		**Hepatologic**
morphological	***biochemical***	**(biochemical)**
RBC count	Glycemia	Glycogen
Leukocyte count	Cholesterolemia	Glucose
Leukocyte formula	ASAT, ALAT, LDH	ASAT, ALAT, LDH
Hematocrit	Hemoglobin concentration	Total cholesterol

5. Effect of *Arthrospira* (*Spirulina)* administration for two weeks on metabolism and hematologic parameters in mice

Experimental protocol

The experiment was performed on laboratory mice (*Mus musculus*, var. *albicans*), obtained from the zoobase of the University of Medicine and Pharmacy of Cluj-Napoca. The animals were divided into 3 groups: the control (**C**), which consumed a nutritionally and energetically equillibrated diet, containing plant and animal carbohydrates, lipids and proteins, minerals and a premix of vitamins; group **F1** (in which the fish flour was replaced by *Arthrospira* (*Spirulina)* powder (3% overall); group **F2** (the fish flour and the soya were replaced by *Spirulina*, at 8% overall). The duration of this experiment was 14 days, after which the animals were sacrificed and blood and liver samples were collected for morphological and biochemical determinations.

Hematologic parameters

The introduction of *Arthrospira* (*Spirulina)* in the animal diet produced an **increase in the number of blood cells** (RBCs and leukocytes), proportionally to the concentration used (Fig. 5.1).

An **increased percent of reticulocytes** (immature red blood cells) was observed, which points to the intensification of hematopoiesis, contributing to the increases observed în the case of the hematocrit and hemoglobin. However, in all the groups, the percent of reticulocytes stayed within the normal limits reported for adult mice, *i.e.* 32-80% (Uray, 1992).

The increase of the leukocyte count in the groups that received a diet containing *Spirulina* correlates with the literature data which signal the immunostimulating effect of this cyanobacterium (Qureshi *et al*., 1996).

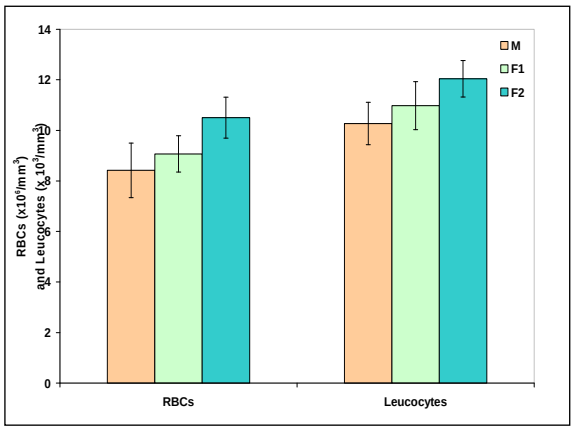

Fig. 5.1. Variation of RBC and leukocyte counts in control mice, and those fed (for 2 weeks) a diet containing *Arthrospira (Spirulina)* powder.

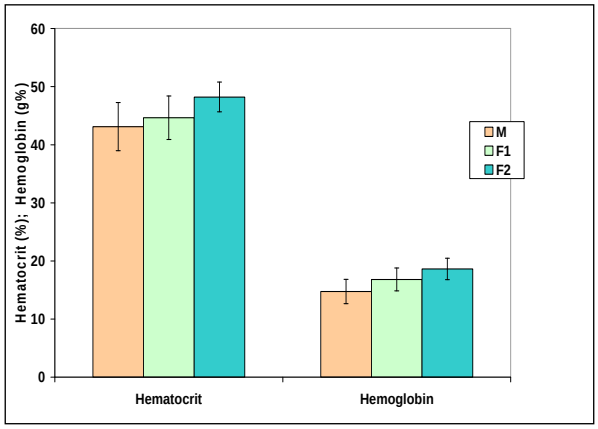

Fig. 5.2. Hematocrit and hemoglobin in control mice, and those fed (for 2 weeks) a diet containing *Arthrospira* (*Spirulina)* powder.

As can be seen from Fig. 5.2. above, the **hematocrit** did not suffer statistically significant variations, although a slight increase can be detected, more evident in the case of the F2 group, where *Spirulina* represented 8% of the total food. This increase can be ascribed to either an increase in the RBC count or to an increase of the mean RBC volume (MEV). Since our data recorded a reduction of MEV and an increase of the total hemoglobin concentration, it means that the hematocrit changes are due to an increase of RBC count.

Hepatologic (hepatic biochemical) parameters

In our experiment, **glycemia** increased slightly, but statistically significant, in the groups F1 and F2, as compared to the control. All the increases, however, are within the physiological limits, so this should be considered a modulating and not a hyperglycemic effect. An explanation for such an effect could be found if we take into consideration that in the alcoholic extract of *Arthrospira* (*Spirulina) platensis*, Babaev *et al*. (1979)

evidenced chromatographycally the presence of certain plant analogues of the thyroid hormons. A few years later, Naliandian and Babaev (1988) also demonstrated the presence in such extracts of the precursors of these hormons: monoiodine thyrosine (MIT) and diiodine thyrosine (DIT), as well as of free iodine.

We also noticed a reduction of **cholesterolemia**, proportional with the percentage of *Arthrospira* (*Spirulina)* contained in the diet. Through its hypocholesterolemic effect, the addition of these cyanobacterium prevents the development of the fatty liver. Thus, *Spirulina* can be credited with hepatoprotective effects în different types of intoxication (Torres-Duran *et al*., 1998; Ble-Castillo *et al*., 2002; Gonzalez de Rivera *et al*., 2003).

Our results overlap to a certain extent with the results of other experimental studies devoted to the demonstration of beneficial effects of *Spirulina*. It is remarkable in this respect that these cyanobacterium do not show toxic effects if administered in optimal doses, adapted to the physiological peculiarities of the organism used for experiments.

6. Physiological effects of the local, wild strain of *Arthrospira* (*Spirulina)* administered to mice for two month

Experimental protocol

This experimental variant is similar to the one described above, having the same type of objectives, but a longer time duration (2 months). It was performed on the same species (*Mus musculus*, var. *albicans*) and the organization of the experiment was identical (*i.e.*, with the 3 groups of mice: C, F1 and F2).

Hematologic parameters

Spirulina administration resulted in the increase of the **RBC and leukocyte counts**, proportionally to the percentage of cyanobacterium used (Fig. 6.1). The reticulocytes, however, increased less in the case of the mice fed a richer *Spirulina* diet. The large proportion of reticulocytes generally observed suggests an intensification of the hematopoiesis. The fact that the number of reticulocytes increases more in the F1 group (with 3% *Spirulina*) could mean that as the number of adult erythrocytes (RBCs) increases (it is the highest in F2) the rate of the formation of new cells tends to a steady state.

The hematocrit shows a statistically significant increase in the F2 group, which received a larger percentage of *Spirulina* (8%). This phenomenon could be caused by an increase of the RBC count, which indeed is the case with F2 (see Fig. 6.2).

The increased number of leukocytes is also revealed by the **leukocyte formula**, which indicates an increase of the percentage of *monocytes* and *lymphocytes* in mice that had a lower concentration of *Spirulina* in the diet. Here, a statistically significant reduction in the percentage of the neutrophils was also observed. It appears that a lower concentration of Spirulina (3%) in the diet stimulates the specific immunity (the

lymphocyte response), whereas the higher concentration (8%) mainly stimulates the unspecific immunity (the phagocytic leukocytes). As a compensatory phenomenon, the percentage of neutrophils, eosinophils and basophils decreased in the groups that received *Spirulina*, as compared to the control.

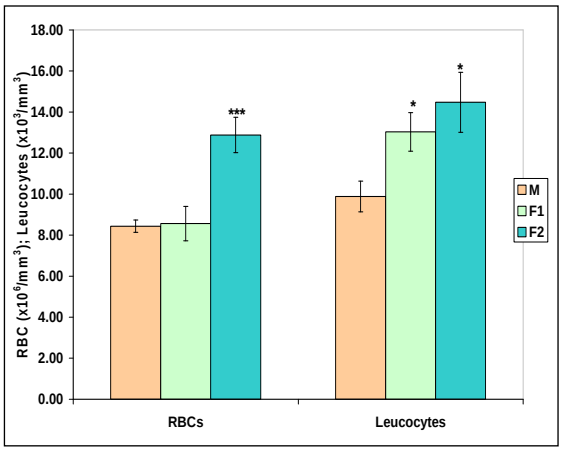

Fig. 6.1. Variation of the RBC and leukocyte counts in control mice and those fed a diet containing *Spirulina* for 2 months.

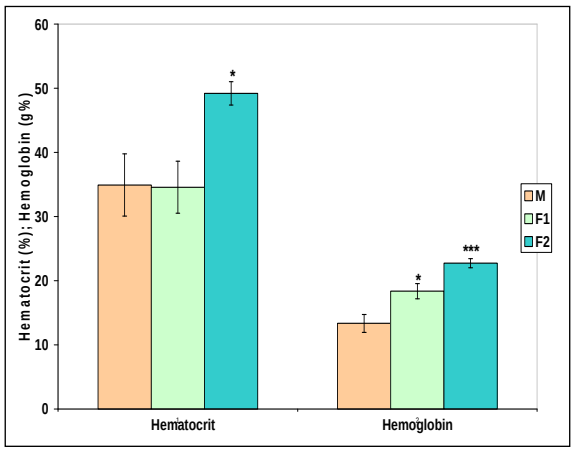

Fig. 6.2. The hematocrit and hemoglobin in control mice and those fed a diet containing *Spirulina* for 2 months.

*- denotes statistically significant differences vs. the control

Glycemia did not suffer statistically significant variations, while the concentration of **blood cholesterol** decreased significantly only in the F1 group.

Our experiment demonstrates that *Arthrospira* (*Spirulina)* has a positive effect in anemias, most likely due to its high content of nutrients and especially of the following substances: vitamin B12, folic acid, essential amino acids, high iron bioavailability.

One should also note the hypocholesterolemic (cholesterol lowering) effect of this cyanobacterium, already recommended for risk reduction in the cardio-vascular diseases (Belay, 2002; Juarez-Operoza *et al*., 2009).

7. Effect of *Arthrospira* (*Spirulina)* on hematopoiesis in rats

Previous studies have also reported that *Arthrospira* (*Spirulina)* can reduce the severity of anemia, inducing an increase of hematologic parameters (Kostic *et al.*, 1993; Zikic *et al.*, 1997; Simsek *et al.*, 2009). Apparently, its content of phycocyanin stimulates hematopoiesis and mimics the effect of endogenous erythropoietin (Epo). The role of Epo consists in stimulating the proliferation, growth and differentiation of erythroid precursors, with the consequent increase of the erythrocyte count.

The objective of the study presented in this section was to test the hematopoietic effects of the locally isolated wild strain of *Arthrospira* (*Spirulina)* added as biomass to

the food of white rats. More exactly, we investigated the stimulating effects of this cyanobacterium on both anemiated and normal/healthy rats.

Experimental protocol

In our experimental variant, white rats, having initially about 150 g each, were monitored for 3 weeks. They were divided into 3 groups: group **C** (control), which was fed for the first week only bread and milk, to which *Spirulina* powder (3% of the food) was added for the last two weeks; group **An** (anemiated), which consumed all the time only bread and milk; group **An-Sp**, also anemiated, but treated with *Spirulina* for the last two weeks, in a similar way to the C group. The anemia in groups **An** and **An-Sp** was induced through 3 blood takings in days 1, 2 and 4. In days 3 and 5, small blood samplings were performed to check for the level of the induced anemia.

At the first/initial blood sampling, the 3 groups displayed very similar/close results, while in the subsequent samplings the results differed statistically significant in a manner suggestive of an antianemic effect of *Spirulina*.

The evolution of the blood parameters in the An group. At the second sampling (after anemia was produced), a highly significant decrease in RBC count was recorded in the An group, as compared to the initial sampling, while at the final sampling this decrease was smaller, but still statistically significant. However, as compared to the second sampling, there was a very significant increase in RBC count at the final sampling. This increase is most likely due to the induction of Epo synthesis and its effect on erythropoiesis, as a reaction of the organism to anemia. As regards the reticulocytes, there is a highly significant increase of their number at both final and second sampling, although the increase is somewhat smaller at the final sampling. This can be attributed to the conversion in time of some reticulocytes into adult erythrocytes, as indicated by the increase of RBC count at the last sampling, as mentioned above and as we have also seen in a previously described experiment performed on mice (the present thesis). Significant increases can also be observed for hematocrit. All these results demonstrate the natural capacity of hematologic recovery for the animals tested.

For the **An-Sp** group, all blood parameters tested show increased values at the final sampling, as compared to the initial one. The same statement is valid if comparison is made with the second sampling, with one notable exception: the reticulocytes. Their count is higher after the induction of anemia (before *Spirulina* addition) than at the final sampling. This can be explained by the fact that phycocyanin has an Epo-like effect, stimulating the hematopoiesis, i.e. the conversion of reticulocytes into RBCs. Indeed, the RBC count is much larger in the An-Sp group than in the An group and even higher than in the control (C) group. Since the control group also received *Spirulina*, the difference should be ascribed to the combined effect of the endogenous Epo (induced by anemia) and the Epo-like effect exerted by *Spirulina*. The value of the other blood parameters is also in line with this conclusion. For example, the hematocrit is significantly increased at the final sampling.

To have a general image of our results, a synthetic comparison across the the 3 groups at all 3 samplings is also illustrated by bar diagrams in Figs.7.1.-7.2. Fig. 7.1. illustrates and compares the reticulocyte count of the 3 groups at the moments of the 3 samplings. As can be seen, there are highly significant increases (***) in both An and An-Sp groups as compared to the control, at the second and third (final) sampling, whereas a very significant decrease (++) can be observed at the final sampling for the An-Sp group as compared to the An group. In Fig. 7.2. we illustrate the evolution of RBC count in the 3 rat groups. As can be seen, the differences that exist between the groups at each sampling do not appear statistically significant, although there are differences among the 3 moments of sampling.

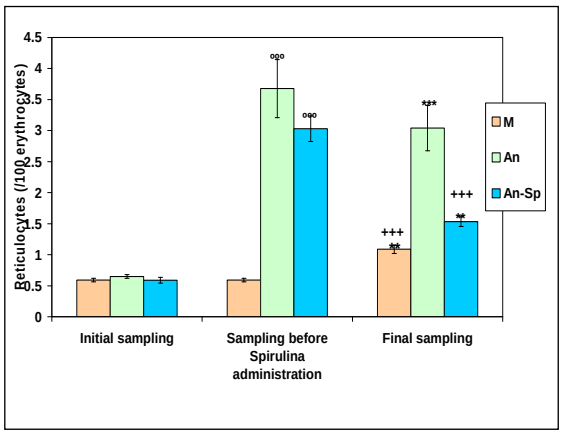

Fig. 7.1. A comparative illustration of the evolution of the reticulocyte count in the 3 groups of rats.

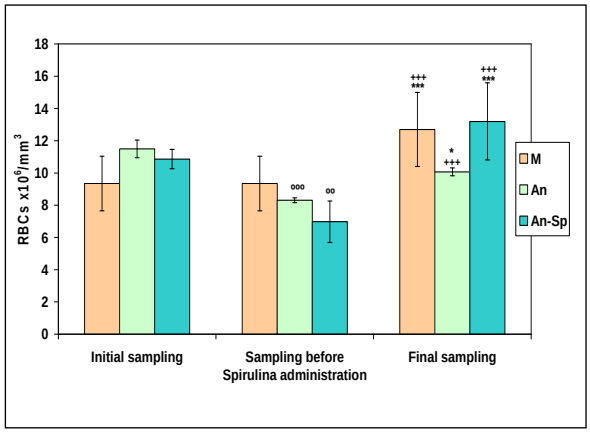

Fig. 7.2. The evolution of the RBC count in the 3 groups of rats.

º - denotes the significance of the differences between the sampling before *Spirulina* administration and the initial sampling; * - denotes the significance of the differences between the final sampling and the initial sampling, while the symbol + is used to show significant differences between the final sampling and the one before *Spirulina* administration.

From the results described above, we can conclude that:

- the hematostimulating effect of our strain of *Arthrospira (Spirulina)* was observed even in group C (control), în the absence of anemia.

- in the rats of the An (anemiated) group, after 14 days since the induction of anemia, the measured parameters (% reticulocytes, RBC count, and hematocrit) partially recovered, as a result of erythropoiesis intensification under the action of the induced endogenous Epo.

- the animals in the An-Sp group were more efficient in restoring the affected parameters, as compared to the An group, because the normal reaction of the organism to anemia was supplemented by the effect of phycocyanin contained by the cyanobacterium *Spirulina.*

8. The effects of *Arthrospira (Spirulina)* administration on the thyroid function of rats

Experimental protocol

The objective of the experiment presented here was to verify the hypothesis that *Spirulina*, through its content of iodine and compounds similar to the thyroid hormones, can stimulate the thyroid function. For the experiment, we used females found at the second gestation period and the new borns (rat pups) of these females (the gestation period usually lasts for 22 days).

The pregnant females were divided into 3 groups, **C**, **T** and **TS**. The animals in the **C** (control) group did not suffer any treatment; the animals in group **T** had the thyroid inhibited with thiourea powder, starting from day 15 of pregnancy until the weaning of the pups; the **TS** group was treated with thiourea as the T group, but the mothers received 5% *Spirulina* powder in the food since the day of birth.

The rat pups taken into study were also distributed into 3 groups: group **C** contained pups from the females of the group **C**; group **T**, with pups coming from mothers that received thiourea during the days 15-22 of pregnancy and during lactation; group **TS**, with pups from mothers that had the thyroid inhibited like group T, but which benefitted of the *Spirulina* supplement, indirectly through the maternal milk, and after weaning directly from the food.

The weight of the pups in the treated groups, as compared to the control, presented important variations, both at birth and during posnatal development. While the pups born by the control mothers are within the normal limits at both ages, the pups born to mothers that received the thyroid inhibitor are lighter/smaller by 25% and their further evolution is also weaker. The pups coming from mothers that had the thyroid inhibited but which also received *Spirulina* registered from the beginning smaller weight differences, but still statistically significant. Since they also consumed *Spirulina* for the last two weeks, before being sacrificed, there was a visible tendency to the reduction of the differences recorded from the control.

Evaluation of the spatial memory through the labyrinth test

The rat pups started with different learning abilities, the group with the supressed thyroid being clerly inferior to the control group. Such a result is expected, considering the profound implication of the thyroid hormons in neurogenesis, formation of synapses, axonal mielinization and the establishment of the neuronal networks. At the first sight, it was a surprise for us that the TS group started with a time almost as good as the control group. However, one should consider that *Arthrospira (Spirulina),* beside iodine and substances similar to the thyroid hormons (Babaev *et al*., 1979; Naliandian and Babaev, 1988), contains a large quantity of essential fatty acids, very necessary during this period for the mielin synthesis (Falch *et al*., 1995).

During the learning period, the pups improved their performance, but differently. The pups in the T group reduced the time needed to go through the labyrinth by 47%, those from the TS group by 78% and the C group by 94%. These results clearly demonstrate the capacity of *Spitulina* to partially fill the necessities of a deprived

organism – a fact that becomes of special importance for children living in geographical areas with iodine deficit or for the malnourished ones.

Evolution of thyroid gland and of the thyroid hormon concentration

Thyroid inhibition usually has multiple and sometimes contrary effects. In adult animals, the inhibition of the thyroid hormon synthesis leads to the stimulation of the TRH secretion, through a negative feed-back at the level of the hypotalamus, and especially the stimulation of adenohypophysis and intensification of TSH secretion.

In our experiment, we observed that, if the administration of the thyroid hormon inhibitor overpasses 30 days, a compensatory phenomenon occurs, *via* TSH, which partially reduces the consequences of thyroid supression. Therefore, we tried to avoid this phenomenon by administering the thiourea in reduced concentrations and only for 30 days.

Thyroid weight of the pups at 30 days was significantly decreased only in the T, and not in the TS group (the one that recieved both thiourea and *Spirulina*) (Fig. 8.1). With all these, the thyroid hormon concentrations stay under the values of the control, especially for the active form (T_3). A comeback tendency was noted only for the T_4 form of the hormon, after te administration of *Spirulina* (Fig. 8.2).

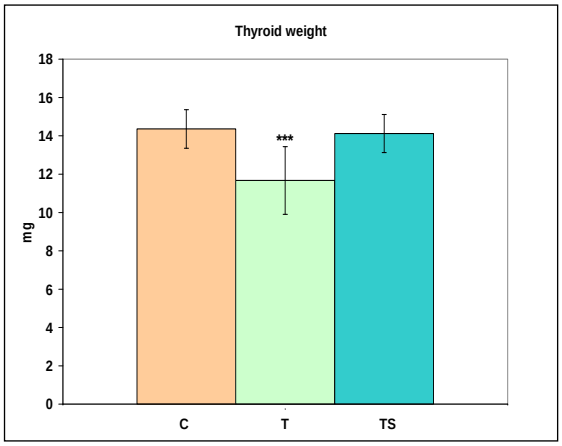

Fig. 8.1. Thyroid weight of rat pups at 30 days.

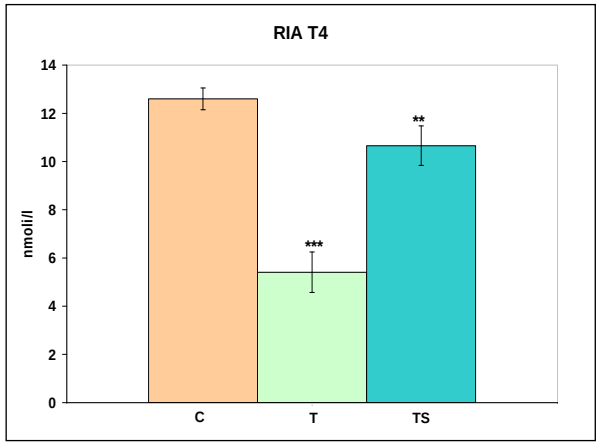

Fig. 8.2. Serum **c**oncentration of thyroxine in rat pups of 30 days.

9. The immunostimulating properties of a local, wild strain of *Arthrospira (Spirulina)*

Experimental protocol

Taking into consideration the work of Russian researchers (Loseva and Dardynskaya, 1993) regarding the radioprotective effect of *Arthrospira (Spirulina)*, we began the study of the immunostimulating proprties of our wild strain. For this purpose, a mixture of immunosuppressing elements was used. It included: Prednison (active

substance - prednison acetate), Cell Cept (active substance - mycofenolate mofethyl), Sandimmun neoral (active substance - cyclosporin). The animals (mice) were divided into 4 groups, each with a different diet: group **C** (control); group **Im** (immunosuppressed), which consumed the same food as C, but the animals received daily an intraperitoneal dose of 20 µl immunosuppressing mixture; group **Im-Sp** (immunosuppressed and treated with *Spirulina*), which recieved food supplemented with 8% *Spirulina* powder and were also daily injected with the immunosuppressing mixture as the Im group; group **Sp,** which recieved food supplemented with 8% *Spirulina* powder, but without immunosuppressing treatment.

Hematological and biochemical parameters

An **increase of the RBC count** was noted in all groups, as compared to the control, although this increase was statistically significant only for the Sp group (Fig. 9.1).

As regards the **leukocytes**, one can notice a statistically significant decrease in the Im group as comparaed to the C group. In the Im-Sp group, there is a decrease when compared to C, but an increase in comparison to the Im group, while in the Sp group the lekocyte count is comparable to the control (even slightly higher - by almost 10%). When compared to the immunosuppressed group, the increase is over 60%. This confirms the immunostimulating capacity *of Spirulina*. According to Qishen *et al*. (1988), the stimulation of erythropoiesis may be the consequence of the increased bioavailability of the iron contained by *Spirulina*, in comparison with other food sources, although from a previous experiment described in this thesis (see section 7 of this summary), we know that other mechanisms may also be at work.

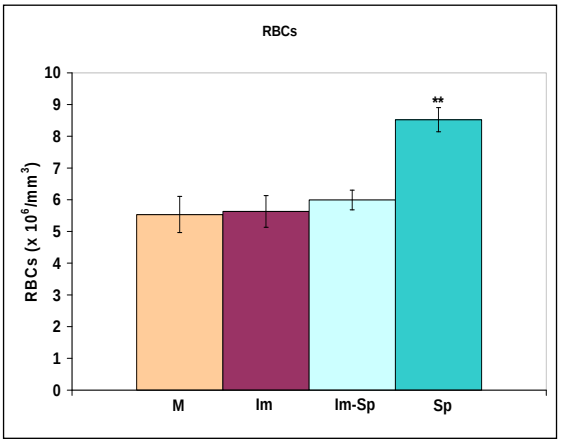

Fig. 9.1. Variation of RBC count in the 4 groups of mice.

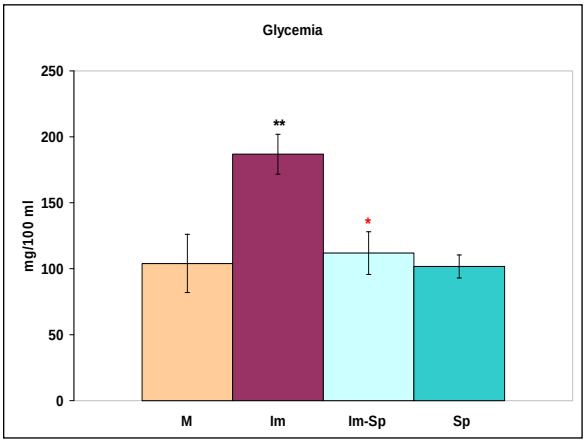

Fig. 9.2. Variation of glycemia in the 4 groups of mice.

Glycemia recorded a statistically significant increase in the Im group as compared to the control, while in the Im-Sp group, a comeback towards normality can be

observed (a 40% decrease when compared to the Im group). This result points to an important hypoglycemic effect of Spirulina (Fig. 9.2).

In the Sp group, the pagocyting activity of macrophages was intense, a fact which may be interpreted that *Spirulina* stimulates the intercellular signalling at the level of the macrophage and antigen presenting cell.

Corroborating our data with the ones existing in the literature, we can state that *Spirulina* improves the macrophage functions and ensures the increase of the natural defense capacity of the organisms having a decreased immunity.

10. The hepatoprotective effect of the cyanobacterium *Arthrospira (Spirulina)* in the chronic ethanol intoxication of rats

Experimental protocol

To investigate the possible hepatoprotective effects of *Spirulina*, we measured certain hematological and biochemical parameters (from serum and hepatic tissue) in normal and ethanol-intoxicated rats, some of the groups also receiving *Spirulina*.

In an experiment lasting 6 weeks, Wistar rats were divided into 4 groups: the control (**C**), which received the standard diet; the ethanol-intoxicated group (**EtOH**), which recieved in addition ethanol (6 g/kg body weight); the **EtOH-Sp** group, which was treated as the EtOH group, but their diet contained 8% Spirulina powder; the **Sp** group, which was similar to the control, but recieved Spirulina (8%).

Hematological and biochemical parameters

It is known that ethanol induces RBC lysis through free radical generation and their aggressive behaviour (Ballard, 1997). Consequently, in our experiment, we see a significant decrease of RBC count in the EtOH group. The administration of *Spirulina* to ethanol-intoxicated rats led to an evident increase of RBC count (although not statistically significant). The highest RBC count was recorded in the Sp group, but again, the increse from the control does not appear statistically significant.

The number of leukocytes decreased significantly only in the EtOH group and increased significantly only in the Sp group. Nevertheless, in the EtOH-Sp group, this count has been restored, its value being actually higher than in the control, although this increase does not appear statistically significant (as compared to the control). Thus, we can say that the immunosuppression induced by ethanol was totally removed by *Spirulina*.

The hematocrit recorded a slight decrease in the ethanol group, which paralleled the decrease in RBC count. The administration of *Spirulina,* along with ethanol, lead to a significant increase of the hematocrit. **Hemoglobin** did not show significant changes, as compared to the control.

The introduction of ethanol in the diet determined **a significant hyperglycemia**. (glucose increased by 35%). This is the result of alcohol metabolism, which generates acetaldehyde and changes the cell redox ratio towards a more reduced state (Lieber,

1993). On the background of this alcoholic hyperglycemia, *Spirulina* additon restored glycemia to almost normal values.

Hepatic glucose and glycogen concentrations had a parallel evolution, both decreasing significantly under the influence of ethanol. However, in the EtOH-Sp group, their values approached those of the control.

The concentration of serum proteuins was significantly reduced in the EtOH group, but brought back to the level of the control by the addition of *Spitulina*, as can be seen in Fig.10.1 for the EtOH-Sp group.

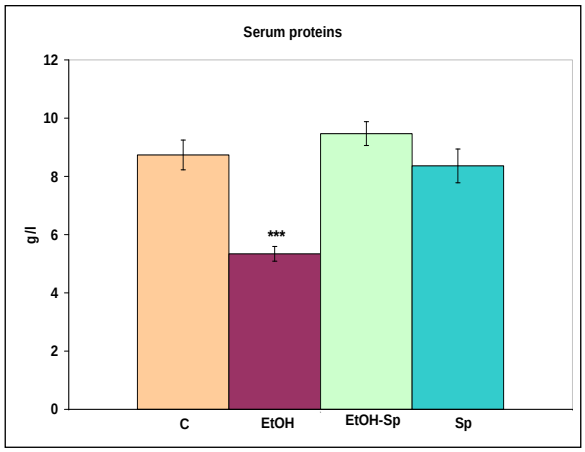

Fig. 10.1. Variation of serum protein concentration in ethanol-intoxicated rats, treated with *Arthrospira (Spirulina)*

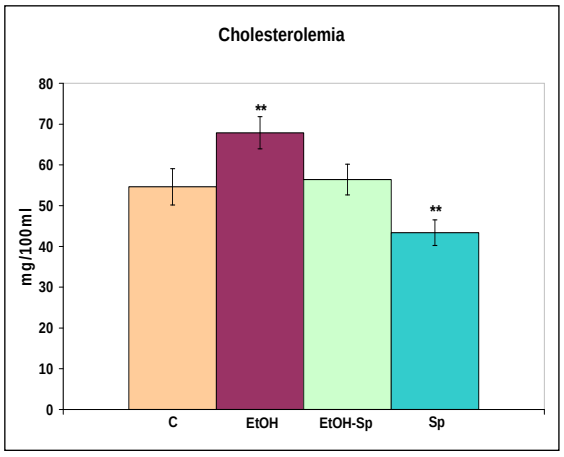

Fig. 10.2. Variation of serum cholesterol (cholesterolemia) in ethanol-intoxicated rats, treated with *Arthrospira (Spirulina)*

We also verified whether under our experimental conditions we can detect a cholesterol lowering action of *Spirulina*. We observed an increase of plasma cholesterol in the EtOH group and a decrease in the groups that recieved *Spirulina*. Cholesterol increase induced by ethanol administration is associated with other changes in lipid metabolism that are known in the literature (Lieber, 1993; Navder *et al*., 1997). More importantly, however, is that the hypercholesterolemic effec of ethanol has been completely prevented/reversed by the administartion of *Spirulina,* as can be seen from Fig. 10.2.

In conclusion, we can say that in our experiment, the administration of *Spirulina*, both in the absence and especially in the presence of ethanol intoxication, lead to a significant decrease in the concentration of hepatic glucose and glycogen. The cholesterol lowering effect was also evidenced in our experiment. Ethanol administration decreased significantly the concentration of serum proteins, both the synthesis and the export of proteins being affected. The use of *Spirulina* powder led to an increase in the level of serum proteins, restoring in the EtOH-Sp group the export capacity for hepatic proteins.

11. Ultrastructural studies on the effects of *Spirulina* administration in the chronic intoxication of rats with ethanol

Experimental protocol

The structural and ultrastructural studies were made in collaboration with the Electron Microscopy Center of the Babes-Bolyai University, Cluj-Napoca. Female Wistar rats were divided into 4 groups, exactly as in the preceding experiment (section 10) and submitted to a similar protocol of work, with two exceptions: the study lasted for 10 weeks and the groups intoxicated with ethanol (groups noted here **E** and **ES**) recieved ethanol in the drinking water (10% v/v). At the end of the experiment, the animals were sacrificed by bleeding, under anesthesia. Samples were taken from the hepatic tissue for electron microscopy preparations and the rest of the liver was homogenized for mitochondrial preparations (see the next experiment - section 12).

Ultrastructural studies on the liver of the control rats (group C)

The examination of the normal hepatic tissue reveals a parenchimal structure with hepatocytes arranged in regular cell plates. Every hepatocyte has one spherical or oval nucleus with a diameter of about 6-7 μm and contains 1-3 nucleoli in the kariolymph. Often, the nucleoli are disposed near the nuclear envelope, suggesting an intense activity that involves exchanges between the nucleus and the cytoplasm. In the vicinity of the nucleus there are numerous mitochondria of normal electrondensity (Fig.11.1). The endoplasmic reticulum is represented mainly by narrow profiles of granular endoplasmic reticulum (GER), arranged in packs of parallel profiles or around mitochondria. The lipid droplets are rare in the cytoplasm, because they are transported immediately into the blood capillaries or deposited in the so-called Ito cells (Ito and Shibasaki, 1968).

Ultrastructural studies on the liver of the ethanol-intoxicated rats (group E)

In the hepatocyte cytoplasm of these rats, found mainly close to the centrolobular zone, one can observe mitochondria with a rarefied matrix, of low electrondensity, an aspect which suggests a decrease of their participation to the energy metabolism. It can also be seen that GER has dilated canaliculi and there is a dilated perinuclear space, suggesting a decrease of the protein synthesis (Fig.11.2). The Kupffer cells, which are the liver macrophages, have a relatively intense activity for capturing and distroying the structures altered by alcohol. In the lumen of the sinusoid capillaries, neutrophils can occasionally be seen, their presence indicating a clear ihflammatory process.

Ultrastructural studies on the liver of the ethanol-intoxicated rats, treated with *Spirulina* (group ES)

Concomitant administration of *Spirulina* and ethanol leads to a certain degree of protection against the toxic effect of ethanol, the ultrastructural images from the liver of these animals (Fig. 11.3) being close to those of the control group. Most of the hepatocytes are normally structured, with 1 or 2 predominantly euchromatic nucleoli, GER is present as narrow profiles with a parallel disposition around normally electrondense mitochondria, which is helpful in protein synthesis. The lipids are missing from Ito cells, a fact which indicates a good metabolization of these substances, close to the normal state. The presence of glycogen microparticles is also evident, in some

hepatocyte forming patches or rosettes. There are also several centers of glycogen synthesis and numerous peroxisomes. Peroxisomal proliferation is one of the indicators of increased cell capacity to oppose to the alterations caused by reactive oxygen species (ROS). It is obvious that this peroxisomal proliferation is a consequence of the food supplementation with *Spirulina*.

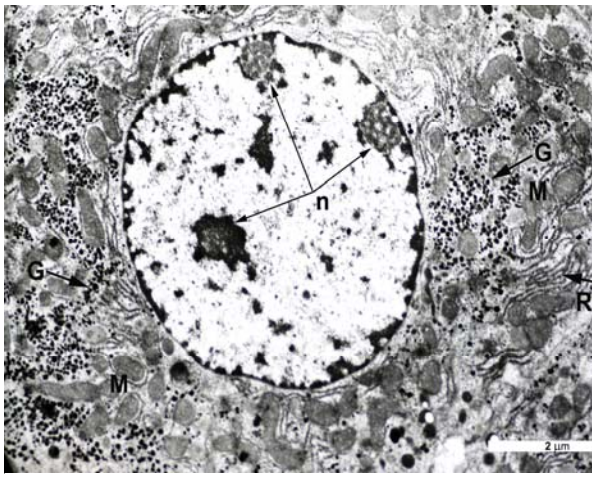

Fig. 11.1. Control group (C): hepatocyte with a spherical, euchromatic nucleus and 3 nucleoli (n). In the cytoplasm, one can observe parallel profiles of granular endoplasmic reticulum (REG) and several glycogen patches (G). The bar is 2µm.

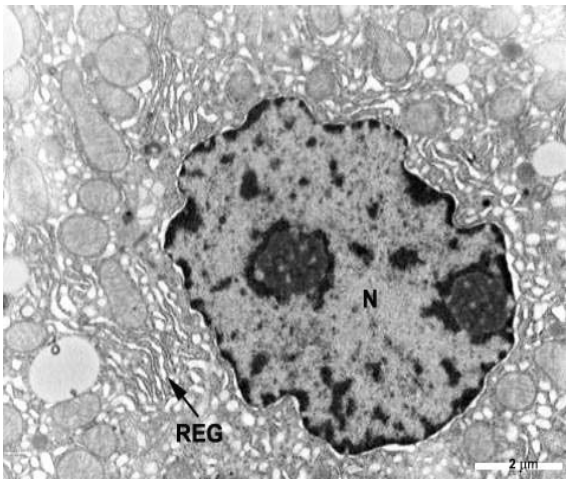

Fig. 11.2. Group E: hepatocyte from the centrolobular zone. The nucleus (N) has an irregular form, the REG profiles are dilated and the mitochondria have low electrondensity. The bar is 2 µm.

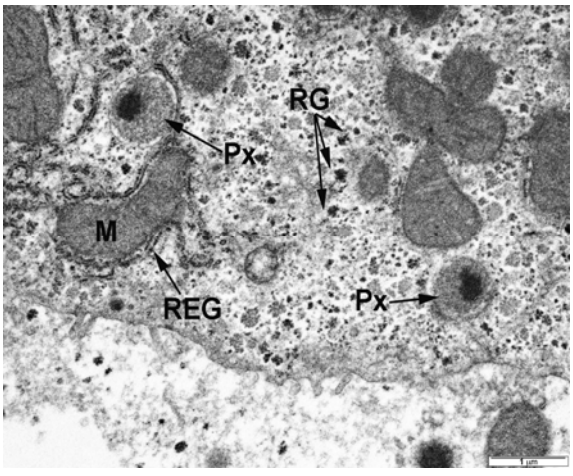

Fig. 11.3. Group E-Sp: numerous peroxisomes (Px) and glycogen rosettes (RG) can be seen in the hepatocytes . M – mitochondrion; REG – granular endoplasmic reticulum. Bar – 1µm.

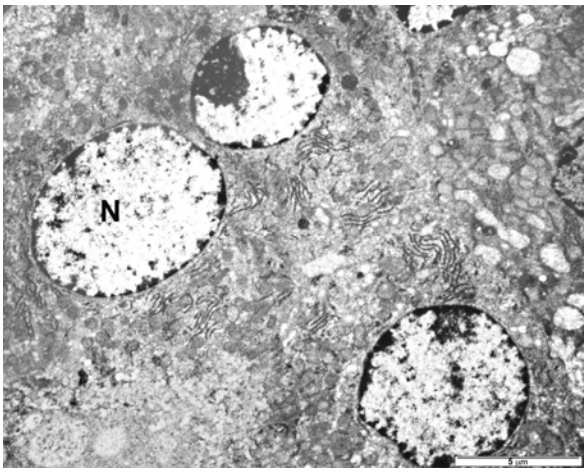

Fig. 11.4. Group Sp: hepatocytes with normal aspect; spherical nuclei (N) with a regular outline; normally electrondense mitochondria and parallel REG profiles. Bar – 5 µm.

Ultrastructural studies on the liver of the *Spirulina-treated* rats (group S)

The ultrastructural aspects described for the liver of the control rats are in general also present here (Fig.11.14).

The abundence of the granular endoplasmic reticulum indicates an intense metabolic activity, especially directed toward the protein synthesis. Mitochondria are in general spherical, with an electrondense matrix, pointing to an active energy metabolism. The biliary canaliculi are normally constituted, with a narrow diameter, delimited by microvilli. Around the biliary canalicului there are present lysosomes as peribiliary corpuscles, involved in bile synthesis. The Kupffer cells are active, with many lysosome ready to get into activity.

12. Effects of *Arthrospira (Spirulina)* administration to rats, chronically intoxicated with ethanol, on hepatic mitochondria; monitoring the permeability transition and the associated phenomena (mitochondrial swelling, ΔΨ collapse and calcium efflux)

The purpose of the present study was to establish whether Spirulina has a protective effect on liver mitochondria of both normal and ethanol-fed rats with regard to the permeability transition and associated phenomena (matrix swelling, ΔΨ collapse and calcium efflux). The animals used in this study are the same as those used in the preceding experiment and the notations used for the 4 groups is identical (see section 11). After liver homogenization, the mitochondria were prepared by differential centrifugation as described by Berlean and Tarba (20011). The details of the measurements of the bioenergetic parameters which are of utmost importance for assesing the integrity and the efficiency of energy metabolism of these organelles has also been described there,

Membrane potential (ΔΨ), calcium fluxes and matrix swelling were followed spectrophotometrically, in some cases by parallel recordings, using a diode array instrument, while ΔΨ was also recorded fluorimetrically. The respiration and all the associated phenomena monitored by us were triggered by the addition of succinate in the presence of rotenone. From the comparison of the spectrophotometric recordings, we observed a good correlation between the concentration of calcium added to the mitochondrial suspension, the moments of ΔΨ collapse, swelling and calcium release (massive efflux), as shown in Fig.12.1 and Fig.12.2. Given this observation, for simplicity, in later experiments, we routinely used spectrophluorimetric recordings of membrane potential to quantify the sensitivity of mitochondria to calcium. This was achieved by adding 10-μM pulses until ΔΨ collapsed. This value served as a reliable index of comparison between groups.

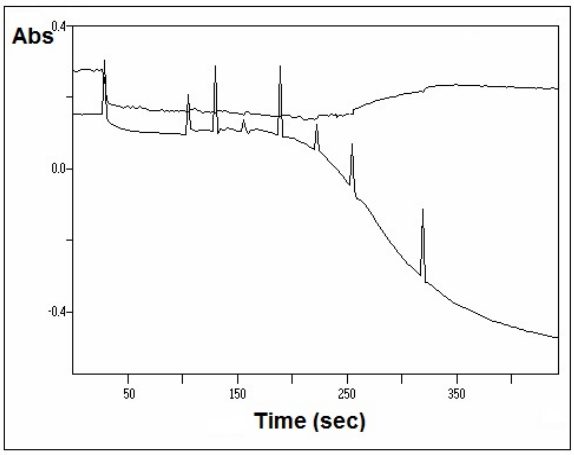

Fig. 12.1. Membrane potential (upper trace) and mitochondrial swelling (lower trace) in mitochondria of control (C) rats.

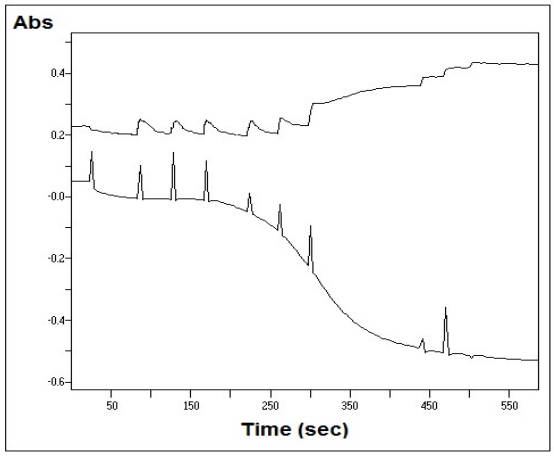

Fig. 12.2. Calcium (Ca^{2+}) fluxes (upper trace) and matrix swelling (lower trace) in mitochondria of C rats.

Mitochondria of the control (C) rats. Fig.12.3 presents a recording of fluorescence changes (λex= 622 nm; λem= 670 nm) associated with the generation and collapse of the membrane potential following succinate addition and repeated calcium pulses, respectively. Fluorescence recording has the advantage of not being sensitive to spectral changes associated with swelling, while all the additions are made in only one cuvette. We can quantify the capacity of mitochondria to resist to calcium-induced stress just by inspecting the membrane potential recordings with the fluorescence method.

Mitochondria of the ethanol-fed (E) rats. Fig. 12.4 illustrate the kinetics of ΔΨ respectively, of a representative preparation in the E group of 5 rats. It can be seen that mitochondria of this group are more sensitive to calcium-induced stress than the control ones, as demonstrated by the smaller number of 10-μM calcium pulses added until the collapse of ΔΨ. These results point to the fact that the mitochondrial membranes of the alcoholic rats and their transport systems are more fragile, ready to give up to slight or moderate metabolic stresses and release the components of the intermembrane space and probably even of the matrix, despite the apparent well-being of the animals.

Mitochondria of the double-treated (ES) rats. Representative behaviour of the mitochondria isolated from rats treated with both ethanol and *Spirulina* are shown in Fig. 12.5. Their behaviour is practically indistinguishable from that of the control mitochondria, a statement which is supported by the statistical analysis. Nevertheless, it is apparent from the recordings that *Spirulina* supplement has a protective effect on the liver of ethanol-fed rats.

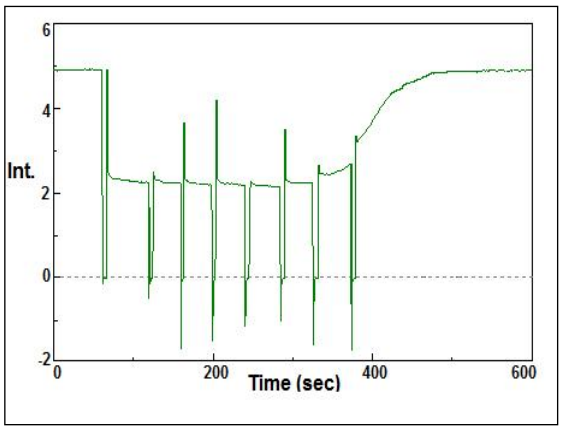

Fig. 12.3. Spectrofluorimetric recording of membrane potential kinetics upon addition of succinate and several 10-µM calcium pulses in control (C) mitochondria.

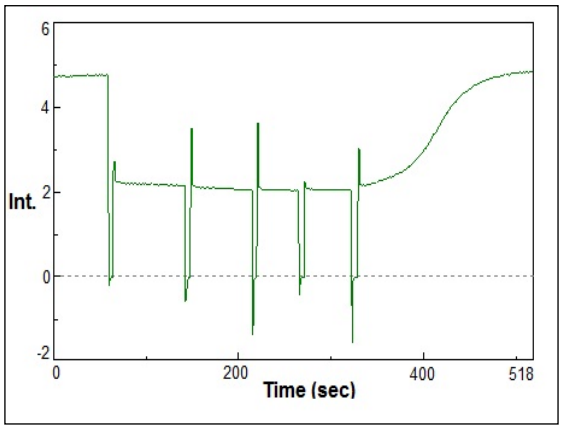

Fig. 12.4. Spectrofluorimetric recording of membrane potential generated by succinate-induced respiration in mitochondria of ethanol-fed (E) rats.

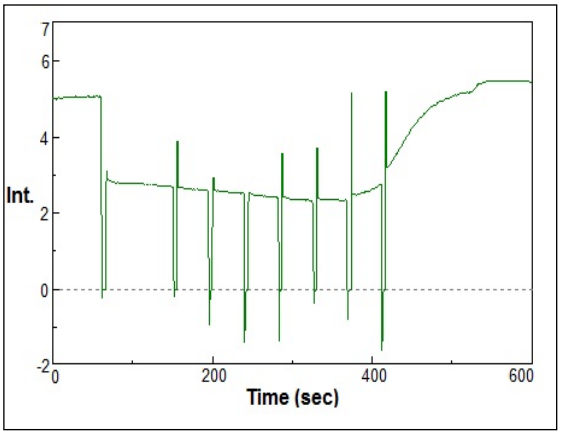

Fig. 12.5. Spectrofluorimetric recording of membrane potential generated by succinate-induced respiration in mitochondria of ethanol-fed *Spirulina*-treated (ES) rats.

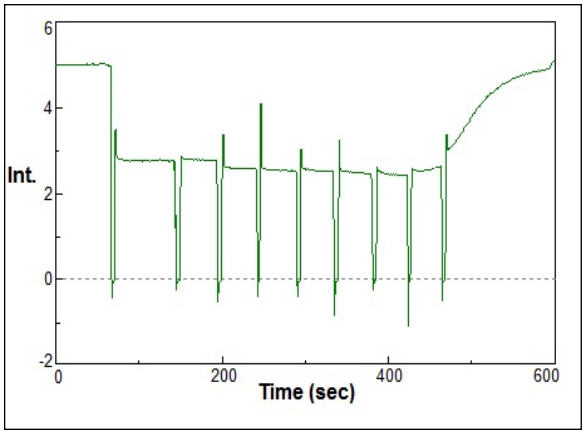

Fig. 12.6. Spectrofluorimetric recording of membrane potential generated by succinate-induced respiration in mitochondria of *Spirulina*-treated (S) rats.

Mitochondria of the Spirulina-treated (S) rats. Representative illustration for the behaviour of mitochondria isolated from this group are presented in Fig.12.6 (for ΔΨ). One can see that mitochondria of this group accumulate around 6-7 calcium pulses before the induction of the permeability transition (PT), amounting to a total of 60-70 µM, or 60-70 nmoles/mg protein, the individual values varying between 45 and 90 nmoles/mg, with a mean of 66.1 nmoles/mg mitochondrial protein. Under the conditions

used by us, in which inorganic phosphate (Pi) has a higher concentration than magnesium, this performance is quite remarkable, since it is known that Pi promotes calcium-induced PT, while magnesium has an opposite effect (Bernardi and Pietrobon, 1982; Racay, 2008). This means that *Spirulina* added as a nutritional supplement increases the resistance of liver mitochondria to calcium-induced stress.

Our present results confirm the beneficial effects of *Spirulina* administration to both normal and ethanol-fed rats. We demonstrate that *Spirulina* enhances the resistance of liver mitochondria to calcium induced stress, postponing $\Delta\Psi$ collapse and permeability transition, factors involved in cell death by both apoptosis and necrosis. This is very likely an important mechanism (if not the most important one at the subcellular level) through which *Spirulina* exerts its hepatoprotective effects reported in the literature.

Conclusions

Following the present study, 7 conclusions are formulated in this summary, which demonstarate that in all the xperimental models used (anemia, hypothyroidism, ethanol intoxication), the majority of the parameters investigated were favourable changed by the administration of *Arthrospira* (*Spirulina)* in the food of the animals (mice and rats). The conclusions are:

1. The external aspect of the animals, the aspect of their internal organs as well as the values of the parameters investigated demonstrate that the administration of *Spirulina* powder (3 and 8%) in the animal diet, for either a shorter (2 weeks) or a longer period (6-10 weeks) had no toxic effects;
2. Our study demonstrated that *Spirulina* has a positive effect in anemia, most likely due to its high density of nutrients and especially of the following substances: vitamin B12, folic acid, essential aminoacids, high bioavailability iron. In all the animals tested, the hematologic parameters (morphological, biochemical and enzymatic) were partially restored/improved as a result of hematopoiesis stimulation under the action of the endogenous Epo. The animals in the anemiated group which was treated with *Spirulina* (An-Sp group) were more efficient in restoring the affected parameters as compared to the An group (anemiated but without receiving *Spirulina*);
3. The tests applied to verify the hypothesis that *Spirulina*, through its content of iodine and compounds similar to the thyroid hormones can stimulate the thyroid function, in animals which had this function chemically supressed, have confirmed this supposition;
4. *Spirulina* improves the macrophage functions and assures the increase of the natural defense capacity of the organism;
5. Administration of *Spirulina*, alone or in combination with ethanol, produced a significant decrease of the hepatic glucose and glycogen. A cholesterol lowering effect was also observed in our experiment. Ethanol administration decreased significantly both the synthesis and the export of the hepatic proteins, while use of *Spirulina* powder generally induced an increase in the level of serum proteins and restored the capacity of protein export at the level of the hepatic membrane in the ethanol-intoxicated animals;
6. The electron microscopic studies of rat liver demonstrated that the ultrastructural aspects found in the normal liver can generally be observed in the liver of ethanol-intoxicated rats if they received *Spirulina* in their food, while those that did not recieve this supplement presented serious alterations, thus confirming the hepatoprotective effect of this cyanobacterium against hepatic toxins;
7. Moreover, we have observed the beneficial effects of *Spirulina* in mitochondria isolated from ethanol-intoxicated rats which recieved this supplement in their food. We demonstrated that a series of bioenergetic aspects (membrane potential, calcium fluxes. permiability transition), which are involved in the survival of the cell, are much better regulated in rats that received *Spirulina.*

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