5. Discussion

There is a large body of published evidence, which suggests that green algae, such as *Chlorella vulgaris*, may have the ability to positively influence many biological systems in mammals. In this study we have assessed the effects of different algal processing methods (electroporation and ultrasonication prior to spray drying) on the efficiency with which rats can metabolise algal protein. It has also been attempted to define the effect of *C. vulgaris* on the reproductive parameters of mice. Animals in both of these experiments were carefully monitored to detect any deleterious effects of feeding with algae. Furthermore, additional experiments were conducted to investigate the safety of algal feeding.

5.1. Evaluation of efficacy of novel technological processing of micro-algae in nitrogen balance study on rats

The composition of the *C. vulgaris* C1 used in our study was not affected by the different processing methods employed prior to spray-drying, except for changes in the crude ash and crude fiber content. The crude ash content [assessed on a dry matter (DM) basis] was 5 % higher in the electroporated samples compared to the other groups. The crude fiber content was 2 % higher (in terms of DM) in the electroporated group than in the simply spray-dried sample and 4 % higher than in ultrasonicated and spray-dried *C. vulgaris*. During the extraction process used in the Weender method fiber determination, part of hemicelluloses and lignin is washed out. It is possible that during electroporation chemical reactions take place leading to a change of the structure and stronger binding of these substances that are then more stable during the analytical process and therefore the determined crude fiber amount increases. There is no obvious explanation for the high crude ash content determined in the electroporated and spray-dried micro-algae other than of an analytical artifact.

The analysis of crude components of the *C. vulgaris* preparations used in our study differed from those reported by Trubachev et al. (1976). Amino acids levels were comparable to data obtained by Saleh et al. (1985) who used an outdoor culture of *C. vulgaris* K5. Our values were lower than those determined by Komaki et al. (1998) for raw *Chlorella* biomass, it is worth noting that no cultivation method was mentioned by these authors. This is of importance when comparing the crude components in micro-algal cells, as the cultivation method, strain of micro-algae used and the technical processing can all influence the final

levels of nutrients in the algal cells (Oh-Hama & Miyachi, 1993; Priestley, 1975; Spoehr & Millner, 1948).

Saleh et al. (1985) determined the amounts of RNA and DNA in drum-dried *C. vulgaris* to be 4 % of DM, Trubachev et al. (1978) reported 3.9 % of nucleic acids in DM of continuously cultivated *C. vulgaris* with RNA/DNA ratio of 4.43. In our case the determined amount of nucleic acids was 1.3 – 1.5 % of DM, with the RNA/DNA ratio of 1.64 - 1.71. These results are very surprising and most probably are due to the applied analytical method of extraction of the nucleic acids. Further investigations are necessary to prove this fact.

To evaluate the nutritional value of the proteins in the three differently treated *C.vulgaris* preparations we accomplished a nitrogen balance study according to method initially described by Mitchell (1924) and standardized over many years in our Institute (Bock, 1966). In this method young albino rats are fed with feed containing a fixed amount of nitrogen-free mixture (containing oil, vitamins, minerals), the investigated source of protein at 150 mg of protein N per 100 g of body weight (which allows maximum growth rates), and starch (e.g. maize starch) being added to 100% of the diet. Animals are kept in metabolism cages allowing separation and collection of leftover, feces and urine on a daily basis. Nitrogen is then measured in all components. Nitrogen measurement is the biggest concern and weakest point of this method. Urine is filtrated and leftover feed are dried before measurement but there is no way to totally separate feed residues from the feces. Therefore, in final calculations there is always an error of some degree. This error is in all groups, so it does not affect the comparison between control and experimental groups, but makes the comparison between literature findings difficult. On the other hand, to obtain exact endogenous and metabolic nitrogen values, they have to be determined in N-free studies on animals of the same species, the same strain (or line) and age. Ideally, determination of NMR should be done for each animal with new genetic material; it is every 3-4 years for one strain of rats – don't understand this. Because of the doubt associated with these data, the fact that feeding N-free feed leads to increased catabolism (which cannot be assumed as physiological) and to reduce the number of animals used a regression formula for rats was calculated (Bock, 1966), where the daily maintenance nitrogen requirement and nitrogen lost with feces depends on the dry matter intake and body weight of the animal used for the study. The values obtained in these formulas are then used to calculate true crude protein digestibility, biological value and net-protein-utilization. Therefore, results obtained for these parameters bear the biggest error.

Apparent protein and amino acids digestibility was determined in our study using a method basing on measurement of nitrogen in fecal samples. Inaccuracy with this approach has been recognized in studies on pigs (Low, 1982) and apparent ileal digestibility based on N flow measured at the terminal ileum is now more often used in protein utilization evaluation studies (James et al., 2002). In order to determine ileal N flow in rats the animals have to be killed to obtain enough ileal digesta for analyses. The feeding regime, together with the killing time chosen for this purpose is of great importance (Butts et al., 2002; James et al., 2002). In our study we opted to use the established N-balance technique as in this case the animals can be used several times for repetitions, what reduces the number of animals used. In this manner the number of replicates necessary for statistical analysis can be easily achieved.

The control group of animals was fed on casein; all these animals performed well showing that the experimental set-up did not adversely affect the animals' performance. All diets were isonitrogenic and according to N intake we can state that the micro-algae did not affect the palatability of the feeds. Only electroporated and spray dried *C. vulgaris* seemed to be less palatable, in this group the N-intake was significantly lower than in casein group, nevertheless, did not significantly differ from the other algal groups. As casein covers all the nutritional protein needs of growing rats, we determined that the maximal potential daily weight gain for 145 g rats was 4.3 g/day. None of rats from experimental groups reached this level, and within the algal groups the highest daily weight gain was observed for the US-DA group, twice as high as in ES-DA and 1.5 times higher than in S-DA group.

The apparent digestibility of crude protein of spray-dried *C. vulgaris* determined in the study was equal to 55 %. This is in disagreement with the findings of Bock & Wuensche (1967), who obtained the aPD for *C. vulgaris* to be 44 % (see Table 54 for comparison). Ultrasonication prior to spray-drying led to a significant increase of aPD to 60 %. Electroporation prior to spray-drying significantly decreased the aPD to 45 %. The true crude protein digestibility, which reflects the N-loses with feces, was 66.5 % for ultrasonicated and spray-dried micro-algae, significantly higher than tPD obtained in other algal groups. This pattern of increase of algal protein value in US-DA group and its decrease in the ES-DA group was also observed for apparent digestibility of amino acids and all other nutritional parameters, with the exception of biological value. As already mentioned, biological value bears the biggest calculation error due to use of MNR and IN, both calculated from regression formulas. Considering the internal control casein group, where the BV achieved 106.8 %, we can see the error is of approximately 7%. Normally BV of casein does not exceed and is close

to 100 %. Taking it under consideration, the BV for protein of ultrasonicated and spray-dried micro-algae would be app. 93 – 94 %, compared to the BV of spray-dried *C. vulgaris* of 87 % and of electroporated and spray-dried *C. vulgaris* – 86 %. These values were much higher than the ones reported by Bock & Wuensche (1967) (Table 54) or Saleh et al. (1985). It is obvious from the study that ultrasonication prior to spray-drying increased the nutritional value of spray-dried micro-algal protein, whereas electroporation had the opposite influence.

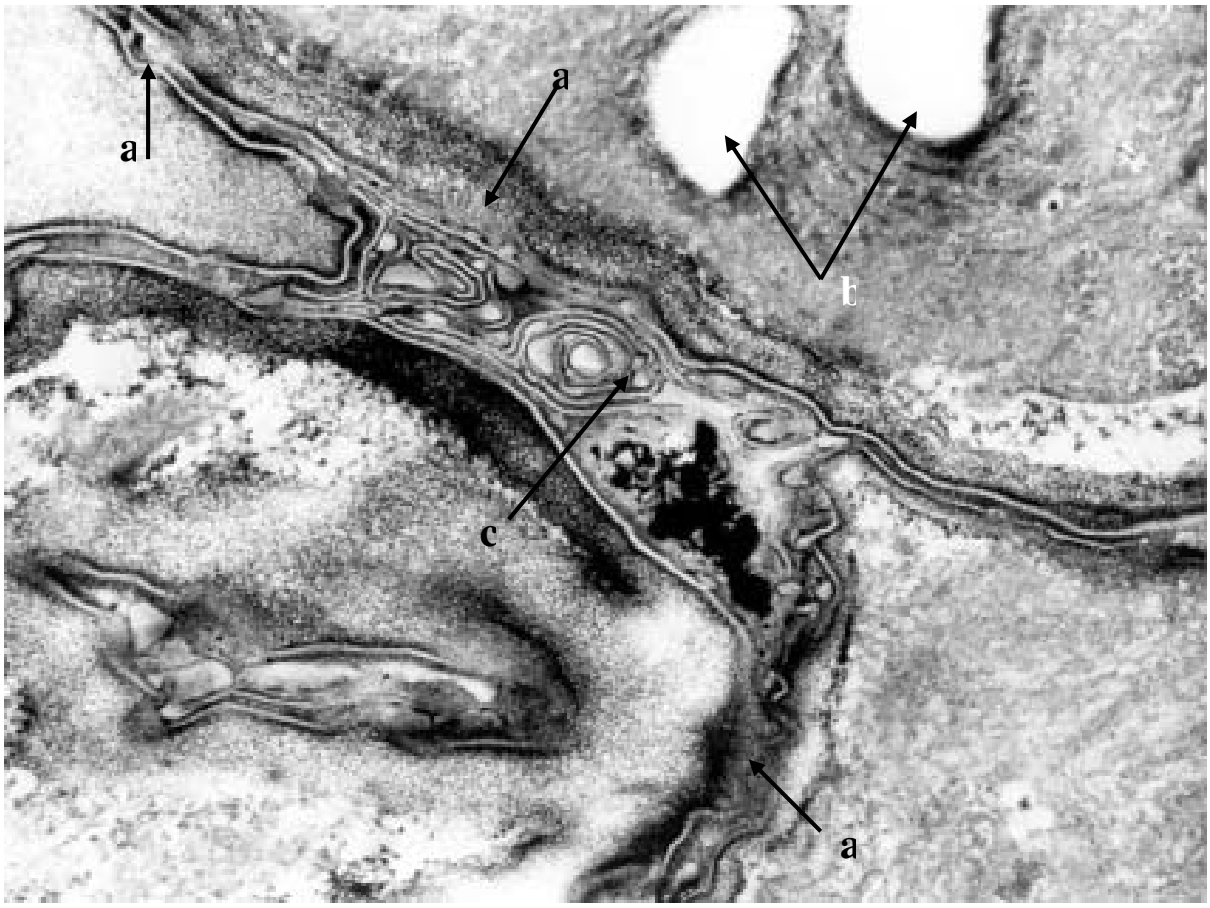
These findings can be supported by ultra microscopic findings. Images of ultrasonicated and spray-dried micro-algal cells show a partially destrupted cell wall, with small perforations (Fig. 23). Treatment with electrical field (electroporation) followed by spray-drying leads to total destruption of the cell wall and cell membrane, in the electron microscope these structures look shrunken and totally changed, the cytoplasm is also fully dried and condensed (Fig. 24).

Table 54. Nutritional indices for *Chlorella vulgaris* and *Scenedesmus obliquus* reported by Bock & Wuensche (1967).

Algae powder	**BV (%)**	**aPD (%)**	**tPD (%)**	**NPU (%)**
Chlorella vulgaris	**52.9 ± 5.4**	**44.1**	**59.3**	**31.4**
Scenedesmus obliquus	47.0 ± 3.5	26.1	32.7	20.1
S. obliquus treated with boiling water	47.9 ± 2.5	36.7	52.9	25.3
S. obliquus heated with stream	40.3 ± 3.0	27.7	44.3	17.9

These changes are not seen in spray-dried micro-algal cells. The only changes that can be observed in these cells are cytoplasm detachment from the cell membrane (Fig. 25). We can speculate that the electrical field or high temperature generated during electroporation process could destroy cell wall structures and some of internal algal components, like vitamins, glycoproteins and others, making them useless for animal. Ultrasonication seems to be a less “harmful” technique, and the changes that arise during this procedure are enough to improve the contact of gut and microbial digestive enzymes with the algal cell components.

Figure 23. *Chlorella vulgaris* cell after ultrasonication and spray-drying (electron microscopy; 33,000-fold)



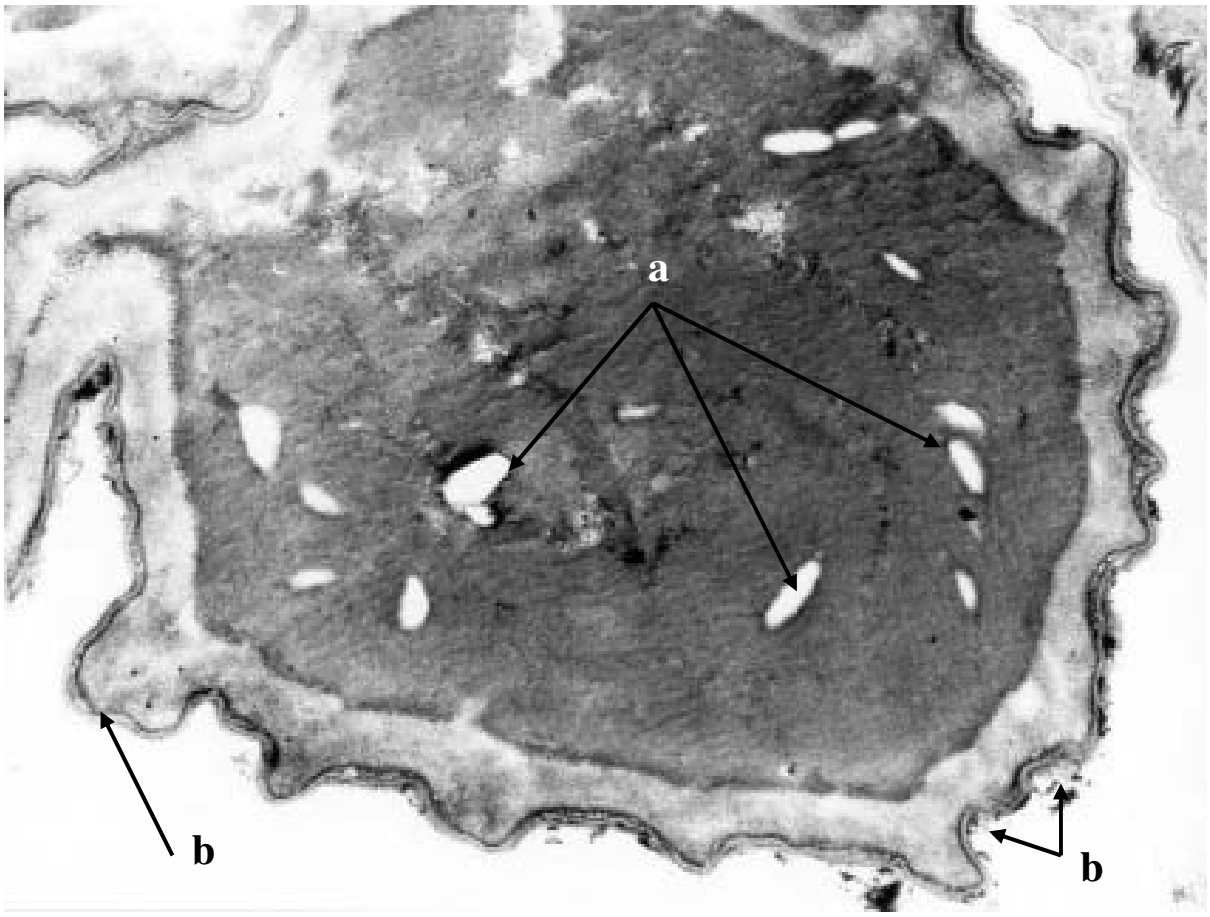
This picture was taken in Institute for Research on Zoo and Wild Animals, Berlin, Germany, 2003

a – perforations of cell wall and cell membrane

b – vacuoles, chloroplast, lipids

c – destroyed cell's components and cell wall/membrane, seen as spiraled form between two micro-algal cells

Figure 24. *Chlorella vulgaris* cell after electroporation and spray-drying (electron microscopy; 21,000-fold)

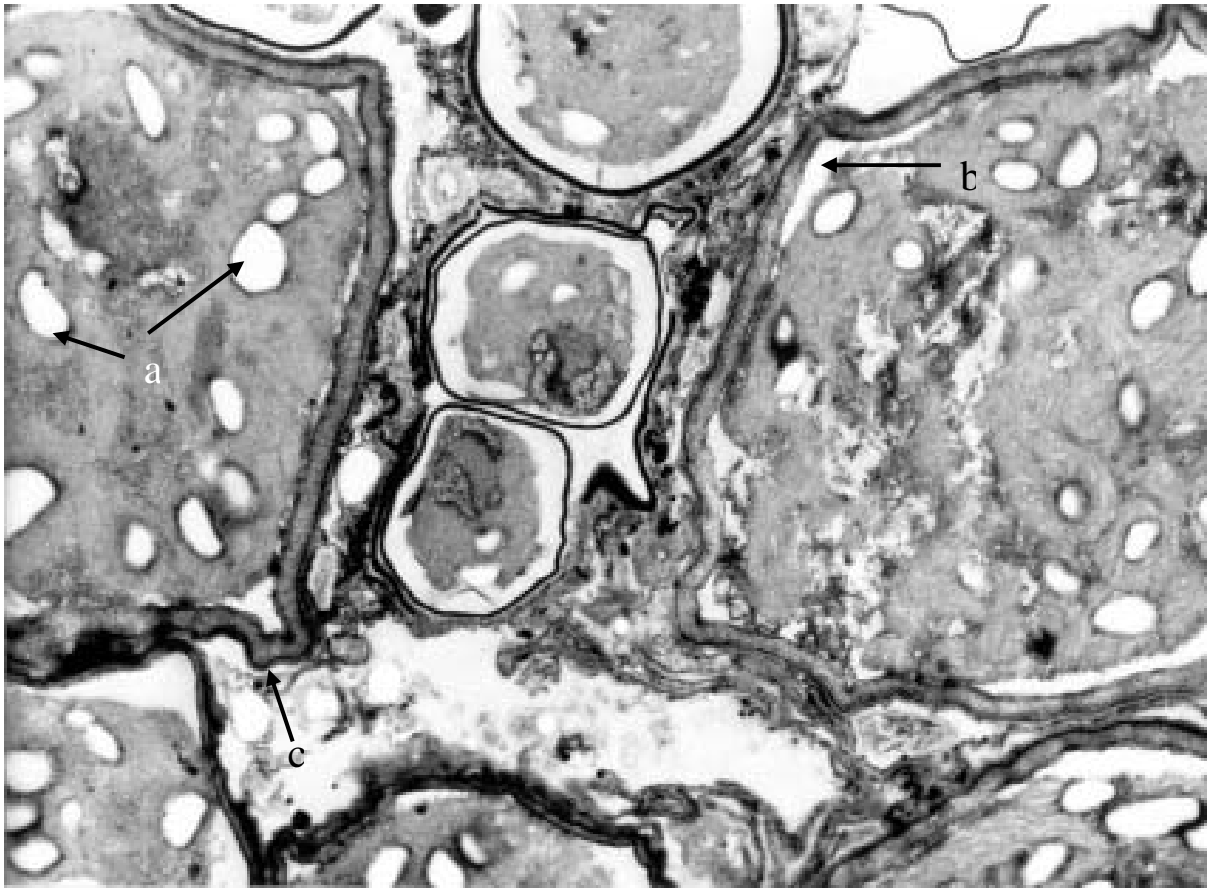


This picture was taken in Institute for Research on Zoo- and Wild Animals, Berlin, Germany, 2003

a – multiple vacuoles (chloroplast, lipids) in the algal cell

b – perforation of cell wall and cell membrane

Figure 25. *Chlorella vulgaris* cell after spray-drying (electron microscopy; 10,600 – fold)



This picture was taken in Institute for Research on Zoo- and Wild Animals, Berlin, Germany, 2003

a – multiple vacuoles (chloroplast, lipids) in the micro-algal cell

b – diffusion of cytoplasm from the cell membrane

c – cell wall and cell membrane well seen, well bound together, without any visible perforations

One of the parameters used to evaluate the digestibility of protein is the protein efficiency ratio (PER), where protein effect on daily weight gain is evaluated. However, this parameter has several weak points. Daily weight gain is assumed over a period of time – the longer the period is, the greater the difference in the daily weight gain. The animal's weight at beginning of the feeding trial is very important as the individual growing curve changes in shape with time. This parameter does not differentiate between protein and fat accumulation in growing rats. Moreover, PER depends on the genetic background of animal being studied – casein had different PERs when fed as the sole protein source to different strains of rats (Lubitz, 1963). In our studies we have confirmed this in the feeding trial over a longer period, where PER of casein differed between weeks of of the study. Starting from day 5 of study (after adaptation period), in first week the PER for casein was equal to 2.1, in the second – 2.9, in the third week reached value of 3.7 and in the fourth week decreased to 3.4. The same pattern was observed for PER of US-DA, the maximal value of 1.8 was achieved in the third week of feeding the micro-algae as sole protein source, when the animals gained beteween 100 g and 109 g. In Experiment 1 older animals were used, and the values of PER obtained for *C. vulgaris* were about 1.40 for S-DA, 0.98 for ES-DA and 2.08 for US-DA.

Comparison of PER for *C. vulgaris* with values cited in literature is very problematic, because of reasons listed above. Moreover, there is very little data considering pure *C. vulgaris*, more authors investigated different micro-algae belonging either to *Chlorella* sp. or other genera. Generally our results are in agreement with others' findings. The PER for *C. vulgaris* reported by Saleh et al. (1985) and determined in N-balance study on 21 day old Sprague Dawley rats was 2.00. Erchul and Isenberg (1968) reported PER for dried *Chlorella sp*., as determined in modified N-balance study on 21 day old Sprague Dawley rats, to be 1.54 – 1.68 or 0.85 (when the micro-algae were heated before drying). Pabst (1974) reported PER for micro-algae to be 2.5 – 3.2, depending on micro-algal species (*Scenedesmus*, *Coelastrum*, *Uronema*).

This is the first time that ultrasonication and electroporation were used prior to spray-drying of the micro-algae in order to enhance the nutritional value of micro-algal protein. In previous studies several other techniques were investigated and only a couple of them was reported to be successful. Cooking micro-algae (*Scenedesmus* with *Chlorella* in ratio 10:1) for 30 minutes, as determined in N-balance study on rats, resulted in a true protein digestibility of 73 % and PER of 1.5 (Cook, 1962). Treating *Scenedesmus obliquus* with boiling water

resulted in apparent protein digestibility of 36.7 % and true protein digestibility of 52.9 %, what exceeded digestibility of untreated micro-algae and *S. obliquus* heated with steam (Bock & Wuensche, 1967). It is noteworthy to mention that the apparent and true digestibility of crude protein of dried *C. vulgaris* obtained by the last mentioned authors was 44.1 % and 59.3 %, respectively, being almost twice as high as for dried *S. obliquus* (26.1 % and 32.7 %, respectively). In contrary, high pressure homogenization during spray-drying reduced the apparent protein digestibility of *C. vulgaris* when compared to only spray-dried micro-algae (Komaki et al., 1998), a similar reduction was seen after 2 hours of boiling the algal mixture (Cook, 1962).

Summarizing, the novel procedure of micro-algal treatment based on ultrasonication followed by standard spray-drying, as determined in N-balance studies on growing rats, was successful and increased the nutritional value of the micro-algal protein when compared to spray-drying alone. On the contrary, electroporation applied before spray-drying leads to decreasing of the nutritional value of micro-algal protein, probably due to the influence of the high temperatures produced during the procedure. The ultrasonication technique could therefore be introduced into the technological process of the micro-algae, but questions of economics still remain, and a solution must be found by the industry.

Another aspect of the use of *C. vulgaris* as a protein source, or as a co-component, improving the quality of foodstuffs, with regard to its physiological properties mentioned in the introduction. Lin Y. (1969) reported that *Chlorella* supplementation of soybean milk improves the quality of the food, stimulating mice weight gain. Also a supplementation of chick mash with 50% *C. vulgaris* stimulated broilers weight gain (Rangachar, 1973). Yap et al. (1982) in trials in early weaned pigs stated that at least one half of the protein supplied by soybean meal (one-third of the dietary protein) can be replaced by the micro-algae, with no influence on growth performance or the health status. Kotrbacek et al. (1994) reported that supplementation of chick mash with 0.5% *C. vulgaris* fed to broilers from the start of their life increased significantly the weight of broilers after 14 days of life but starting from the 21st day, the weight of broilers fed commercial mash and supplemented mash did not differ (till the age of 56 days). Existing data is therefore not enough and there are more large-scale studies needed, including both mammals and poultry as host organisms, to establish the most suitable level of the algae in diet resulting in the best animal growth, production and health performance.

5.2. Influence of C. vulgaris on animals physiological parameters

5.2.1. Metabolic and hematological parameters

In order to investigate the influence of the green micro-algae *C. vulgaris* on the metabolic parameters of rats, we conducted two experiments (Experiment 2 and 3), where adult male Wistar rats (initial weight app. 200 g BW) were fed diet consisted in 20% of the micro-algae and 80% of commercial feed (Experiment 2) and young growing male Wistar rats (initial weight app. 75 g BW) were fed the micro-algae as sole protein source (20% of *C. vulgaris* in diet) (Experiment 3). In both experiment animals were fed the algal diets for longer period of time (28 and 33 days, respectively) and the metabolic parameters were determined in blood and urine.

Influence of feeding the micro-algae on hematological parameters was investigated on growing rats (Experiment 3) and in multi-generation study on pregnant mice. The rats were fed 20% of micro-algae in diet, whereas mice were fed chows supplemented with only 1% of the micro-algae.

5.2.1.1. Uric acid and allantoin

It is known from the literature, that micro-algae contain relative high level of nucleic acids, about 4% of dry matter (Narasimha et al., 1982; Saleh et al., 1985), and therefore a surprising result of our analyzes was finding of only 1.5% of nucleic acids in algal dry matter. This could have been a result of the method applied for nucleic acids extraction in our laboratory that probably did not let fully open the algal cells and release the nucleic acids from the cell.

Intestinal phosphorylases degrade the nucleic acids present in feed to pyrimidyne and purine bases, which are then either absorbed or used by the gut microbiota. After absorption, the bases are further metabolized in liver and pirymidines are catabolised to ammonia and carbon dioxide and in part to acetic or succinic acids (which can be further utilized in Krebs cycle). Purines are further metabolized in liver and kidneys to uric acid which is then oxidized to allantoin by uricase in liver (Chin et al., 1980). The metabolic pathway of purines catabolism in human beings is shorter and finishes on uric acid, as humans lost the uricase in the evolutionary process. Only carnivores and omnivores (scavenger animals) possess this enzyme, and so in rat most of uric acid is metabolized to allantoin which is excreted then with

urine (Pak et al., 1973). Pak et al. (1973) in their experiment on rats of different age fed different protein type and concentration found the excretion of allantoin was independent of the age of animals and of the type of protein consumed. It depended on the weight and weight gain and so indirectly on the protein concentration in diet. Determined daily allantoin excretion in urine was 6 – 19 mg/day for rats 45 – 75 g BW and 22 – 39 mg/day for rats 229 – 275 g BW. For determination of allantoin concentration in urine the method of Young & Conway (1942) was used.

Saleh et al. (1985) determined the uric acid concentration in serum after feeding rats with *C. vulgaris* (in amount of 10 % of the diet) increased to 3.5 mg/100 (equal to 208.25 μmol/L) ml in compare to casein group (2.75 mg/100 mL). Schneegurt et al. (1993) reported total urinary uric acid and allantoin excretion in rats fed diet with 5 % cyanobacterial biomass to be 1.4 mg/day, what was comparable with casein group (1.3 mg/day).

In our studies on rats there was no difference in serum uric acid concentrations between experimental and control animals. When rats were fed 20 % micro-algae mixed with commercial chows, the serum uric acid concentration fluctuated in time but did not significantly exceed the initial value of 201 ± 100 μmol/L and corresponded to values measured be Saleh et al. (1985). When rats were fed US-DA as sole protein source for 33 days, the serum uric acid level was about 125 μmol/L. The values did not differ between algal and control group, in both groups the remained on comparable levels, and the values were comparable with literature findings (Heaf & Davies, 1976). At the beginning of the experiment the initial serum uric acid concentration was determined to be 60 μmol/L in algal and 90 μmol/L in casein group. The reason for so low determined level is most probably due to analytical procedure.

Serum allantoin concentration did not differ between groups when rats were fed algal or casein protein as sole protein source and was in range of 2 – 4 mmol/L. Only fluctuations combined with sampling time were observed resulted from individual fluctuations and analytical processing. The values of serum uric acid and allantoin concentration obtained in our study were comparable to findings of Koguchi et al. (2002; 2003).

Daily production of uric acid and allantoin measured in urine increased in rats fed algae as sole protein source. This increase (uric acid up to 8 μmol/day, allantoin up to 500 μmol/day) was temporary and lasted for about first two weeks. The difference between determined daily excretions in both groups was of 2 – 3 μmol/day for uric acid and 200 μmol/day for allantoin. After this time the amounts of the metabolites excreted in urine in algal group started to decrease and achieved level more or less equal to the amount of these

metabolites produced by rats from control group. All measured values were comparable with findings of other researchers that applied the same analytical methods of allantoin determination (Koguchi et al., 2002; 2003). Pak et al. (1973) determined daily allantoin excretion in rats of this age to be at level of 140 – 250 μmol/day. Differences may be due to allantoin determination method – the method of Young & Conway (1942) used by these authors is very sensitive for time and temperature of heating and reading of the chromophore (Borchers, 1977).

Feeding large amount of nucleic acids may stimulate the enzymatic system at the very beginning, and after a while there might be a stop signal and the purines are not further metabolized in excess, or – on the other hand, microorganisms of the gastrointestinal tract may totally degrade the nucleic acids yet in the gut, before they are even absorbed. Several studies on the nucleic acids degradation by bacteria were done and it was stated, part of dietary purines is completely catabolized in the gut of rodents as rats or mice and the fermentation process is being done in the hind gut (Greife, 1986). As Koguchi et al. (2002; 2003) showed dietary fiber influences RNA-derivatives absorption, where the electric charge in different pH or viscosity play crucial role. One can not exclude, that at least partially, the dietary fiber present in algal cells (first of all in the cell walls) may decrease amount of nucleic acids available for gut enzymes and further absorption, thus reducing uric acid and allantoin production and final excretion in urine.

5.2.1.2. Serum enzymes activities and serum total protein

Increase of activities of enzymes in serum is a result of damages of different organs' cells. Increase of serum activities of aminotransferases is most specific for liver damage. Alanine aminotransferase (ALAT) is located in cell in cytoplasm and so its activity in serum will increase even after short-time cell wall damage. ALAT is also present in cells of many organs as heart, kidney or muscles so its increase will also follow increasing of cell membrane permeability of other organs. Aspartate aminotranferase (ASAT) is located partially in cytoplasm and is mostly associated with mitochondrial membranes, so the activity of this enzyme will increase after long-term and deep cell damage (like necrosis, inflammation). This enzyme is present in substantial concentrations in a wide variety of tissues; the highest concentrations are found in heart, liver, skeletal muscles, brain, and plasma.

Glutamate dehydrogenase (GLDH) is present almost only in mitochondria of central hepatocytes in the liver. Elevation of this enzyme in serum will be seen after major damage of liver cells and in changes in central lobular regions, like in acute blood stasis.

Alkaline phosphatase (ALP) is bound with cell membranes and has three main isoenzymes: liver, bone and intestinal. Its activity in serum will increase after long-term damage to one of these organs, especially after damage of hepatobiliary tract and gut mucosa. Total serum protein reflects the overall protein balance and serves for measurement of liver function without differential evaluation of albumin and globulin components. Its concentration will change in many clinical cases, also depends on hydration status, exercise or "pumping" during blood collection. Drop in serum proteins is seen first of all in chronic liver diseases or malnutrition.

Activities of serum enzymes measured in experiment 2 when adult rats were fed 20% micro-algae in feed and in experiment 3, when young growing rats were fed casein or micro-algae as sole protein source, did practically not differ between groups. What we could observe were small changes of the enzymes' activities in time within groups, what is a physiological situation.

Only in case of alkaline phosphatase some changes were observed. In experiment 2 the determined serum ALP activity increased after 7 days of feeding with the micro-algae to 410 U/L in compare to initial activity of 198 U/L, but thereafter it decreased to initial level. We cannot explain reason for this fluctuation; we consider it as analytical error.

Feeding micro-algae as sole protein led to decrease of the serum ALP activity when compared to the casein group starting from day 12 of experiment, whereas the ALP serum activity remained practically unchanged till the end of experiment. Because of the experimental needs we could not measure the serum enzymes' activities before starting with algal feeding, so we have no comparison to initial activity of ALP in serum of rats used in our study. The lower activity of ALP measured in serum of rats fed algal diet might have resulted from slower growth and thus reduced activity of osteocytes that produce one of isoenzymes of ALP.

Total serum protein was in both groups comparable. We considered the data obtained in the casein group in face of no clinical and histological disturbances as normal physiological ranges, and so all values measured for algae group were in normal physiological ranges. High activity of ASAT measured in rats' serum in both groups might have been caused by heart muscle injury during blood collection and partially due to methods used for estimation.

Generally, there was no observed influence of the micro-algal components on the liver metabolism of rats determined on basis of serum enzymes activities and serum protein level. The decrease of ALP's activity in serum of growing rats fed the green micro-algae is an interesting result, what should be further investigated, as for now we have no explanation for this phenomenon. *Chlorella vulgaris* reduces the lipids peroxidation in cells via increased levels of GST-SH and reduced activity of cytochrome b5 and P450 (Singh et al., 1998; 1999) what could partially explain the reduction of ALP activity in serum, as when less membrane lipids are oxidized, the membranes are more stable and the enzyme remains in the cell.

5.2.1.3. Creatinine and urea

Creatinine is the end product of creatine (which as phosphocreatin stores the energy in muscle cells) being excreted with urine. Urea is the end product of hepatic ammonia detoxification and is eliminated from the organism in about 75% with urine. In general, creatinine and urea concentrations in serum are used for screening of excretory renal functions and their elevation in serum is found as the first sign of renal insufficiency or failure (of any reason).

Our results show no difference between groups, only small fluctuations in concentrations measured on different time points. Serum creatinine level determined in adult rats fed 20% micro-algae was 63 – 78 μmol/L, in young growing rats was 46 - 74 μmol/L in algal and 42 – 84 μmol/L in casein group These values are physiological and well comparable with data found in literature (Yokozawa et al., 1982). Serum urea concentration measured in serum of adult rats was 7 – 9 mmol/L throughout the study, whereas in young rats it was 1.5 mmol/L in casein and 2.3 in algal group. The difference could be age dependent but there could also be laboratory influence on determined levels. Nevertheless, these values are physiological and comparable with literature findings (Yokozawa et al., 1982).

Summarizing, there was observed no negative influence of feeding micro-algae to adult and young rats on renal function as determined on biochemical basis.

5.2.1.4. Hematological parameters

Blood hematology describes in general the hemopoietic function of animal's organism, gives indirect clues for Fe storages and capability of vitamin B_{12} synthesis (which is the basic part of hem in hemoglobin). Hematocrit gives clues for the hydration status of

animal and presence of anemia. Red blood cells count together with hemoglobin level, mean corpuscular volume (erythrocyte volume), mean corpuscular hemoglobin (amount of Hb in red blood cell) and mean corpuscular hemoglobin concentration (concentration of Hb in red blood cell) give clues for anemic status and their background (i.e. low Fe stores). Thrombocytes (platelets) count can help to identify bone marrow failure, infections of endothelium or presence of autoimmune or cancer disease. White blood cells count reflects the immune system status – reaction to infections, but also immune system reaction to physiologic states like stress or pregnancy.

In our experiments we did not see major changes of hematological parameters between rats fed the micro-algae and controls. The only observed changes were related to blood hemoglobin level and platelet count. Young growing rats fed US-DA as sole protein source had significantly more thrombocytes (1672 x 10^3/μL) and less hemoglobin (10.7 mg/100 mL) in blood than rats fed casein (1144 x 10^3/μl and 11.7 mg/100 mL, respectively). MCV in respective groups was 55.8 x 10^{-15}L and 60.8 x 10^{-15}L, but the difference was not significant. These values can be a sign of iron deficit, but on the other hand, the number of erythrocytes did not decrease and was the same in both groups and there was no clinical sign of anemia. Thrombocytes count depends also on the sampling technique, as micro clots can be generated already in the needle, what further results in underestimation of TBC. MCV was calculated on basis of Ht and RBC and so it depends on the determined values. Automatic determination of erythrocyte volume gives exact results but this technique was not available. This could partially explain the obtained differences.

Nevertheless, these results can at least in part be due to sub-clinic development of Fe deficiency, what would be in disagreement with literature findings. Kapoor & Mehta (1993b) investigated the influence of feeding *Spirulina* as 6 % supplementation to casein diet (casein was fed in amount of 15.4 % of diet), where these were the only protein and iron sources, on iron status of growing rats. These authors reported very good development of iron stores in liver, kidneys and hearth that were comparable when rats were fed ferrous sulfate as iron source. In spleen the iron concentration was even two fold higher in compare to the other group. We had no possibility to determine the tissue iron concentrations, so we cannot directly compare the results. But, the micro-algae used in our experiments contained about 172 mg/100g in fresh matter, what gave a daily intake of approximately 4.0 mg Fe from micro-algae/day/rat. Rats received additively mineral and vitamin mixture containing all minerals and vitamins necessary for proper rat's growth, so there could be no dietary Fe insufficiency. Also Matsuura et al. (1991) reported very good influence of feed

supplementation with 5% *Chlorella* on recovery from dietary-induced iron deficiency anemia in rats. Rats fed for 30 days this amount of the micro-algae in feed fully recovered and serum Hb level in these rats achieved 15.6 g/100 mL, Ht – 49 % and RBC – 9.0 x 10^6/µl. For comparison, rats with the dietary-induced iron deficiency anemia had Hb = 5.7 g/100 mL; Ht = 20 % and RBC = 2.9 10^6/µl.

The differences observed in our results are therefore probably, at least in part, due to methodological and laboratory errors. To fully understand what happened, further studies would be needed with feeding different amounts of micro-algae to growing rats and determining Fe storages in liver and spleen together with blood and serum parameters.

5.2.1.4.1. Iron status of pregnant mice

In contrary to these findings, in the experiment 4, when pregnant mice fed feed supplemented in 1.0% of spray-dried *Chlorella vulgaris,* we recorded in their blood less platelet cells with no disturbances in hemoglobin concentration in compare to control animals. Serum Fe concentration was slightly higher in mice fed the micro-algae (mean 19 – 21 µmol/L in F_1 and 22 – 26 µmol/L in F_2) than in control group (mean 14 – 26 µmol/L in F_1 and 18 – 19 µmol/L in F_2). The Fe-binding capacity was lower about 10 µmol/L in serum of mice from algal group in both generations. The Fe-binding capacity is test for measurement of the capability of transferrin to bind iron molecules. Normally 30% of the binding sites are bound with Fe and the higher the iron binding capacity the less iron was bound with the protein, thus anemia from iron insufficiency is seen. Decrease of the serum iron binding capacity is in opposite caused by higher transferrin saturation, what was observed in our study.

More iron release from storages may be due to increased demand of faster developing fetuses in the micro-algal group. Similar results found also Kapoor & Mehta (1998) after feeding pregnant rats with *Spirulina*. But the same authors stated serum iron concentration and total iron binding capacity were not reliable parameters for investigating the hematological responses and changes in iron stores, as they do not react on the variations during pregnancy. According to these authors, it is better to use hemoglobin concentration, hematocrite and serum ferritin concentration for these purposes. Unfortunately we were not able to measure the latter one. We did neither measure changes during the pregnancy, only the hematological status in the final stage of pregnancy.

Liver and spleen are the biggest storages of iron and their weight depends on and reflects the animal iron status (Kapoor & Mehta, 1993b). In our experiment, liver and spleen weights did not differ between groups. Therefore, what we have observed in blood could be a temporary effect of enhanced demands of developing fetuses. Stored Fe was pushed out from liver and spleen into serum leading to increase of total Fe concentration and to saturate of the transferring thus reduced iron binding capacity was determined.

Summarizing, there was no negative influence of supplementation with 1.0% of spray-dried *Chlorella vulgaris* on iron status in pregnant mice as determined at the end stage of the pregnancy. Moreover, all demands of growing fetuses were fully covered and no sign of anemia could be observed. It would be of interest to investigate the changes in iron tissue storage throughout the whole pregnancy in conditions of limited dietary iron intake, when *Chlorella vulgaris* would be offered as supplement, whereas different dosages would be applied. It could be of practical use supplementation of food with green micro-algae in regions where dietary iron sources are not much enough to cover all demands of pregnant organism.

5.2.1.5. Internal organs

In feeding studies in Experiment 3 and 4 influence of feeding the animals on *Chlorella vulgaris* in different amount (sole protein source or 1.0% supplement) was additively investigated. Rats fed the micro-algae as sole protein source developed slower than rats fed casein as sole protein source; therefore almost all internal organs were smaller when absolute weights were compared. But the ratio of internal organs to metabolic weight ($BW^{0.75}$) shows that small and large intestine were heavier in algal group in compare to casein group. These parts of gastrointestinal tract (GIT) were also longer. In case of stomach an opposite finding was observed. This suggests the intestinal tract must have adapted to lower digestibility of the feed or there is a component present in micro-algal cells that stimulated the intestinal growth. This would be in agreement with literature findings, where extract of *C. vulgaris* stimulated growth of bone marrow or spleen cells (Justo et al., 2001; Konishi et al., 1990; Tanaka et al., 1986) and a proposal glycoprotein has already been isolated (Noda et al., 1996). As for stomach, the fact that this part of GIT was longer and heavier in rats from casein group was due to larger feed intake in the last 10 days of experiment. Histological investigation did reveal no visible differences between groups, whereas only general situation was considered.

Unfortunately, the cryopreservation of intestinal samples did not allow to get enough straight intestinal villous for height measurements.

Livers of rats fed micro-algae were smaller, what was probably due to less lipid infiltration of these organs in those animals, what was observed by Okuda et al. (1975) and Sano & Tanaka (1987). In histological evaluation of liver slices no lipid infiltration was seen in livers from rats from casein group, and was neither observed in livers from rats fed the micro-algae, so findings of these authors could have not been confirmed and lipid contents were not responsible for the lower weight of the livers. The spleens were heavier in rats from algal group, what could be a result of increased iron storage (already discussed above) or increased hematopoiesis (Konishi et al., 1985; 1990; Tanaka et al., 1984; 1986). Absolute hearth weights were lower in rats from algal group, but with respect to metabolic BW they were significantly heavier. This was most probably accidental effect of calculations.

No changes between groups for weights of kidneys or testicles with respect to metabolic BW were noticed. The differences between absolute weights of kidneys were most probably due to presence of renal fat that not always could be fully removed before weighing of the organ. No histological changes in renal structure were observed.

Our results are somehow in opposite to findings of other authors. Venkataraman et al. (1979) reported the relative weights of rats' organs (with respect to 100 g BW) after 12 weeks of feeding with 24 % or 36 % of micro-algae *Scenedesmus acutus* were lower than in group fed casein (12 % of diet). These results were because the rats from algal groups gained more weight as the rats from casein group over the experimental period, which was also 3 times longer as in our study. Gastrointestinal tract was not investigated by these authors, so the comparison of GIT results is impossible.

In the experiment on mice, when only 1.0% of the micro-algae were fed as supplement in commercial rodent diet, no differences between absolute weights of organs were observed. The organs' weights were not respected to metabolic body weight, as the mice were pregnant. We consider the single significance for stomach weight as accidental, taking into consideration fact that by so many animals the draining of the organ cannot always be complete and that different technical assistants helped by the procedure. Also the difference of stomach lengths may be due to incorrect measurements. These conclusions are supported by fact that these were the only differences we could observe.

No adverse effect on internal organs could be seen after long time feeding rats and mice with *Chlorella vulgaris*. When micro-algae were fed as sole source of protein stimulation of intestinal growth and spleens growth were observed in compare to rats fed

casein as sole protein source. These effects could be result of influence of the micro-algae on cytokines production and cells growth. Livers from rats fed the micro-algae were smaller and were less infiltrated with lipids, what was most probably result of slower growth and lowering of triglycerides levels by *Chlorella*.

5.2.2. Effect of Chlorella vulgaris on reproduction

Ishibashi (1971b) found that feeding female rats with *Chlorella* led to more cases of continuous estrus, and so he hypothesized presence of estrogenic substances in the algae. Kapoor & Mehta (1993a) observed an increase in the number of mice pups after feeding the dams with *Spirulina* enriched feed. It is worth noting that both authors fed the algae as the only protein source, whereas we used the algae as only 1.0% of the feed. Fevrier et al. (1975) fed pigs with feed containing 5.0% of other algae, *Spirulina maxima*. In this case, the number of piglets delivered in the first reproduction cycle was higher in the algae group than in the control group, however, the number of piglets delivered by the same sows in next cycle decreased. Similarly, Pabst et al. (1978) found that when mice were fed for 6 generations with feed containing *Scenedesmus acutus,* comprising 20.0% of the diet, the number of mice pups decreased with generations, with accompanying weight-increase.

In studies on laying hens, the introduction of *Chlorella* into the diet at 10.0% led to laying of more eggs, which were also bigger and had a more intensive yolk, which was then also richer in vitamins and pigments (carotene and xanthophylls) (Arakawa et al., 1960). Lipstein et al. (1980) fed laying hens with feed containing algae meal (mainly *Chlorella*) and noticed no differences, despite more intensive yellow color of the yolk. Experiments done in the Institute for Cereal Processing Ltd. (IGV, Bergholz-Rehbruecke, Germany) (Anonym 1) showed an increase in the number of eggs laid by hens fed feed enriched in 1.0% of *Spirulina platensis*. The eggs were also bigger and of better quality than those from control hens.

In our experiment, where the mice were fed commercial chows enriched in 1.0% *Chlorella vulgaris* powder, we did not notice any difference in number of fetuses or pups between experimental and control groups. The only observed difference was that the fetuses and pups from mice fed the micro-algae grew slightly better, both to weaning age and the end of the experiment, when compared to the control animals. This difference was not significant when all littermates were taken into the consideration, however, random assortment of two males and two females did show a significant difference in the weights of the pups. This is of interest, as it shows, that *Chlorella* may stimulate the pre- and postnatal growth of mice. The

feed intake could have not been measured in this experiment, but the data obtained in parallel experiment on growing mice fed the same feeds (Experiment 5) did not show any difference in daily feed intake between groups. We therefore conclude the effect observed in the mice development to be caused by algal supplementation.

The results of the experiments done worldwide up to date are equivocal. Various authors have used different species of micro-algae, in varying amounts. It is therefore not easy to compare our results with the literature data. It seems that algae may have more positive effects on bird reproduction than on mammalian reproduction. It is possible that the amount of algae used in this study (1.0 %) was insufficient to cause measurable changes in mouse physiology whereas higher levels may be efficacious. It has been noticed that increased concentrations of amino acids, particularly lysine and threonine, have positive influence on the number of rats' pups (Jansen et al., 1977). The supplementation of the feed with 1.0% *C. vulgaris* did not change the composition of amino acids. The commercial feed used in this experiment may meet all the nutritional demands required for optimal reproduction of mice; this may have masked any positive effect of the micro-algae on reproduction. On the other hand, the spray-drying used for destroying of algal cell could have not been sufficient enough to let the algal cell ingredients be useful for the host.

In summary we have observed that females fed a *C. vulgaris*-supplemented diet grew a slightly better than females given control feed, although this was not statistically significant. This observation is in agreement with some previous experiments (Ishibashi, 1971b). In agreement with Pabst et al. (1978), we observed a slightly better growth of the pups from mice fed *C. vulgaris*, this suggests that the algal components stimulating the offspring's growth are present in the milk. It is in agreement with Singh et al. (1998), who found existence of perinatal influence of *C. vulgaris* on hepatic metabolizing enzymes and lipid peroxidation in studies on mice.

With respect to live-born pup numbers, we have found that there are fewer littermates lost in the algae-supplemented group compared to the control group. This difference is not significant, but the positive trend on reducing pre-weaning mortality rate should be noted. Although a small effect, if this response could be replicated in food animals, e.g. swine, there could be great implications for production costs. Further studies are needed to prove this hypothesis.

The aim of this study was to investigate, if supplementation with algal powder in amount of 1.0% would increase the reproduction rate of mice and influence the mice development. According to previous studies discussed above, such action could be expected. The only influence we could observe was a positive, but not statistically significant, effect on the growth rate. We believe there are more experiments needed to fully investigate the green micro-algae. The important factors that have to be taken under the consideration are the dosage of algae in the feed, investigated species and number of animals, the methods used for algal cell destroying and also costs of supplementation. There is still an open question, if the algal influence on reproduction is not limited only to birds and if not, would the supplementation for other animals be economically practical.

5.2.3. Influence of micro-algal supplementation on nutrients' digestibility and utilization on mice

The supplementation of commercial rodent chows with 1.0 % spray dried *Chlorella vulgaris* did not change the composition of the feed. Daily feed intake was not affected and daily nitrogen intake was higher in algal group but it was caused by higher nitrogen excretion with urine and feces, so that as result the nitrogen balance was lower in this group. Nevertheless, daily weight gain was slightly higher in the algal group, and so the apparent and true crude protein digestibility of the feed was also higher in this group. The actual value of protein shown as net protein utilization was better for feed without algal supplementation. This is in agreement with our previous study (Experiment 1). Also biological value of protein decreased when feed was supplemented in 1.0 % of *C. vulgaris*. Apparent digestibility of other crude components was not affected by the algal supplementation. Koehler & Kallweit (2000) reported no differences between feeding piglets on feed with 1.0% or without *C. vulgaris*, what confirms our observations. But when rabbits were fed 3.0 % of *C. vulgaris* in diet, obtained feed efficiency ratio was 5-times higher and daily weight gain was 27% better than for animals from control group (Anonym 1).

Supplementation of mice diet with 1.0% micro-algae had no influence on the palatability of the feed, so there is no contraindication of supplementation of feed with the green micro-algae. It decreased the protein value of the feed, what in case of farm animals can lead to increase of production costs. This is still of question, what level of *C. vulgaris* would be the most efficient when fed to farm animals and more studies both on model laboratory and on farm animals are needed to solve this problem.