



The Geomagnetic Field Affect the Manifestation of Activity of Synthetic Biologically Active Substances

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ABSTRACT

We compared the influence of aqueous solutions of plant growth regulator (PGR) melaphen (MF), made under normal conditions and in conditions of low level electromagnetic field of the Earth (in permalloy container) on the functional state of 6-day-old pea seedlings mitochondria. In a concentration range from 2×10^{-5} to 2×10^{-9} M melaphen that were cooked in various ways, had the same effects to the maximum rates of oxidation of NAD-dependent substrates and the fluorescence intensity of peroxidation products which were caused by the "aging" of mitochondria. However, in the concentration range of 2×10^{-10} M and below MF solutions, which were prepared in normal conditions and in conditions of low intensity of electromagnetic fields have a different impact on the rates of oxidation of NAD-dependent substrates and the fluorescence intensity of lipid peroxidation products. It can be assumed that the biological effects of the drug are associated with the formation of a nanoassociate for the occurrence of which requires the presence of electromagnetic fields. However, after the processing of pea seeds 2×10^{-12} M melaphen prepared in various ways, observed identical protective effect for pea seedlings that are in conditions of insufficient watering and moderate cooling. It is assumed that the processing of seeds outside of the container (on the table) leads to the formation of nanoassociates.

Keywords

Ultra-low doses; biologically active substances; mitochondria; nanoassociates; plant growth regulators; atomic force microscopy.

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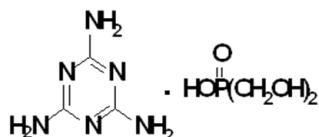
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1. INTRODUCTION

Many natural and synthetic biologically active substances (BAS) exhibit their activity in a range of low (10^{-10} - 10^{-4} M) and ultra-low concentrations (10^{-20} - 10^{-11} M). The level of biological organization, at which the effect of ultra-low doses (ULD) is observed is very diverse: from macromolecules, cells, organs and tissues to animal and plant organisms and even populations [1-3]. It does not follow from the aforesaid that the effect was observed at ultra-low doses of any one of biologically active substances on any one of biological objects. The observable effect at the substance concentrations 10^{-13} - 10^{-17} M and lower can not be attributed to any definite structure or a level of biological organization [4]. The effects of ultra-low doses of biologically active substances have common characteristics that do not depend on the substance nature. These characteristics manifest themselves most visibly in studies on dose dependences. In some cases, the dependence is bimodal: the effect increases at ultra-low doses of a preparation, then, the effect decreases, as the dose is increased and is succeeded by a "dead zone" and increases again. As an example, consider the concentrational dependence of the effect of α -tocopherol on the activity of protein kinase C. Tocopherol inhibits the activity of the enzyme in intervals 10^{-16} - 10^{-12} and 10^{-4} - 10^{-5} M, while in between these concentrations almost no effect on the enzyme activity. Sometimes, the dose dependence has a stage of "a change of sign". For example, if an inhibiting activity was observed in the region of ultra-low doses, it changed for a stimulating one as the concentration was inhibited, and then again an increasing effect was observed: isobruphen 10^{-11} - 10^{-14} M concentrations increases the activity of the enzyme of prostaglandin synthetase, but at a concentration of 10^{-6} M significantly reduces its [5]. There are known cases when the effect did not depend on a dose within a wide concentration range. In particular, in one of the work, wherein the effect of a herbicide of the class of hydro peroxides on a plant cell culture was studied, it was discovered that the preparation has an equal effect at doses that are six orders different (10^{-13} - 10^{-7} M) and the effect is absent in the range of intermediate concentrations [4]. The nature of the dependences may be accounted for by the fact that BAS ultra-low doses have common targets. These targets may be cell and sub cellular membranes that play a key role in the cell metabolism [6].

There exist a great number of hypotheses as to the mechanisms of the effect of ultra-low concentrations of BAS in the literature. Using methods of dynamic light scattering and scanning electron microscopy, S. Samaland, K. Geckeler discovered in aqueous solutions of various substances (in concentration range 10^{-4} - 10^{-2} M) structures ranging in size from 200 to 5 thousand nm [7]. Further studies showed that these structures (clusters) compose of solute and water. They are formed as a result of aggregation, depending on the temperature, time and nature of the solute. It was later proven that these patterns are not "nanoparticles" gases present in the solution [8]. Using the complex physico-chemical methods (dynamic light scattering, conductivity, tenzometric pH-metry) investigated the structures formed in aqueous solutions of biologically active substances (BAS) in a concentration range of 10^{-20} - 10^{-6} M. It was found that solutions of the substances studied in low and very low concentrations are disperse systems comprising the nanoscale (100-300 nm) particles (nanoassotsiates) having an electrical surface charge. Changes in the concentration of dissolved substances leads to the nonlinear changes in the properties of the system, which affects the size, an electrokinetic potential (ζ -potential) nanoassotsiates, as well as the physico-chemical properties of the solutions, such as electrical conductivity χ , surface tension σ , pH. These systems retain stability i.e. a certain monomodal nanoassociates size distribution for several days depending on the nature of the solute and its concentration [9]. Concentration dependences for a sizes and electrokinetic potential (ζ -potential) of nanoassociates formed from solutions of BAS at low and ultra-low concentrations are comparable with the biological effects of the preparations as published previously [10]. This suggested that the biological effects of bioactive substances in low and ultra low concentrations were associated with the formation and the restricting nanoassotsiates [11]. Due to the using of permalloy container that shields its contents from electromagnetic fields (the induction of the geomagnetic field is reduced inside the container more than a thousand times), it turned out that in the range 10^{-7} - 10^{-2} M, the formation of nanoassotsiates does not depend on the external fields, but at lower concentrations the formation of nonassociative depends on external fields: in the low-level electromagnetic fields (permalloy container) nonassociates not formed [12-13]. Moreover Palmira NP et al showed that antioxidant solutions of potassium phenozan cooked in vivo have polymodal effect on the microviscosity of lipid components of biological membranes, showing maxima at concentrations of 10^{-6} , 10^{-12} , 10^{-15} M. storage these solutions in the permalloy container lead to the disappearance of biological effects in concentrations 10^{-12} , 10^{-15} M. Based on this it has been suggested that the biological effects associated with the presence in solutions nanoassotsiates. In this context, the aim of our work was a comparative study of the biological effects of solutions with low concentrations of biologically active substances (BAS), in particular of plant growth regulator of Melaphen (melamine salt of bis(hydroxymethyl)phosphinic acid), in natural conditions and with a significant decrease in the level of the external geomagnetic field ((in permalloy container):



Regulators of plant growth and development (PGR) improve plant tolerance to biotic and abiotic stresses, in particular to deficit of water. These biologically active substances must be safe for human health and the environment. Such preparation is melaphen (LD₅₀ -2000 mg/kg for mice; has not been identified and DNA -damaging activity in any of the studied concentrations of melaphen and this drug showed no mutagenicity in the Ames test in experimental variants with and without metabolic activation it) [15]. The solutions of melaphen were prepared by method of successive decimal dilutions from the initial solution 1×10^{-3} M [16].

The growth and development of plants and their resistance to stress factors, primarily dependent on energy metabolism, in this connection the aim of our work was to study the influence of the plant grows regulator melaphen upon the functional



state of mitochondria of 6 day etiolated pea seedlings in natural conditions and with a significant decrease in the level of the external geomagnetic field in conditions of the insufficient watering and moderate cooling.

The choice of this model is related to the fact that plants grow in constantly changing environmental conditions and undergo combined effects of various abiotic and biotic natural factors. In this case, quite often there arise critical situations when in the time of insufficient water availability (after a snowless winter) occur; even a small lowering of temperature (which the usual in spring time of year).

2. Material and methods

2.1. Plant material

The study was carried out on mitochondria isolated from pea seedlings (*P. sativum*), variety Flora-2 obtained in standard conditions and in the conditions of insufficient watering.

2.2. Germination of pea seeds

Pea (*Pisum sativum* L., cv. Flora 2) seeds were washed with soapy water and 0.01% KMnO₄. Control seeds were then soaked in water, experimental seeds – in 10⁻¹² M melaphen (or in 10⁻¹² M melaphen, designed in permalloy container within 24 hours) for 1 hour. Thereafter, seeds were transferred into covered trays on moistened filter paper in darkness for 2 days. After 2 days, half of control (water deficit + moderate cooling) (WD+MC) and melaphen (water deficit + moderate cooling + melaphen) (WD+MC+MF) or (water deficit + moderate cooling + melaphen, designed in permalloy container within 24 hours) (WD+MC+MF+PC) treated seeds were transferred in the open trays on dry filter paper where they were kept at 14 °C for 2 days. After two days of water deficit with the moderate cooling, seeds were transferred into covered trays on wet filter paper, where they were kept over a period two days at temperature of 22°C. Another half of control plants were retained in closed trays on wet filter paper, where they were kept for 6 days at 22°C. On the sixth day, mitochondria were isolated from seedling epicotyls.

2.3. Isolation of mitochondria

Isolation of mitochondria from 6-day-old epicotyl of pea seedlings (*P. sativum*) performed by the method [17] in our modification. The epicotyls having a length of 1.5 to 5 cm (20-25 g) were placed into a homogenizer cup, poured with an isolation medium in a ratio of 1:2, and then were rapidly disintegrated with scissors and homogenized with the aid of a press. The isolation medium comprised: 0.4 M sucrose, 5 mM EDTA, and 20 mM KH₂PO₄ (pH 8.0), 10 mM KCl, 2 mM 1, 4-Dithio-di-theiritol, and 0.1% fatty acids-free (FA-free) BSA. The homogenate was centrifuged at 25000g for 5 min. The precipitate was re-suspended in 8 ml of a rinsing medium comprised: 0.4 M sucrose, 20 mM KH₂PO₄, 0.1% FA-free BSA (pH 7.4) and centrifuged at 3000g for 3 min. The supernatant was centrifuged for 10 min at 11000 g for mitochondria sedimentation. The sediment was re-suspended in 2-3 ml of solution contained: 0.4 M sucrose, 20 mM KH₂PO₄ (pH 7.4), 0.1 % FA-free BSA and mitochondria were precipitated by centrifugation at 11000 g for 10 min.

2.4. Rate of mitochondria respiration

Respiration in mitochondria we recorded polarographically (an LP-7 polarograph, Czech Republic) using Clarke oxygen electrode. Pea sprout mitochondria were incubated in a medium containing: 0.4 M sucrose, 20 mM HEPES-Tris buffer (pH 7.2), 5 mM KH₂PO₄, 4 mM MgCl₂ and 0.1% BSA.

2.5. The Level of Lipid Peroxidation

The level of lipid peroxidation (LPO) was evaluated by the fluorescence method [18]. Lipids were extracted by the mixture of chloroform and methanol (2:1). Lipids of mitochondrial membranes (3--5 mg of protein) were extracted in the glass homogenizer for 1 min at 10°C. Thereafter, equal volume of distilled water was added to the homogenate, and after rapid mixing the homogenate was transferred into 12-mL centrifuge tubes. Samples were centrifuged at 600 g for 5 min. The aliquot (3 mL) of the chloroform (lower) layer was taken, 0.3 mL of methanol was added, and fluorescence was recorded in 10-mm quartz cuvettes with a spectrofluorometer (FluoroMaxHoribaYvon, Germany). The excitation wavelength was 360 nm, the emission wavelength was 420--470 nm. The results were expressed in arbitrary units per mg protein. The using of this method permits recording both fluorescence of 4-hydroxy-2, 3-nonenals (HNE) and the fluorescence of MDA. The emission wavelength depends on the nature of the Schiff's bases: the Schiff's bases formed by 4-hydroxy-2, 3-nonenals have fluorescence wavelength 430-435 nm; those formed by MDA, 460-470.

2.6. Atomic force microscopy (AFM)

Mitochondrial morphology was investigated by atomic force microscopy (AFM). Mitochondrial samples were fixed with 2% glutaraldehyde for 1 hour, followed by washing with water by the centrifugation. Precipitation mitochondria were applied to the polished surface a silicon wafer and dried in air. The study was performed on a SOLVER P47 SMENA at a frequency of 150 kHz in tapping mode. NSG11 used cantilever with a radius of curvature of 10 nm. An analysis of mitochondria AFM images under study permits us to determine the volume of individual mitochondria. The volume of a mitochondrial image is equal to the product of the sectional area of the mitochondrial image and the average height of the image in the region of section and is calculated by an Image Analysis program to the coordinate data and scanning pitch. The section was made at a height of 30 nm. Volume of image drugs mitochondria corresponded to the product of the area of the cross section image of mitochondria multiplied on the medium altitude-of this image in area of section. In the analysis and processing the data file, there was used Statistica 6. In the analysis there were used individual mitochondria.

2.7.Reagents:

sucrose, Tris, FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone), malate, glutamate, succinate, EDTA, ADP, glutaraldehyde (Sigma-Aldrich, USA), BSA (Sigma, USA), HEPES 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (MP Biomedicals, Germany), chloroform, methanol (Merk, Germany), 1, 4-Dithio-di-theiritol (Fluka, Germany).

3. Results and discussion

To determine the influence of solutions melapen, which were prepared in natural conditions and under a low level of geomagnetic field (a permalloy container), we used the model of the "aging" of mitochondria, i.e. prolonged incubation (35 min) of pea seedling mitochondria in hypotonic medium containing 1 mM KH_2PO_4 . The incubation of mitochondria in the hypotonic medium promoted the generation of ROS, which manifested itself in 2- to 3-fold increase in the intensity of fluorescence of LPO products (fig.1).

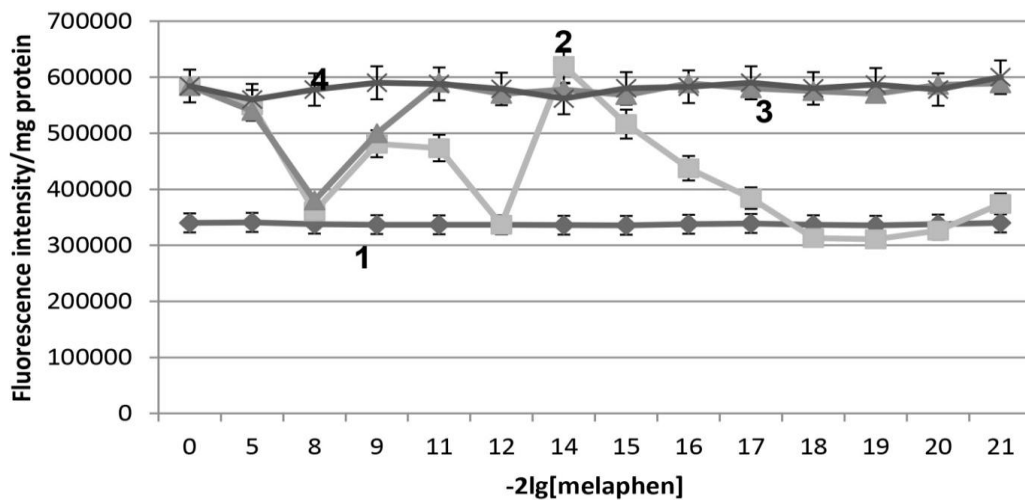


Fig.1.The fluorescence intensity of LPO products after introduction of different concentrations of melapen, which was prepared in natural conditions, or melapen, which was in the permalloy container, into the incubation medium of the pea seedling mitochondria. Y-axis: fluorescence intensity /mg protein; X-axis: the concentration of melapen. 1-control (without introduction of melapen into the incubation medium); 2- fluorescence intensity in samples containing different concentrations of melapen which was prepared in natural conditions; 3-fluorescence intensity in samples containing different concentrations of, which was in the permalloy container, 4-“aged” mitochondria (without introduction of melapen).

The introduction of melapen into the incubation medium of mitochondria caused the reduction of LPO intensity, which shows dependence from dose. Melapen decreased the intensity of fluorescence of LPO products in membranes of “aged” mitochondria in concentrations of 2×10^{-7} ; 2×10^{-12} and 2×10^{-18} - 2×10^{-21} M. A different pattern was observed when administered in the incubation medium of mitochondria melapen, which was in permalloy container within 24 hours: in the concentration range of 2×10^{-5} - 2×10^{-9} M the effects of melapen, which was in permalloy container, did not differ from the effects melapen, solutions of which were in the vivo. However, in the concentration range of 2×10^{-10} - 2×10^{-21} M melapen, which was in the permalloy container, had no significant effect on the fluorescence intensity of lipid peroxidation products “aged” mitochondria.

Changes of physico-chemical properties of membranes of mitochondria, likely would entail changing and lipid-protein interactions and, consequently, the activity of membrane-bound enzymes, in particular, enzymes of the mitochondrial respiratory chain. Indeed, the introduction melapen to the incubation medium mitochondria led to a change in their bioenergetics characteristics (fig.2).

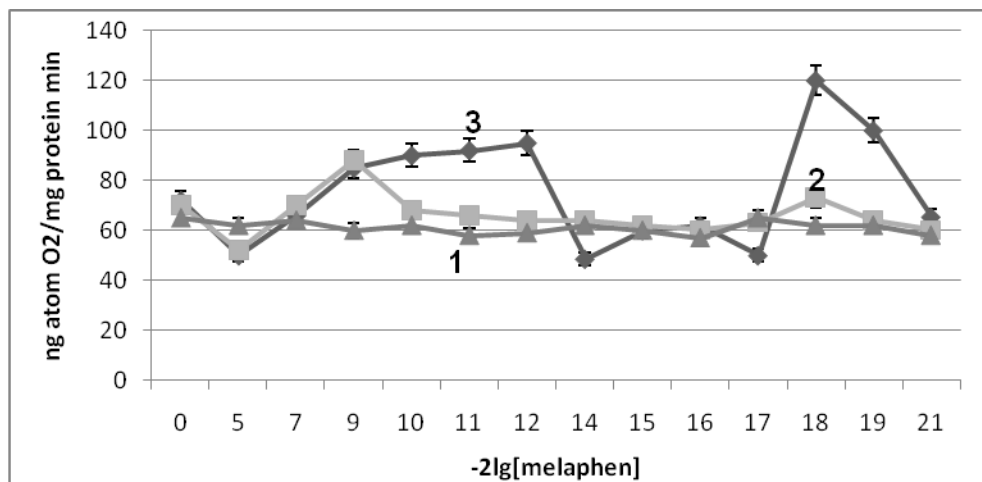


Fig.2 Effects of melaphen, which was prepared in natural conditions, or melaphen, which was in the permalloy container, on the rate of NAD-dependent substrate oxidation by mitochondria isolated from pea seedlings. Y-axis: rats of malate+ glutamate oxidation in the presence FCCP in ng. atom O₂/mg protein×min; X-axis:- the concentration of melaphen. 1-contro l(without introduction of melaphen into the incubation medium); 2- the rate of NAD-dependent substrate oxidation in samples containing different concentrations of melaphen, which was in the permalloy container; 3-the rate of NAD-dependent substrate oxidation in samples containing different concentrations of melaphen, which was prepared in natural conditions.

Has been observed changes in maximum rates of oxidation of NAD-dependent substrates by mitochondria depending on the melaphen concentration. The drug concentrations in 2×10^{-5} ; 2×10^{-7} ; 2×10^{-14} - 2×10^{-17} and 2×10^{-21} M almost no exert influence upon the rates of oxidation of NAD-dependent substrates in the presence of FCCP. Other effects exert the drug in concentrations of 2×10^{-9} - 2×10^{-12} and 2×10^{-18} M. In these concentrations, the drug stimulated the electron transport in the mitochondrial respiratory chain in the presence of ADP at 36-44% and 32-66% in the presence of FCCP. Introduction melaphen, which was 24 hours in permalloy container into the incubation medium of mitochondria, had other impact upon bioenergetics characteristics of these organelles. In the concentration range of 2×10^{-5} - 2×10^{-9} M drug also affects the maximum rates of NAD-dependent substrate oxidation as melaphen, who was in natural conditions. However, in the concentration range of 2×10^{-10} M and below these solutions of melaphen did not affect the maximum rate of oxidation of NAD dependent substrates that, possible, may indicate that, in this concentration range under lower intensity of the geomagnetic field nanoassotciates not formed [13, 14]. To determine the influence of the geomagnetic field at the emergence in highly diluted solutions of BAS nanoassotciates and their effects on metabolic processes we compared the effects of treatment of pea seeds (*P. Sativum*, variety Flora-2) with solutions melaphen (which were prepared in normal conditions, and the solutions, which prepared using permalloy container) on the morphological characteristics of mitochondrial pea seedlings were subjected to stress. In quality exposure of stress, we used a model of insufficient watering, combined with a moderate cooling. The choice of this model is connected with the fact that in nature the plant is exposed to influence at once several factors of environment. The study of metabolic conversion in the plant cell at the combined action of several abiotic factors is actual in particular the combined effects of insufficient moisture and moderate cooling. Marked that in the critical period for the survival of the plant, which can be considered as the start of the intensive growth seedling, plant metabolism shows particularly high demands on the provision of moisture and energy, but not always natural factors favor this, for example, in the spring when happens the short-term decrease in temperature and the lack of availability water (after a snowless winter). We have previously shown that the processing of peas 2×10^{-12} M solutions of melaphen prevents changes to the morphological characteristics of mitochondria associated with an increase in ROS generation in these conditions [19]. Indeed, as can be seen from Figure 3, the combined effects of insufficient watering and a moderate cooling upon the sprouts of pea had led to a change in the morphology of mitochondria. Appeared a large number of swollen mitochondria (fig.3b). Statistical analysis of the amount preliminarily fixed with the glutaric aldehyde mitochondria testify about the emergence in this group of seedlings of the solitary and larger volume of mitochondria (V aver. = $115.1(\mu\text{m})^2 \times \text{nm}$) (fig. 3b) compared with the control group (V aver. = $80.7(\mu\text{m})^2 \times \text{nm}$) (fig.3 (a)

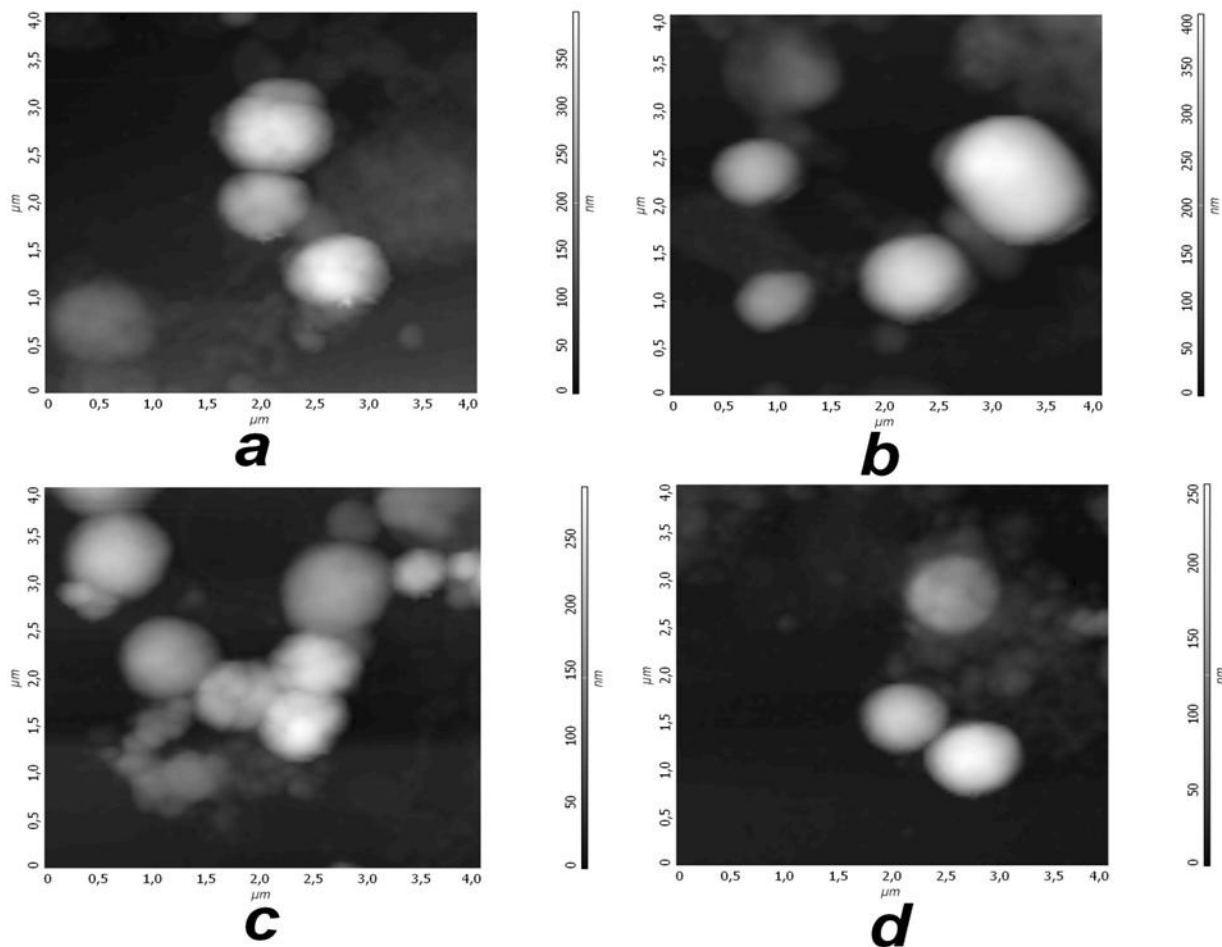


Fig.3. The two-dimensional AFM image of mitochondria ($10 \times 10 \mu\text{m}^2$) isolated from 6-day pea seedlings in the Control group (a), in (the water deficit + moderate cooling) (WD+MC) group (b), in (the water deficit + moderate cooling + 2×10^{-12} M melaphen) (WD+MC+MF) group (c), in the (water deficit + moderate cooling + 2×10^{-12} M melaphen, designed in permalloy container within 24 hours)(WD+MC+MF+PC)group (d).

Processing of peas with 2×10^{-12} M solution melaphen, which was prepared under normal conditions, allow to preventing changes in the morphology of mitochondria. The geometric parameters of the AFM images of mitochondria remained similar to those of the control group (fig.3 c). Note, that the 2×10^{-12} M melaphen, which 24 hour remained in permalloy container had influenced on the morphology of mitochondria is the same as melaphen solution, which was prepared in the usual conditions, i.e. also allow to preventing the swelling of mitochondria (fig. 3 d). As in the first and in the second case was a protective effect melaphen. Thus 24 hour stay of the drug in permalloy container did not reduced the effectiveness of the protective effect of melaphen on the morphology of mitochondria of pea sprouts in a stressful environment.

3.1. Conclusion

In model experiments determined the effects of the geomagnetic field on the effects of low and ultra-low concentration of melaphen on the functional state of mitochondria of pea sprouts. We can assume that bioeffects low and ultra low concentrations of the drug may be associated with the formation or absence of nonassociates that self-organizes under normal conditions but not in low geomagnetic conditions. However, experiments carried out on the whole plant organisms (pea seedlings), had revealed other rules 2×10^{-12} M solution of melaphen, who was in gipogeomagnetic conditions its biological effects did not differ from the biological effects the same solution of the drug, who was made in natural. Probably, at concentrations 10^{-10} M and below the nonassociates in the absence of an electromagnetic fields

are not formed. It is possible that for the formation of nonassociates enough 1 hour stay 2×10^{-12} M solution of melaphen outside the container, as evidenced by data on the effect of soaking seeds peas 2×10^{-12} M solution of melaphen upon morphology of mitochondria of pea seedlings.

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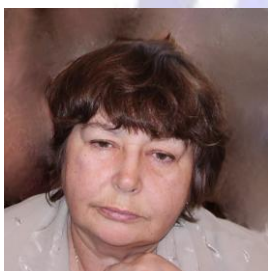
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