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Uncoupler of Oxidative Phosphorylation Prolongs the Lifespan of *Drosophila*

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Abstract—The effect of a moderate ("soft") uncoupling of mitochondria on the lifespan and some parameters of biological age of *Drosophila melanogaster* strain Oregon was studied. Addition of the uncoupler 2,4-dinitrophenol (DNP) to the nutritional mixture of larvae significantly increased the average lifespan of the flies without changing their maximal lifespan. DNP significantly increased the rate of oxygen consumption by isolated mitochondria and tissue homogenates of the flies in state 4 (of Chance). DNP also decreased the activity of alcohol dehydrogenase (a parameter of flies' biological age) in the tissue homogenates, especially on octanol as the reaction substrate. However, being deprived of food the DNP-treated flies displayed a markedly decreased viability as compared to the control flies. On the whole, the results suggest that "soft" uncoupling of mitochondria may increase the lifespan.

Key words: 2,4-dinitrophenol, *Drosophila melanogaster*, lifespan

A considerable aging of the population is now occurring in economically developed countries, i.e., the fraction of elderly persons has increased especially during the last quarter of the XX century [1]. On one hand this indicates essential achievements of mankind in health care and solution of social problems, but on the other hand it is associated with many problems which require fundamental understanding of aging mechanisms.

Unfortunately, at present there is no unique and universally accepted theory of aging. The free radical concept is one of the leading hypotheses because it rather satisfactorily explains many observations of modern gerontology. It is established that generation of reactive oxygen species (ROS) is physiologically important and seems to play an important role in mechanisms of cell proliferation, differentiation, aging, and the appearance of some diseases. Mitochondria are believed to be the major source of ROS, although 98% of oxygen consumed by mitochondria is converted into water and only 2% produce ROS during parasitic chemical reactions at the beginning and in the middle of the chain of respiratory enzymes [2]. The rate of ROS generation in the respiratory chain also depends on the membrane potential, and with increase in this potential above a certain threshold the ROS generation dramatically increases [3]. Based on these observations, it has been suggested that a "soft" uncoupling should be a mechanism preventing excess production of ROS [4] and as a result (in the framework of the free radical aging theory) prevent shortening of the lifespan. However, direct data on the cell or organism level in favor of this hypothesis are still insufficient [5].

Thus, the purpose of the present work was to study the effect of a moderate ("soft") uncoupling of mitochondria on the lifespan and some parameters of biological age of *Drosophila melanogaster*.

MATERIALS AND METHODS

A laboratory population of Oregon strain *Drosophila melanogaster* was used. Immediately after hatching from pupae, the flies (males and females separately) were placed into vessels (no more than 20 flies per vessel) containing food of full value and kept under standard conditions at 24°C. The flies were replaced onto fresh nutritional medium every two days [6, 7].

The uncoupler 2,4-dinitrophenol (DNP) was introduced into the nutritional mixture only at the stage of lar-

Abbreviations: ROS) reactive oxygen species; DNP) 2,4-dinitrophenol; Hsp70) 70-kD heat shock protein; EGTA) ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid.

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vae to the final concentration of 0.8%. Higher concentrations of this xenobiotic were unfavorable for viability of the flies and their larvae.

Fly tissue homogenate was prepared as follows. The flies were immobilized by cooling on ice and homogenized (Teflon/glass) in medium containing 250 mM sucrose, 5 mM Tris-HCl, 2 mM EGTA, 1% (w/v) BSA (pH 7.4) at 4°C [8]. A weighed sample of the flies and the medium were taken at the ratio of 1 : 3. The resulting homogenate was filtered through two layers of nylon tissue and used in experiments. Mitochondria were prepared as described in [8]. From 500 mg of flies about 2 mg mitochondria (in 1 mg protein) was obtained having respiratory control of 2.5-3.0.

Oxygen consumption by mitochondria and fly tissue homogenates was determined by polarography with a Clark-type electrode [9] as follows: the homogenate or mitochondria (0.4-0.6 mg protein) was placed into a cell thermostatted at 30°C which contained: 100 mM sucrose, 10 mM pyruvic acid, 75 mM KCl, 10 mM KH_2PO_4, 2 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.4) [10]. The cell was also supplemented with 1 mg BSA and 1 mM EGTA.

The alcohol dehydrogenase activity of the fly tissue homogenates was evaluated by the rate of reduction of exogenous NAD^+ [11] in medium which contained 100 mM Tris-HCl (pH 9.6), 100 mM butanol or octanol, 0.5 mM NAD^+, and homogenate (1 : 5, homogenization in 100 mM Tris-HCl buffer (pH 9.6)). The incubation temperature was 30°C. The enzyme activity was calculated using the molar extinction coefficient $6.22 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ and expressed in nmol NADH/min per mg protein.

The protein content in the homogenates was determined by a modified Lowry method [12] with human serum albumin (HSA) from Reanal (Hungary) as the standard.

The means in the samples with normal distribution were compared using Student's *t*-test. The difference was considered significant at $p \leq 0.05$.

NAD^+, Tris(hydroxymethyl)aminomethane, and HSA were from Reanal; BSA (fraction V) and EGTA were from Sigma (USA); other reagents were of domestic production (Reakhim, Russia) of analytical purity, chemical purity, or special purity.

RESULTS AND DISCUSSION

Addition of DNP into the nutritional mixture of *Drosophila* larvae increased the weight of flies hatching from the pupae by more than 30%. Thus, weights of the control and experimental females were, on average, 1.26 and 1.71 mg, respectively.

The insects treated with the uncoupler during the larval stage had longer lifespan. This is well manifested by survival curves of *Drosophila* males (Fig. 1). On the 26th day about 50% of the flies were dead in the control, whereas in the experiment no more than 35% of the flies had died. This corresponded to a significant increase in the average lifespan of the flies treated with the uncoupler: it was 32.8 ± 1.7 as compared to 27.3 ± 1.3 days in the control. However, the maximal lifespans (calculated as the average lifespan of the last 10% of flies) were not different: they were 41.1 ± 1.9 and 40.8 ± 3.3 days for the control and experimental populations, respectively.

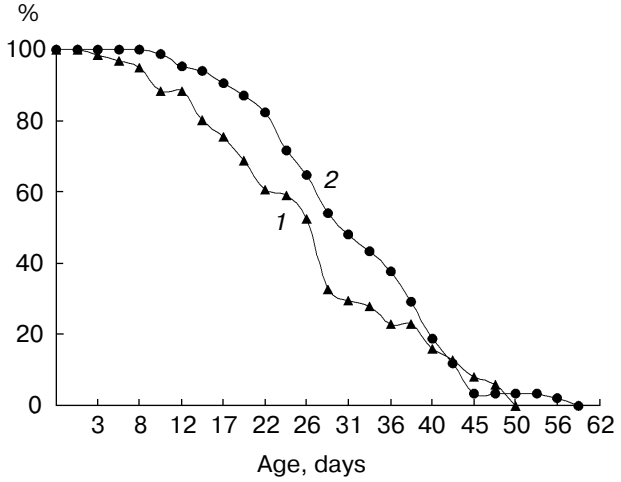

Fig. 1. Survival curves of *Drosophila melanogaster* males in the control (*1*) and after addition of DNP to the nutritional medium (*2*). The survival of flies on the standard medium and in the presence of the uncoupler was determined on samples of 20 males per vessel. The living flies were counted daily; the food was changed once every two days. The initial number of flies was taken as 100%.

In the only available published work [13], 0.1% DNP prolonged by 12.3% the average lifespan of *Drosophila*. In various series of our experiments, the increase in the average lifespan was no less than 20%. Recently DNP has been shown to also increase the lifespan of *Saccharomyces cerevisiae* [14].

Rates of oxygen consumption by mitochondria and tissue homogenates from the control and experimental flies are presented in the table. Certainly, on addition of the uncoupler data on oxygen consumption in the "rest state" under conditions of exhausted ADP (state 4 of Chance) are the most interesting. The rates of oxygen consumption by mitochondria and tissue homogenates from the experimental flies were significantly higher (table), and this suggested a noticeable uncoupling in the mitochondria of the insects.

Obviously, studies on biochemical parameters, which are markedly changing during ontogenesis in the control flies, can present important information about metabolic processes in the tissues of experimental flies. In particular, the alcohol dehydrogenase activity of

Rates of oxygen consumption in state 4 of Chance by mitochondria and tissue homogenates from *Drosophila* males (ng-atom O_2/mg protein per min; M ± m; n = 5-6)

Experimental conditions	Homogenate	Mitochondria
Control	22.5 ± 1.3	32.3 ± 1.6
2,4-DNP	29.1 ± 2.1*	40.0 ± 2.7*

* $p \leq 0.05$ compared to control.

Drosophila tissues characterizes the biological age of the flies [15, 16].

Alcohol dehydrogenase (EC 1.1.1.1) in insects is a cytosolic enzyme and has several isoforms [16] that are different in substrate specificity; thus, substrates of one isoform are ethanol or butanol, whereas the best substrate of another isoform (the so-called octanol dehydrogenase) is octanol [16].

Considering the above-mentioned data, the aldehyde dehydrogenase activity of tissue homogenates from *Drosophila* hatched from the larvae treated with DNP was studied (Fig. 2). This activity was significantly increased in the control flies from the 2nd to 15th day of their life. In the experiment the aldehyde dehydrogenase activity in the 2-day-old flies was either unchanged or decreased (with butanol or octanol as substrate, respectively), whereas in the 15-day-old flies it was decreased on all substrates (most pronouncedly on octanol) (Fig. 2).

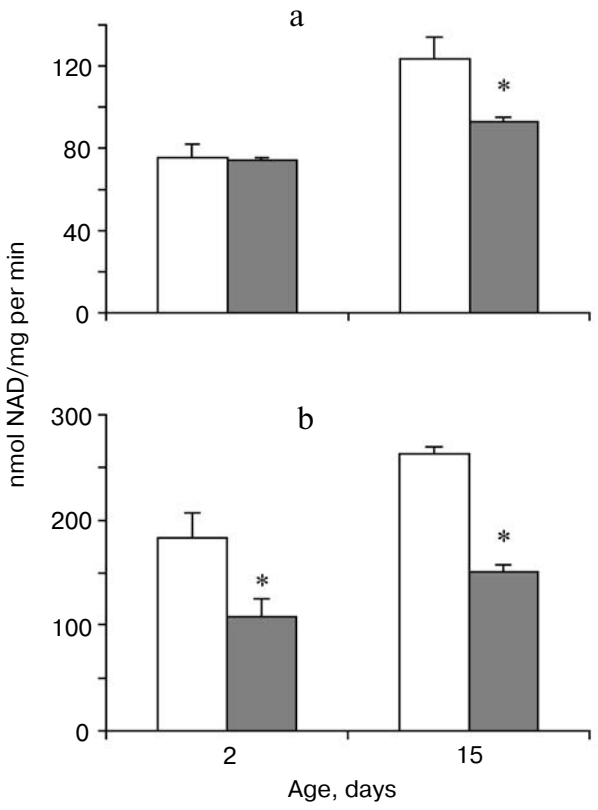

Fig. 2. Alcohol dehydrogenase activity of homogenates from fly tissues in control (light columns) and in the presence of DNP (dark columns): a) on butanol as a substrate; b) on octanol as a substrate; * indicates that differences with respect to the control are significant.

Based on the literature data on the substrate specificity of different isoforms of alcohol dehydrogenase, it was suggested that the difference in the isoenzyme sets in young and adult flies should manifest itself under conditions of our experiment. The pool of alcohol dehydrogenase isoforms was shown to qualitatively change with age of insects [15] and also on addition of some xenobiotics to the nutritional mixture [17].

The association of the alcohol dehydrogenase activity with the age of insects was discussed in [18]. The author found that some mutations of *Drosophila* (*yellow*, *ebony*) significantly increased this activity but reduced the lifespan of the flies. Thus, the increase in the survival negatively correlated with the alcohol dehydrogenase activity of the tissues. These findings seem to be explained by data of work [19]. These authors obtained in the flies an increased number of copies of the gene encoding Hsp70 and suggested that this should be associated with changes in the specific activities of enzymes because of regulation of the cell metabolism by heat shock proteins. In fact, this manipulation decreased the activity of alcohol dehydrogenase. Perhaps the decrease in the alcohol dehydrogenase activity observed by us was associated with the increased synthesis of heat shock proteins, because DNP is known to increase the level of stress proteins in tissues. In particular, the treatment of insects with elevated temperature and DNP resulted in the same response to the level of chromosome puffing [20].

The data of the present work are in agreement with the literature data on the possibility of a geroprotective effect of certain concentrations of xenobiotics as a result of changing (retardation?) of normal aging. Uncouplers cannot only induce the synthesis of heat shock proteins, but also determine subsequent events: the soft uncoupling of mitochondria is accompanied by preventing the excess production of ROS by the mitochondrial respiratory chain, and this can be associated with a significant decrease in the amount of cell reserves of NADH- and $FADH_2$-providing substrates and ATP. This should decrease the total intensity of metabolic processes; moreover, just the increased consumption of O_2 without production of ATP lowers the level of free molecular oxygen capable for producing superoxide anion [2, 4, 21-23]. This is likely to reduce oxidative damage of the cell and increase the lifespan.

Certainly, such flies can exist with relative comfort only under favorable environmental conditions and will lack resistance to extreme stress situations, as occurred in

our experiments with starving insects. In the complete absence of food, the control population of adult (15-day-old) *Drosophila* died within 78 h, while the experimental flies lived for only 48 h.

The findings of this work seem to confirm the reasonability of further studies on the role of uncoupling in the regulation of development and aging of organisms.

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