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Seasonal Changes in Microsomal Fraction Enriched with Na,K-ATPase from Kidneys of the Ground Squirrel *Spermophilus undulatus*

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Abstract—The Na,K-ATPase activity in microsomal fraction isolated from kidneys of winter hibernating ground squirrels was found to be 1.8-2.0-fold lower than that in active animals in summer. This is partially connected with a decrease in Na,K-ATPase protein content in these preparations (by 25%). Using antibodies to different isoforms of Na,K-ATPase α-subunit and analysis of enzyme inhibition by ouabain, it was found that the decrease in Na,K-ATPase activity during hibernation is not connected with change in isoenzyme composition. Seasonal changes of Na,K-ATPase α-subunit phosphorylation level by endogenous protein kinases were not found. Proteins which could be potential regulators of Na,K-ATPase activity were not found among phosphorylated proteins of the microsomes. Analysis of the composition and properties of the lipid phase of microsomes showed that the total level of unsaturation of fatty acids and the lipid/protein ratio are not changed significantly during hibernation, whereas the cholesterol content in preparations from kidneys of hibernating ground squirrels is approximately twice higher than that in preparations from kidneys of active animals. However, using spin and fluorescent probes it was shown that this difference in cholesterol content does not affect the integral membrane microviscosity of microsomes. Using the cross-linking agent cupric phenanthroline, it was shown that Na,K-ATPase in membranes of microsomes from kidneys of hibernating ground squirrels is present in more aggregated state in comparison with membranes of microsomes from kidneys of active animals. We suggest that the decrease in Na,K-ATPase activity in kidneys of ground squirrels during hibernation is mainly connected with the aggregation of proteins in plasma membrane.

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Key words: hibernation, Na,K-ATPase, membrane microviscosity, protein aggregation, kidney, ground squirrel *Spermophilus undulatus*

Many small mammals of northern areas (rodents, insectivores, chiropterans) spend winter season in the state of deep torpor. Winter torpor of these animals (hibernation) is a natural adaptation that allows them to survive under the conditions of low temperatures and the absence of food and water [1, 2]. Entering into hibernation is accompanied by almost 100-fold decrease in metabolism in comparison with normothermia [3] and decrease in oxygen consumption about 45-150-fold [4]. The body of the animal is cooled as a result of decrease in metabolism but even under these conditions the hibernators still control the body temperature and do not allow the temperature to drop down below the freezing point of body fluids [5, 6]. The decrease in metabolism during hibernation is connected with complex changes of physiological and biochemical processes which are directed to saving of energy sources [7-9]. As a result, energy consumption is decreased by 80-90% (even assuming the energy costs of periodical arousals between torpor bouts) in comparison with energy consumption necessary to maintain the constant body temperature at the same low ambient temperature [2, 10].

During hibernation reduction of breathing and heart rates and suppression of nervous activity and other physiological processes take place [4]. Nevertheless, because metabolism still occurs at the low level the accumulation of waste metabolites in the blood takes place. However, urine is not formed during hibernation [11]. Homeostasis is restored only during short spontaneous arousals

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between torpor bouts when formation of urine and urination occur [4]. In the kidneys where energy consumption per mass unit is much higher than that in any other body organ the Na,K-ATPase is the main consumer of ATP: in the cells of renal tubules this enzyme uses up to 80% of the ATP [12]. The thick ascending tubules of Henle's loops located mainly in outer medulla and partially in renal cortex are especially rich in Na,K-ATPase, which provides the reabsorption of ions, metabolites, and water in renal tubules [12]. Under hypometabolic conditions when both glycolysis [13, 14] and oxidative phosphorylation [15, 16] are suppressed, the production and, correspondently, consumption of ATP are restricted. Therefore, the study of peculiar properties of Na,K-ATPase functioning in the kidneys of hibernators under conditions of normothermia and hypothermia has special interest.

Although such studies were carried out earlier and it was shown that the activity of Na,K-ATPase (measured at 37°C) in the kidneys of hibernators is significantly decreased during hibernation, the reasons of the activity decrease are still not clear. For example, Charnock and Simonson [17] found that the activity of Na,K-ATPase in microsomes from renal cortex of ground squirrels is decreased during hibernation more than 2.5-fold. However, Bennis at al. [18] did not find such differences in enriched with Na,K-ATPase microsomal fractions isolated from renal cortex of jerboa. At the same time, in microsomes obtained from outer medulla of jerboa kidneys the activity of Na,K-ATPase was decreased during hibernation more than 2-fold [18]. The authors of both research groups concluded that the observed decrease in Na,K-ATPase activity during hibernation is connected with the decrease in enzyme content in the kidneys. This conclusion was made because the ratio of bound ^{3}H-labeled ouabain to Na,K-ATPase activity in preparations from kidneys of active and hibernating animals did not differ significantly [17, 18]. However, in the Na,K-ATPase molecule the binding sites for ATP and ouabain are located on the opposite sides of the membrane and the most part of plasma membrane fragments during isolation forms sealed vesicles both with right-side out and inside out orientation [19]. Therefore, in the sealed vesicles only one type of binding sites (for substrate or for inhibitor) is available for the ligand (ATP, ouabain) present in the reaction medium. To provide the availability of all binding sites for the corresponding ligands in such preparations it is necessary to use detergents and/or ionophores [19-22], but in the above studies they were not used [17, 18], this raising serious doubts about the conclusions the authors made.

In this connection, the main goal of the present study was to investigate seasonal changes of Na,K-ATPase activity in microsomes from kidneys of the ground squirrel *Spermophilus undulatus* and to evaluate the possible reasons for these changes.

MATERIALS AND METHODS

Adult ground squirrels *S. undulatus* were collected by live trapping in Yakutiya and were maintained in the animal facility of the Institute of Cell Biophysics of the Russian Academy of Sciences (Pushchino) in individual cages at 20-25°C in natural daylight with standard feeding. In November, the animals were put into a dark room at temperature 2-4°C. Active ground squirrels (body temperature 37°C) were used in experiments during April-July and hibernating animals (body temperature 2°C) were used in February-March in the middle of torpor bout. For transportation the kidneys of ground squirrels were frozen in liquid nitrogen and were kept at –80°C before use.

Microsomal fraction from plasma membrane was obtained from outer medulla of ground squirrel kidneys by differential centrifugation [23] with the addition of protease inhibitor cocktail (Amresco, USA) at ratio 1 : 100 (v/v). The preparations were frozen in liquid nitrogen and kept at –80°C before use. Protein concentration was measured according to Lowry et al. [24] in the presence of 1% sodium deoxycholate using BSA as a standard.

Na,K-ATPase activity was measured using a coupled enzyme system as described in full details earlier [25] in medium containing 130 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 50 mM imidazole-HCl, pH 7.2, 2.5 mM ATP, 2 mM phosphoenolpyruvate, 0.3 mg/ml NADH, 5 IU/ml pyruvate kinase, 10 IU/ml lactate dehydrogenase, 1.5 μg/ml microsome protein, and 10 μg/ml ionophore alamethicin. The measurements were carried out using an Ultrospec 2100 spectrophotometer equipped with Multitemp III thermostat (GE Healthcare, USA) in temperature range from 2.5 to 37°C.

SDS-PAGE was carried out according to Laemmli [26] using 5% stacking and 8-20% gradient running gels [27]. As protein markers, the kit (SM1811) of proteins with molecular masses from 10 to 250 kDa from Fermentas (Lithuania) was used.

Immunochemical staining of proteins immobilized on nitrocellulose membrane was carried out according to Towbin et al. [28] as described earlier [29] using mouse monoclonal antibodies against Na,K-ATPase α1-subunit (Millipore (USA) 05-369, Clone C464.6, 0.5 μg/ml), rabbit polyclonal antibodies against Na,K-ATPase α2-subunit (Millipore AB9094, 1 μl/ml), and mouse monoclonal antibodies against actin (JLA20, 0.4 μg/ml) and secondary antibodies conjugated with horseradish peroxidase (Sigma (USA) A0168 and A0545).

The presence of phosphorylated proteins in microsomal preparations was evaluated using a fluorescent dye for phosphoproteins in gel Pro-Q Diamond (Invitrogen, USA). Gels were fixed in solution of 50% methanol and 10% acetic acid (100 ml, 30 min, 2 times), washed by water, and stained in 10 volumes (in comparison with gel volume) of dye (35-45 ml) for 1 h to avoid bright background. The bands of phosphorylated proteins were visu-

alized using a BioSpectrum (UVP, USA) under top UV-illumination with registration of fluorescence at 570-640 nm (diaphragm 1.2, speed 3.5 sec). After that the dye was removed from gels with 50 mM sodium acetate buffer, pH 4.0, containing 20% acetonitrile (100 ml, 3 times for 30 min), and the gels were washed with water and stained with Coomassie R-250 for estimation of the protein content.

Phosphorylation of microsomal proteins by endogenous protein kinases was studied by autoradiography as described earlier [30]. For this purpose the preparations of microsomes were incubated for 10 min at 37°C in medium containing 15 mM Tris-HCl, pH 7.4, 1 mM DTT, 1 mM EGTA, 5 mM $MgCl_2$, 0.1 mM Na_3VO_4, 5 mM NaF, 1 mM pNPP, 1 : 100 (v/v) protease inhibitor cocktail (Amresco M221), 1 mg/ml protein, and 1 mM [γ-^{32}P]ATP (5 MBq/μmol) [31, 32].

Proteins were identified by liquid chromatography/tandem mass spectrometry (LC-MS/MS) after trypsinolysis as described earlier [33]. LC-MS/MS was done using an Ultimate Plus chromatograph (LC Packings, USA) connected with an Esquire 6000 mass spectrometer (Bruker Daltonics, Germany).

Preparations of microsomes were treated with cupric *o*-phenanthroline at 37°C as described earlier [25]. Into sample buffer for SDS-PAGE, β-mercaptoethanol was not added.

Fatty acid composition of lipids and cholesterol content in membranes of microsomes was evaluated by hybrid gas chromatography/mass spectrometry (GC-MS). For this purpose 15 μl of microsomal fraction (about 85 μg of protein) was dried after addition of 15 μl of methanol, and acid methanolysis of the sample was carried out (1 M HCl in methanol, 400 μl) for 1 h at 80°C. After that methyl esters of fatty acids, aldehydes, and sterols were extracted with hexane (400 μl) for 5 min. The hexane extract was dried and the dry pellet was treated with N,O-bis-(trimethylsilyl)-trifluoroacetamide (20 μl) for 15 min at 80°C to obtain trimethylsilyl esters. To the mixture of esters 80 μl of hexane was added and 1-2 μl of the resulting solution was loaded into the injector of the GC-MS [34].

EPR spectra of spin probes 5-doxylstearate and methyl ester of 16-doxylstearate incorporated into membranes of microsomes were recorded on a Varian E-4 EPR spectrometer (USA) with constant working frequency 9.2 GHz at temperature range from –60 to 40°C. Spectra were registered at scan width of constant magnetic field 40 Oe, modulation amplitude 2 Oe, microwave power 10 mW, recording speed 2 min, and time constant 0.1 sec. The medium for spectral measurement contained 25 mM imidazole-HCl, pH 7.0, 250 mM sucrose, 5 mg/ml protein, and 0.1 mM spin probe. Order parameter *S* for 5-doxylstearate and rotational correlation time τ_c (nsec) for methyl ester of 16-doxylstearate were calculated using known formulae [35, 36].

Fluorescence of hydrophobic probe pyrene was measured in the temperature range from 5 to 40°C using a Varian Cary Eclipse fluorescence spectrophotometer (USA) equipped with a thermostat as described earlier [37]. Slit width was 5 nm. The medium for fluorescence measurement contained 130 mM NaCl, 20 mM KCl, 30 mM PIPES-NaOH, pH 7.0, 0.2 mg/ml protein, and 7 μM pyrene. Fluorescence of the probe was excited at 285 nm ("induced" fluorescence) or 335 nm (intrinsic fluorescence) and fluorescence intensity of monomers and excimers was registered at 394 and 470 nm, respectively.

All experiments were carried out at least three times using at least three different preparations of microsomes, and after that the mean values and standard errors shown on figures were calculated. The number of preparations used is shown in parenthesis. To estimate the significance of data difference for two samples, Student's *t*-test for independent samples with unequal dispersions with two-sided distribution was applied.

RESULTS AND DISCUSSION

Because of the majority of microsomal membranes forms sealed vesicles [19] that are impermeable for ATP and ions, Na,K-ATPase activity was measured in the presence of the ionophore alamethicin, after the addition of which the enzyme activity increased 2-3-fold. Under these conditions Na,K-ATPase activity in microsomes from outer medulla of kidneys of active and hibernating ground squirrels at 37°C was equal to 146 ± 9 and 82 ± 4 μmol/h per mg protein, respectively ($p < 0.00001$). In the absence of alamethicin the values of Na,K-ATPase activity in preparations of microsomes from kidneys of active and hibernating ground squirrels were not significantly different (53 ± 3 and 44 ± 3 μmol/h per mg protein, respectively, $p > 0.1$) and were comparable with the values of enzyme activities in the kidneys of active and hibernating jerboa [18] that were measured also in the absence of ionophores.

The temperature dependence of Na,K-ATPase activity in the preparations in Arrhenius plots has a typical character with the break at 20°C (Fig. 1). Such character of temperature dependence is well known for may membrane enzymes, in particular for Na,K-ATPase and Ca-ATPase [38, 39], and the breaks on Arrhenius plots are usually explained as being due to structural rearrangements of the membranes [39]. It was shown that at temperatures above 20°C Na,K-ATPase activity in microsomes from kidneys of hibernating ground squirrels is 1.8-2.0-fold lower in comparison with enzyme activity in microsomes of active animals. Arrhenius plots in this temperature range are practically parallel; therefore, the activation energies of Na,K-ATPase from kidneys of hibernating and active ground squirrels are the same

(about 11.5 kcal/mol). Decrease in temperature from 20 to 2.5°C leads to less expressed difference in the Na,K-ATPase activity. Under these conditions the activation energy of Na,K-ATPase in preparations from kidneys of hibernating ground squirrels is about 7 kcal/mol lower in comparison with preparations from kidneys of active animals (27.9 and 35.0 kcal/mol, respectively). Probably such decrease in Na,K-ATPase activation energy during hibernation has a special meaning and allows the enzyme to be involved more rapidly in the functioning of kidneys of ground squirrels during awakening, when homeostasis should be restored very quickly.

Decrease in enzyme content in plasma membranes could be one of the reasons for the decrease in Na,K-ATPase activity during hibernation [17, 18]. The content of Na,K-ATPase in preparations of microsomes was evaluated by the most reliable and direct method of immunochemical staining of Na,K-ATPase catalytic subunit (95 kDa) using antibodies against α1-isoform of the enzyme (Fig. 2). It was found that the content of Na,K-ATPase in microsomes from kidneys of ground squirrels during hibernation is decreased by only 25% ($p < 0.0015$). The bands of membrane bound actin (43 kDa) visualized using specific antibodies were used as an internal control; seasonal differences in actin content were not found ($p > 0.15$).

It was shown using antibodies against α2-subunit of Na,K-ATPase that this enzyme isoform is absent in

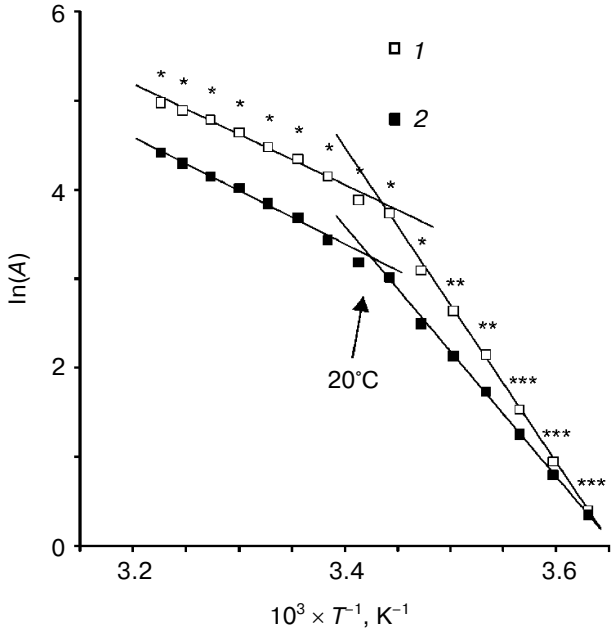

Fig. 1. Arrhenius plots for Na,K-ATPase activity (A, μmol/h per mg protein) in preparations of microsomes from kidneys of active (*1*, $n = 14$) and hibernating (*2*, $n = 16$) ground squirrels; n is the number of preparations used. The arrow indicates the area of structural transition. * $p < 0.0005$, ** $p < 0.05$, *** $p > 0.5$.

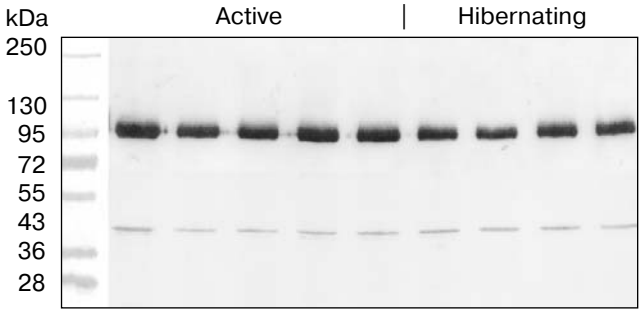

Fig. 2. Immunochemical staining of Na,K-ATPase α1-subunit (95 kDa) and actin (43 kDa) using monoclonal antibodies.

microsomes isolated from kidneys of both active and hibernating ground squirrels (data not shown). This is in good agreement with the data that only α1-isoform of Na,K-ATPase catalytic subunit is present in all segments of the kidneys [40]. It is known that different isoforms of Na,K-ATPase α-subunit have different sensitivity to ouabain. The study of enzyme inhibition by ouabain in preparations of microsomes from both groups of animals showed that during hibernation the sensitivity of Na,K-ATPase in kidneys of ground squirrels to this inhibitor was not changed (I_{50} = 12-13 μM). This support our conclusion that the isoenzyme composition of Na,K-ATPase is not changed during hibernation.

It is known that the change in activity of many enzymes during hibernation is connected with their phosphorylation by protein kinases [41]. Phosphorylation of plasma membrane proteins in skeletal muscles of ground squirrels results in decrease in activity of Na,K-ATPase during the winter [42]. Na,K-ATPase itself (α-subunit) also could be phosphorylated by different protein kinases (A, C, and G), which results in decrease in enzyme activity as described in many publications [43-48].

The fluorescent dye for phosphoproteins Pro-Q Diamond stains only 10 protein bands from more than 30 protein bands on electrophoregrams of the studied preparations of microsomes (Fig. 3). Five protein bands are stained the most intensively, including the band which corresponds by its electrophoretic mobility to α-subunit of Na,K-ATPase. Because intensity of fluorescence of dye bound with phosphoprotein depends on the amount of protein in the corresponding band, the ratio of intensities Pro-Q/Coomassie was calculated (data not shown). Significant seasonal differences of this parameter for phosphoprotein bands were not found ($p > 0.1$) with the exception of one protein band of high molecular mass protein (365 kDa), the phosphorylation of which is increased during hibernation by about 16% ($p \approx 0.05$).

Autoradiographic study of phosphorylation of microsomal proteins by endogenous protein kinases in the presence of [γ-^{32}P]ATP does not reveal phosphorylation of Na,K-ATPase α-subunit *in vitro* (Fig. 4). The most inten-

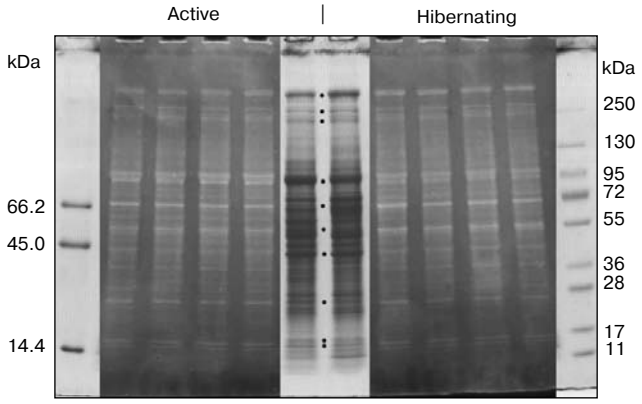

Fig. 3. Identification of phosphorylated proteins in the gel using fluorescent dye Pro-Q Diamond. Fragments of electrophoregram stained by Coomassie (two lanes in the middle and the lanes with protein markers) are given for comparison. The points indicate the most intensive bands.

sively phosphorylated protein bands on autoradiograms and on gels stained by Pro-Q Diamond were identified using mass spectrometry (data not shown). This analysis did not reveal potential regulatory proteins of Na,K-ATPase among these phosphorylated proteins. In a 365-kDa band, the protein megalin (glycoproteid 330, gp330) was identified, which belongs to the family of LDL receptors and is involved in lipid exchange and endocytosis. Therefore, phosphorylation of Na,K-ATPase is probably not involved in the regulation of enzyme activity in kidneys of ground squirrels during hibernation. The observed differences in the level of megalin phosphorylation are probably connected with some changes in lipid phase of plasma membrane during hibernation that in turn could affect Na,K-ATPase activity.

Na,K-ATPase is an integral membrane enzyme whose activity depends on the lipid microenvironment [49-52]. Therefore, the change in membrane microviscosity during hibernation can be a mechanism of the regulation of membrane enzyme activities. It is known that the membrane microviscosity is an integral parameter and is increased with increase in content of saturated fatty acids, cholesterol, and protein in membranes [53]. The fatty acid composition and cholesterol content in membranes of microsomes from kidneys of ground squirrels were studied by GC-MS (table). It was found that five fatty acids typical for lipids from plasma membrane [54] are present in the large amounts in membrane lipids. The content of these fatty acids is about 85%. It was also found that the mole percents of several fatty acids (16:0, 18:0, 20:4, 20:3) are slightly decreased during hibernation and the mole percents of other fatty acids (14:0, 18:2, 18:1) are slightly increased. The calculations showed that total mole percent of unsaturated fatty acids in preparations from kidneys of hibernating ground squirrels is higher only by 3.3% in comparison with preparations from kidneys of active animals. At the same time, the total level of unsaturation of fatty acids is not changed significantly during hibernation (table).

The lipid/protein ratio in preparations of microsomes was calculated using data of GC-MS (table). The tendency to decreased protein content in microsomes from kidneys of ground squirrels during hibernation was observed. However, because of large variation of the data the seasonal difference in lipid/protein ratio is not significant and it is unlikely that the decrease in protein content in preparations from kidneys of hibernating ground squirrels in comparison with preparations from active animals affects significantly the microviscosity of membrane lipid phase during hibernation. At the same time, it was shown that the content of cholesterol in membranes of microsomes from kidneys of hibernating ground squirrels is 1.9-fold higher than that in preparations from active animals ($p < 0.05$). Therefore, among the three factors that can affect membrane microviscosity (level of unsaturation of fatty acids, protein content, and cholesterol content) only cholesterol content is changed significantly during hibernation. To elucidate the influence of the change in cholesterol content on membrane microviscosity, the properties of membrane lipids were studied using spin probes and the fluorescent probe pyrene.

The spin probe 5-doxylstearate allows characterization of the properties of surface area of membranes where in particular cholesterol is located. Arrhenius plots for A_{max} value and for order parameter S, which are calculated from positions of extrema on EPR spectra of 5-doxylstearate, did not reveal seasonal changes in probe mobili-

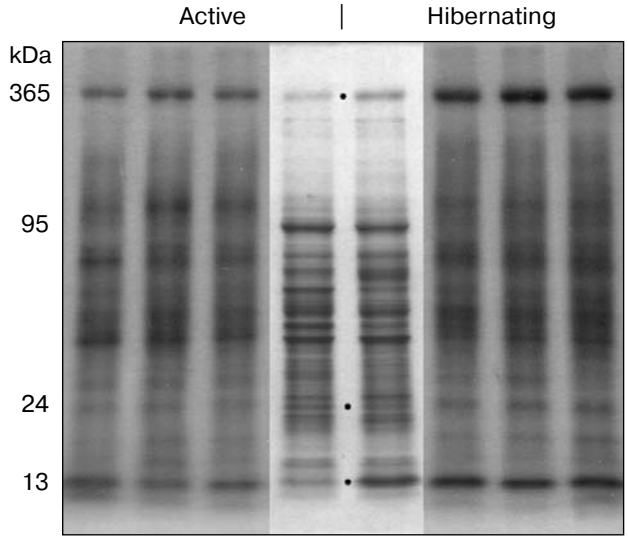

Fig. 4. Autoradiogram of microsomal proteins phosphorylated by endogenous protein kinases. Fragments of electrophoregram stained by Coomassie (two lanes in the middle) are given for comparison. The points indicate bands that are the most different in intensity.

ty in membranes of microsomes from kidneys of active and hibernating ground squirrels. Arrhenius plots for A_{max} value are characterized by two breaks at 0 and −19°C, which are connected with structural transitions in membranes (Fig. 5a). Arrhenius plots for the order parameter *S*, which characterizes structural state of the membrane, had one break at 20°C (data not shown). The break at the same temperature is present on Arrhenius plots for Na,K-ATPase activity. Therefore, despite of higher content of cholesterol in membranes of microsomes from kidneys of hibernating ground squirrels this spin probe does not reveal changes in microviscosity in comparison with preparations from kidneys of active animals. This probably is connected with location of cholesterol in membranes of microsomes in clusters (rafts), which are inaccessible for this spin probe.

The second spin probe, methyl ester of 16-doxylstearate, does not have a polar group and therefore penetrates deep into the lipid bilayer. Arrhenius plots for rotational correlation time τ_c of methyl ester of 16-doxylstearate in membranes of microsomes from kidneys of active and hibernating ground squirrels are also practically the same and have breaks at −30°C (Fig. 5b). Only at temperatures above 0°C insignificant decrease in rotational correlation time in preparations from kidneys of hibernating ground squirrels could be observed, which is connected with more unrestricted movement of the probe in comparison with preparations from active animals. Therefore, slight decrease in microviscosity of microsomal membranes during hibernation is observed that most likely is connected with the small decrease in protein content in membranes that was discussed above.

The hydrophobic fluorescent probe pyrene penetrates into the deep areas of lipid bilayer where the fatty acid chains of lipids are located, and parameters of pyrene fluorescence allow characterization of microviscosity of probe surrounding and hydrophobic volume of the membrane [55, 56]. The excimerization level of pyrene – the ratio of fluorescence intensity of excimers (dimers) and monomers of the probe – is the most sensitive parameter because formation of pyrene dimers at its particular concentration is controlled by the rate of diffusion, i.e. depends directly on microviscosity and hydrophobic volume of the membrane. Moreover, pyrene allows estima-

Fatty acid composition and cholesterol content in preparations of microsomes from kidneys of active and hibernating ground squirrels

Parameter	Active (n = 8)	Hibernating (n = 8)
Molar fraction, %		
fatty acids and aldehydes		
16:0 palmitic*	23.5 ± 0.4	21.1 ± 0.3
18:2 linoleic*	15.0 ± 0.8	17.7 ± 0.7
18:1 oleic*	12.3 ± 1.0	15.7 ± 0.8
18:0 stearic*	17.6 ± 0.4	16.0 ± 0.3
20:4 arachidonic**	18.0 ± 1.1	15.6 ± 0.9
14:0 myristic*	0.7 ± 0.1	2.0 ± 0.4
16:1 hexadecenic**	2.6 ± 0.5	3.1 ± 0.2
16a palmitic aldehyde*	1.5 ± 0.2	1.0 ± 0.1
18a stearic aldehyde***	3.1 ± 0.5	3.4 ± 0.2
20:3 eicosatrienoic**	1.9 ± 0.3	1.1 ± 0.3
others in total*	3.8 ± 0.2	3.2 ± 0.1
unsaturated fatty acids in total*	51 ± 3	54 ± 2
Total level of unsaturation of fatty acids, number of double bonds per molecule of fatty acid***	1.22 ± 0.03	1.20 ± 0.03
Ratio		
lipid/protein, mg/mg**	1.1 ± 0.4	1.4 ± 0.4
cholesterol/lipid, mg/g*	40 ± 9	75 ± 4

* $p < 0.05$.
** $p > 0.1$.
*** $p > 0.5$.

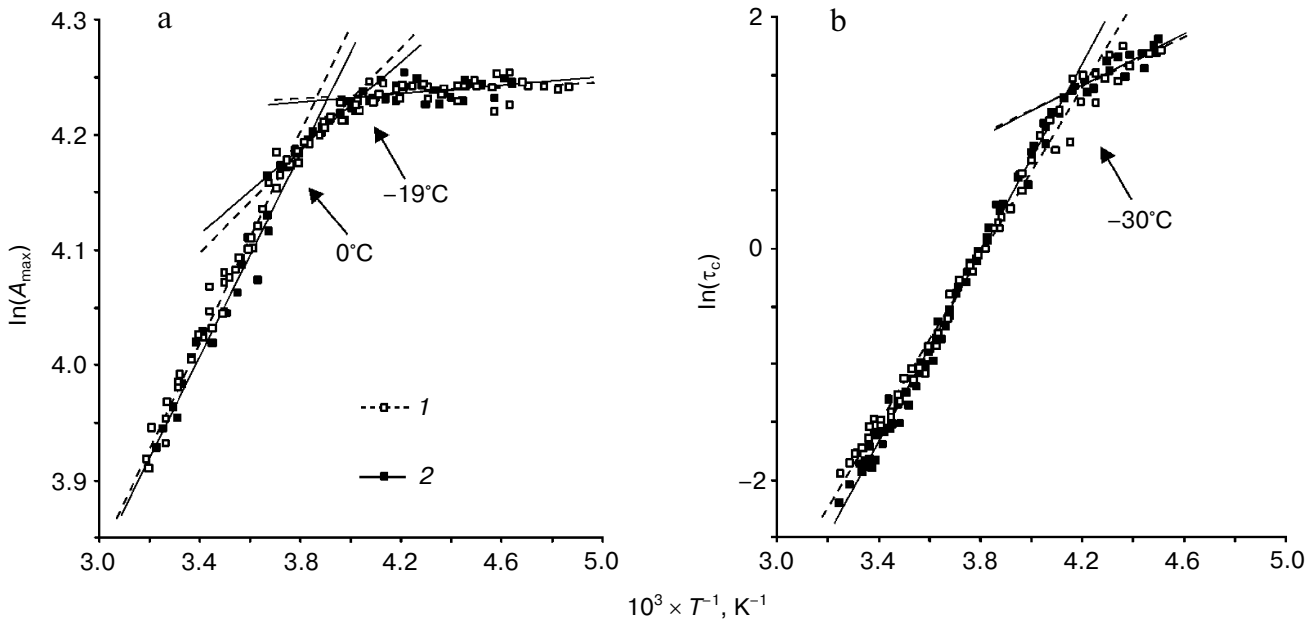

Fig. 5. Arrhenius plots for A_{max} value (a) and τ_c (nsec) (b) calculated from EPR spectra of 5-doxylstearate and methyl ester of 16-doxylstearate, respectively, in membranes of microsomes from kidneys of active (*1*, $n = 3$) and hibernating (*2*, $n = 3$) ground squirrels. The arrows indicate the areas of structural transition.

tion of the properties of annular lipids, which are located in close proximity to hydrophobic areas of integral membrane proteins because non-radiative resonance fluorescence energy transfer is possible for the tryptophan–pyrene pair. After excitation of tryptophan residues in hydrophobic domains of protein molecules, some part of the energy is transmitted to probe molecules located in the range of the Forster radius, which results in appearance of so-called "induced" pyrene fluorescence [37, 55, 56].

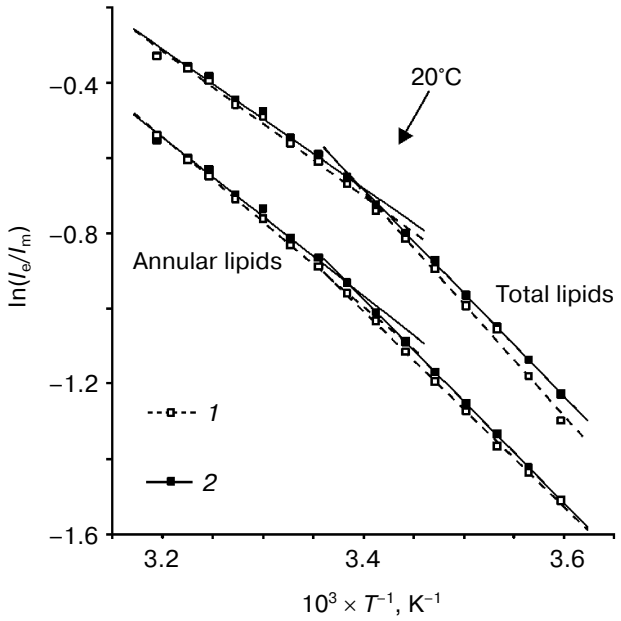

Fig. 6. Arrhenius plots for excimerization level of pyrene (I_e/I_m) in membranes of microsomes from kidneys of active (*1*, $n = 7$) and hibernating (*2*, $n = 4$) ground squirrels. The arrow indicates the area of structural transition.

We did not find significant differences in excimerization level of pyrene incorporated into microsomal membranes from kidneys of active and hibernating ground squirrels for both total and annular lipids that are located in the range of Forster radius from the membrane proteins ($p > 0.5$). Arrhenius plots for excimerization level of pyrene in microsomes from kidneys of active and hibernating ground squirrels have breaks at the temperature of about 20°C (Fig. 6). At the same time, the excimerization level of pyrene for annular lipids is lower by 20-25% than that for total membrane lipids in microsome membranes of both active and hibernating ground squirrels, which is probably connected with structural influence of protein on membrane lipids or could be explained by location of proteins in lipid rafts that have higher contents of saturated fatty acids and cholesterol.

Therefore, the data obtained using GC-MS and spin probes and the fluorescent probe pyrene indicate that significant changes in composition and structural state of membrane lipid phase in kidneys of ground squirrels, which could lead to observed significant decrease in Na,K-ATPase activity, do not occur during hibernation. However, other changes that could affect activity of integral membrane enzymes could occur in membranes during hibernation. In particular, among these changes the lateral separation of proteins and lipids takes place during hibernation that was described for endoplasmic reticulum

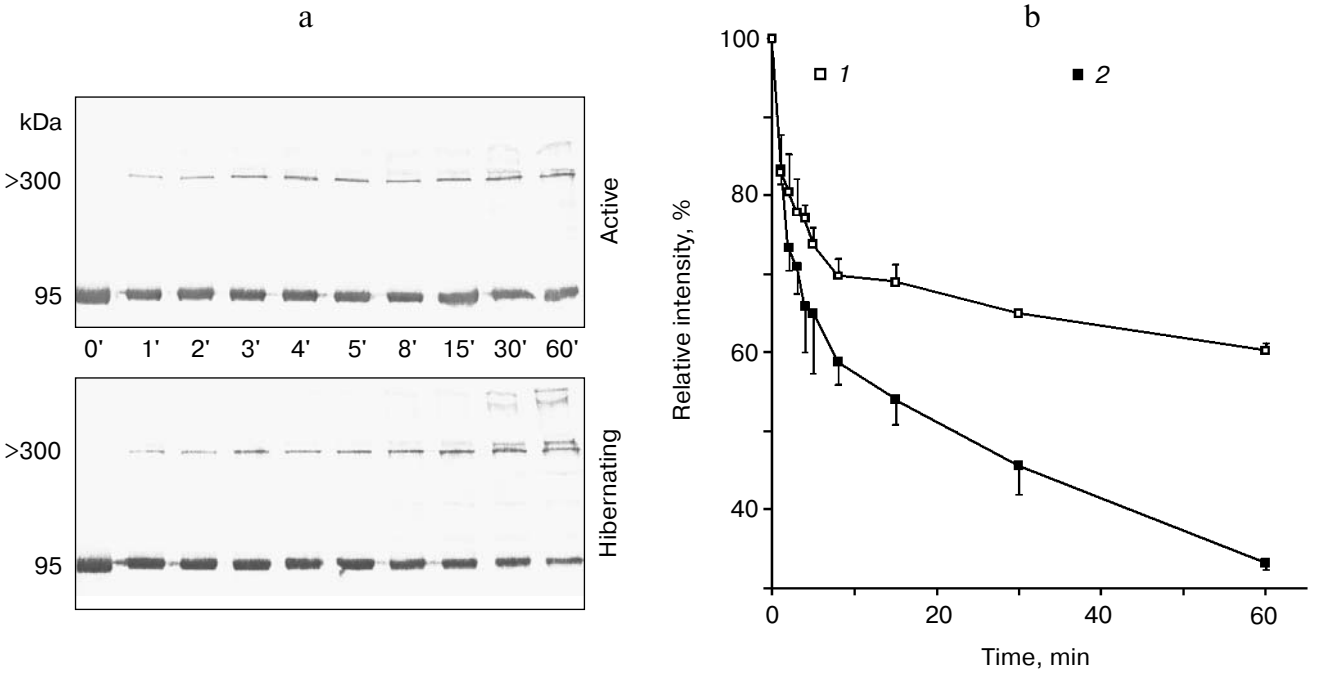

Fig. 7. Immunochemical staining of Na,K-ATPase α1-subunit in preparations of microsomes from kidneys of ground squirrels treated by cupric *o*-phenanthroline (a) and the decrease in monomeric form of Na,K-ATPase α1-subunit (95 kDa) upon time of incubation of preparations with the cross-linking agent (b). *1*, *2*) Active ($n = 3$) and hibernating ($n = 3$) ground squirrels, respectively.

of nervous tissue of ground squirrels [57]. Such separation of membrane components should be accompanied by protein clusterization and aggregation that was described earlier for sarcoplasmic reticulum Ca-ATPase from skeletal muscles of ground squirrels, the activity of which is decreased during hibernation mote than twofold [37]. It was already suggested that the formation of aggregates of ion-motive ATPases can result in their inhibition [58, 59].

The decrease in energy migration from tryptophan residues to pyrene via resonance energy transfer can be considered as indirect indication of protein aggregation in membranes because after aggregation a significant part of aggregated proteins becomes "inaccessible" for pyrene. The value of energy migration is calculated as the ratio of "induced" pyrene fluorescence (excitation via tryptophan residues at 285 nm) to intrinsic pyrene fluorescence (excitation at 335 nm). In fact, we found that resonance energy transfer from tryptophan residues to pyrene in preparations from kidneys of hibernating ground squirrels is significantly lower (by 11-14%, $p < 0.01$) than that in preparations from kidneys of active animals. This indicates the possibility of aggregation of proteins, in particular, Na,K-ATPase, during hibernation. After the treatment of studied preparations by cross-linking agent cupric *o*-phenanthroline, which catalyses the formation of covalent S–S-bonds between neighboring protein molecules [27, 60], a progressive decrease in intensity of the protein band of the monomeric form of Na,K-ATPase α-subunit and appearance of oligomeric protein bands visualized by immunochemical staining were observed (Fig. 7a). The relative number of enzyme molecules cross-linked during 1 h was 1.7-fold higher in preparations from kidneys of hibernating ground squirrels ($p < 0.001$) in comparison with preparations from kidneys of active animals (Fig. 7b).

Therefore, during hibernation in kidneys of ground squirrels Na,K-ATPase is present in more aggregated state, which in combination with observed decrease in protein content in membranes is probably the main reason for the decrease in Na,K-ATPase activity in kidneys of the hibernating animals.

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REFERENCES

1. Lyman, C. P. (1982) *Hibernation and Torpor in Mammals and Birds*, Academic Press, New York.
2. Kalabukhov, N. I. (1985) *Hibernation of Mammals* [in Russian], Nauka, Moscow.

3. Galster, W. A., and Morrison, P. (1970) *Am. J. Physiol.*, **218**, 1228-1232.
4. Anufriev, A. I. (2008) *Mechanisms of Winter Hibernation of Small Animals in Yakutiya* [in Russian], SO RAN Publishing, Novosibirsk.
5. Anufriev, A. I., Akhremenko, A. K., and Arkhipov, G. G. (1986) *Abstr. XI All-Union Symp. Adaptation of Humans and Animals* [in Russian], Yakutsk, pp. 69-70.
6. Anufriev, A. I., and Akhremenko, A. K. (1990) *Ekologiya*, **21**, 68-72.
7. Geiser, F. (1988) *J. Comp. Physiol.*, **B158**, 25-37.
8. Heldmaier, G., and Ruf, T. (1992) *J. Comp. Physiol.*, **B162**, 696-706.
9. Geiser, F., and Ruf, T. (1995) *Physiol. Zool.*, **68**, 935-966.
10. Schmidt-Nielsen, K. (1982) *Animal Physiology: Adaptation and Environment* [Russian translation], Mir, Moscow.
11. Popova, N. K. (1979) in *Ecological Physiology of Animals*, Pt. I [in Russian], Nauka, Leningrad, pp. 187-198.
12. Berezov, T. T., and Korovkin, B. F. (2002) *Biological Chemistry* [in Russian], Meditsina, Moscow.
13. El Hachimi, Z., Tijane, M., Boissonnet, G., Benjouad, A., Desmadril, M., and Yon, J. M. (1990) *Comp. Biochem. Physiol.*, **B96**, 457-459.
14. Brooks, S. P. J., and Storey, K. B. (1992) *J. Comp. Physiol.*, **B162**, 23-28.
15. Fedotcheva, N. J., Sharyshev, A. A., Mironova, G. D., and Kondrashova, M. N. (1985) *Comp. Biochem. Physiol.*, **B82**, 191-195.
16. Bronnikov, G. E., Vinogradova, S. O., and Mezentseva, V. S. (1990) *Comp. Biochem. Physiol.*, **B97**, 411-415.
17. Charnock, J. S., and Simonson, L. P. (1978) *Comp. Biochem. Physiol.*, **B60**, 433-439.
18. Bennis, C., Cheval, L., Barlet-Bas, C., Marsy, S., and Doucet, A. (1995) *Pflugers Arch.*, **430**, 471-476.
19. Walter, H. (1975) *Eur. J. Biochem.*, **58**, 595-601.
20. Jorgensen, P. L., and Skou, J. C. (1971) *Biochim. Biophys. Acta*, **233**, 366-380.
21. Besch, H. R., Jr., Jones, L. R., Fleming, J. W., and Watanabe, A. M. (1977) *J. Biol. Chem.*, **252**, 7905-7908.
22. Bonnafous, J. C., Dornand, J., and Mani, J. C. (1979) *Biochem. Biophys. Res. Commun.*, **86**, 536-544.
23. Jorgensen, P. L. (1974) *Meth. Enzymol.*, **32**, 277-290.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265-275.
25. Shorina, E. A., Dolgova, N. V., Rubtsov, A. M., Storey, K. B., and Lopina, O. D. (2004) *Biochim. Biophys. Acta*, **1661**, 188-195.
26. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
27. Shorina, E. A., Mast, N. V., Lopina, O. D., and Rubtsov, A. M. (1997) *Biochemistry*, **36**, 13455-13460.
28. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350-4354.
29. Dolgova, N. V., Kamanina, Yu. V., Akimova, O. A., Orlov, S. N., Rubtsov, A. M., and Lopina, O. D. (2007) *Biochemistry (Moscow)*, **72**, 863-871.
30. Kondrashev-Lugovskii, A. S., Malysheva, A. N., Storey, K. B., Lopina, O. D., and Rubtsov, A. M. (2004) *Biol. Membr. (Moscow)*, **21**, 491-497.
31. Hawkins, C., Xu, A., and Narayanan, N. (1994) *J. Biol. Chem.*, **269**, 31198-31206.
32. Pelosi, M., and Donella-Deana, A. (2000) *Biochemistry (Moscow)*, **65**, 259-266.
33. Kondrashev-Lugovskii, A. S. (2009) Ph. D. Thesis [in Russian], MSU, Moscow.
34. Osipov, G. A. (2010) in *Chemical Analysis in Medical Diagnostics* [in Russian], Nauka, Moscow, pp. 293-368.
35. Marsh, D. (1981) in *Membrane Spectroscopy* (Grell, E., ed.) Springer Verlag, Berlin, pp. 76-81.
36. Fung, L. W.-M., and Johnson, M. E. (1984) *Curr. Top. Bioenerg.*, **13**, 107-115.
37. Malysheva, A. N., Storey, K. B., Ziganshin, R. Kh., Lopina, O. D., and Rubtsov, A. M. (2001) *Biochemistry (Moscow)*, **66**, 918-925.
38. Charnock, J. S., and Bashford, C. L. (1975) *Mol. Pharmacol.*, **11**, 766-774.
39. Boldyrev, A. A. (1982) in *Advances in Science and Technology. Series Biological Chemistry* [in Russian], Vol. 17, PIK VINITI, Moscow, pp. 75-146.
40. Barlet-Bas, C., Arystarkhova, E., Cheval, L., Marsy, S., Sweadner, K., Modyanov, N., and Doucet, A. (1993) *J. Biol. Chem.*, **268**, 11512-11515.
41. Storey, K. B. (1997) *Comp. Biochem. Physiol.*, **A118**, 1115-1124.
42. McDonald, J. A., and Storey, K. B. (1999) *Biochem. Biophys. Res. Commun.*, **254**, 424-429.
43. Middleton, J. P., Khan, W. A., Collinsworth, G., Hannun, Y. A., and Medford, R. M. (1993) *J. Biol. Chem.*, **268**, 15958-15964.
44. Fisone, G., Snyder, G. L., Fryckstedt, J., Caplan, M. J., Aperia, A., and Greengard, P. (1995) *J. Biol. Chem.*, **270**, 2427-2430.
45. Blanco, G., and Mercer, R. W. (1998) *Am. J. Physiol.*, **275**, 633-650.
46. Blanco, G., Sanchez, G., and Mercer, R. W. (1998) *Arch. Biochem. Biophys.*, **359**, 139-150.
47. Chibalin, A. V., Pedemonte, C. H., Katz, A., Feraille, E., Berggren, P. O., and Bertorello, A. M. (1998) *J. Biol. Chem.*, **273**, 8814-8819.
48. Chibalin, A. V., Zierath, J. R., Katz, A. I., Berggren, P. O., and Bertorello, A. M. (1998) *Mol. Biol. Cell*, **9**, 1209-1220.
49. Grisham, C. M., and Barnett, R. E. (1973) *Biochemistry*, **12**, 2635-2637.
50. Charnock, J. S., Cook, D. A., Almeida, A. F., and To, R. (1973) *Arch. Biochem. Biophys.*, **159**, 393-399.
51. Kimelberg, H. K., and Papahadjopoulos, D. (1974) *J. Biol. Chem.*, **249**, 1071-1080.
52. Boldyrev, A., Ruuge, E., Smirnova, I., and Tabak, M. (1977) *FEBS Lett.*, **80**, 303-307.
53. Goroshinskaya, I. A., Golotina, L. Yu., Gorlo, E. I., Rovda, T. A., and Bordyushkov, Yu. N. (1999) *Vopr. Med. Khim.*, **45**, 53-57.
54. Blyuzdin, Yu. A., Osadchaya, L. M., and Boldyrev, A. A. (1986) *Biokhimiya*, **51**, 1499-1505.
55. Vladimirov, Yu. A., and Dobretsov, G. E. (1980) *Fluorescent Probes in the Study of Biological Membranes* [in Russian], Nauka, Moscow.
56. Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum Press, New York.
57. Azzam, N. A., Hallenbeck, J. M., and Kachar, B. (2000) *Nature*, **407**, 317-318.
58. Mahaney, J. E., and Thomas, D. D. (1991) *Biochemistry*, **30**, 7171-7180.
59. Voss, J. C., Mahaney, J. E., and Thomas, D. D. (1995) *Biochemistry*, **34**, 930-939.
60. Geimonen, E. R., Batrukova, M. A., and Rubtsov, A. M. (1994) *Eur. J. Biochem.*, **225**, 347-354.