EXPERIMENTAL BIOLOGY

Development of Laboratory Rats Receiving Silver-Enriched Ration for a Long Time

E. Yu. Il'icheva, P. S. Babich, V. V. Barishpolets, N. V. Tsymbalenko, L. V. Puchkova, and N. S. Sapronov

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 153, No. 3, pp. 359-365, March, 2012
Original article submitted December 27, 2010.

We studied the effect of silver ions on the status and metabolism of copper in rats receiving Ag-diet from the first day of life and for 6 months. The effect of silver ions on copper metabolism was assessed by body weight, relative weight of organs (body weight/organ weight), oxidase activity, content of immunoreactive ceruloplasmin and copper concentration in blood serum, by the expression of copper-transporting protein genes in the liver, and copper and silver distribution in liver and brain cells. Brain functions were evaluated by open-field behavior and passive avoidance conditioning. No acute deficiency of ceruloplasmin-associated copper was observed in rats receiving silver-enriched diet starting from the early postnatal period; copper metabolism in the liver did not change, psychoemotional state and memory corresponded to the control. However, Ag-diet almost 2-fold decelerated the growth of experimental rats. We hypothesize the existence of an unknown mechanism of copper delivery to organs in rats that is activated during the early ontogeny under conditions of ceruloplasmin-associated copper deficiency.

Key Words: *copper; silver; copper deficiency during ontogeny; copper metabolism*

Copper plays a role of structural and catalytic cofactor in a variety of vital enzymes due to its unique property, existence of two stable oxidation states Cu(I)–Cu(II) [12]. Moreover, it participates in cell signaling [9]. At the same time, copper, similarly to iron, can induce the formation of reactive oxygen species (ROS) acting on cells like ionizing radiation [13]. Toxic effects of copper is prevented by special systems consisting of proteins binding Cu(I) ions and safely transport them within the cell. Many elements of this system are identified, cloned, and studied and the relation between copper imbalance, copper-induced ROS generation, and development of some severe diseases such as cancer, neurodegenerative diseases, amyloidoses, *etc*. is demonstrated. [10]. At the same time, various aspects of copper metabolism remain unknown, especially the relationships between intracellular copper metabolism (transport and distribution of copper ions in cells, their delivery to copper-containing enzymes, recycling, and excretion from cells) and its extracellular turnover (absorption of dietary copper, its distribution between extracellular pools, delivery to different cell populations, and excretion from the body). The absence of long-living radioactive copper isotopes and available methods of using its heavy isotopes impedes these studies, while the lack of information hampers the development of therapeutic approaches for diseases related to abnormal copper status. At the same time, silver ions

Institute of Experimental Medicine, North-Western Branch of the Russian Academy of Medical Sciences, St. Petersburg, Russia. ***Address for correspondence:*** puchkovalv@yandex.ru. L. V. Puchkova

Ag(I) can be used for the study of some aspects of copper metabolism. Silver ions Ag(I) are electron twins of Cu(I) ions; they bind to copper-transporting proteins and are transported in the same pathways as copper ions. Their transport in the body and cells can be traced similarly as it can be done for radioactive isotopes.

We have previously demonstrated [3] that in rats receiving AgCl with fodder, silver ions enter the liver and are incorporated into ceruloplasmin (CP), the main serum copper-transporting protein exhibiting also ferroxidase activity [8]; this leads to deficiency of CP-associated copper and disturbances in iron transport [3].

In the liver, the central organ controlling copper homeostasis in the body, the types of copper metabolism are changed during ontogeny [5,11]. After birth, the embryonic type of copper metabolism is retained; it is characterized by low CP content in the blood, copper accumulation in the liver, and the absence of its excretion with bile. During this period, copper is excreted with urine. In hepatocytes, a gene encoding Menkes ATPase (ATP7A) is expressed (this gene is not expressed in adult mammals). Activity of Wilson ATPase (ATP7B) and CP is suppressed. Transition to adult-type copper metabolism (in rats on day 13 of life) is associated with induction of *ATP7B* and *CP* gene expression and suppression of *ATP7A* gene. Simultaneously, copper excretion with bile is started, copper concentration in the liver decreases, while the concentration of CP and copper increases. The effect of silver ions on copper metabolism during ontogeny in mammals was not studied. This was the aim of our study. Here we present the data on the effect of silver ions on copper metabolism in the liver and brain, the major copper-utilizing organs. The obtained results can be useful for the development of therapeutic approaches to the treatment of pathologies related to disturbances in copper metabolism. The results can also help to evaluate the consequences of silver accumulation in human body. Silver does not belong to biogenic elements, *i.e.* there are no silver-dependent enzymes or physiological process [14]. At the same time, the use of silver as the antimicrobial agent increases from year to year (in accordance with WHO recommendations). Since silver is transported by copper-transporting proteins, the presence of this metal in food, water, clothes can led to its accumulation in human tissues.

MATERIALS AND METHODS

The study was performed on outbred rat pups obtained from Rappolovo nursery. Starting from the first day of life, the pups were divided into 4 groups. Group 1 comprised controls. Group 2 consisted of pat pups fed by dams receiving AgCl starting from the first day of lactation (Ag-dams) and transferred to standard ration starting from day 23 of life. Group 3 rats received Ag-diet starting from day 23 of life. Group 4 pups were fed by Ag-dams and then (starting from day 23) received food supplemented with AgCl. AgCl concentration in the food was 50 mg/kg body weight per day. All animals had unrestricted access to food (GOST R0258-92) and tap water. Copper content in the ration of all groups remained standard. The animals were maintained at 25°C, ~60% humidity, and 12-h dark:light regimen (automatically controlled). In 5, 20, 40, and 180 days, 5-10 rats from each group were weighed, decapitated with a guillotine, the blood, organs, and urine were collected. Blood serum was separated by centrifugation after clot formation. A weighed sample of stomach content was resuspended in 2 volumes of physiological saline, incubated 30 min at 0°C, centrifuged at 10,000*g* and 4°C for 10 min, and the supernatant was collected. Brain activity in 6-month-old rat pups was evaluated by open-field behavior and passive avoidance conditioning [1]. The cell homogenate was fractionated by differential and equilibrium centrifugation, oxidase activity of blood serum was determined by the method of Ravin or by direct test in PAAG [3]. RT-PCR analysis and immunoblotting were previously described in details [2,3]. The concentrations of silver, copper, and iron were measured by atomic adsorption spectrometry [3].

The data were processed using integrated SPSS 9.0 software (StatSoft Inc.) by two-way ANOVA (General Linear Model) at $P<0.05$. Intergroup comparisons (Tukey and Dunnet tests) were performed in cases when significant effect was revealed by ANOVA. If the data do not fit the normal distribution law and equality of dispersions was not proven, the data were processed using Kruskal–Wallis test.

RESULTS

First, we tested whether silver ions appear in the breast milk and enter newborn rats and whether copper metabolism is changed under these conditions. The study was performed on 10-day rats fed by dams receiving AgCl with food (AgCl(10)-rats) starting from the first day of lactation. Ten-day-old rats born at the same time and fed by dams receiving standard diet served as the control. By day 10 of life, the rats of these groups did not differ by the markers of physical development (fur development, rising, tail rotation, pinna detachment). In the liver and brain of these rats, the concentrations and intracellular localization of silver and copper ions were determined and the relative content of mature gene transcripts of copper-transporting proteins and copper-containing enzymes was measured. All measurements in 10-day-old rats were repeated twice, each tissue, blood, and urine sample consisted

of aliquots taken from 3 animals. In Ag-females, CP-associated copper deficiency developed [3]: the level of oxidase CP in the blood decreased (Fig. 1, *a*), but immunoreactive CP polypeptides were detected in the circulation (Fig. 1, *b*). The polypeptides differed by electrophoretic mobility: one corresponded to oxidase CP and the other moved more slowly. None of them exhibited oxidase activity. In the serum of Ag(10)-rats, oxidase activity also decreased (Fig. 1, *a*), while immunoreactive CP was present in the same forms as in adult rats (Fig. 1, *b*). In stomach content of Ag(10)-rats, in contrast to controls, oxidase CP was not detected, but CP polypeptides were present (Fig. 1, *a*, *b*). Subcellular distribution of silver and copper ions in the liver cells of Ag(10)-rats was similar: both metals were located in the cytosol and mitochondria (Fig. 1, *c*). We studied the distribution of silver and copper in the body of Ag-females and pups fed by them (Table 1). Silver entered breast cells of lactating Ag-females and then was detected in stomach content and blood serum of pups fed by them. No decrease in copper concentration was observed in stomach content and blood of Ag(10)-rats. Body weight in Ag(10)-rats was lower than in controls by about 20%, but this difference was insignificant. In newborn rats, similarly to adults, silver was primarily accumulated in the liver and in far lower amounts was detected in the brain. No significant changes in copper concentration in these organs were revealed. Copper concentration in the liver of Ag(10)-rats increased similarly to that in 10-day-old controls and ~5-fold surpassed copper concentration in the liver of adult rats. Both silver and copper were excreted with urine. The profile and level of the expression of copper-transporting proteins (high-affinity copper transporter CTR1 that transports also Ag(I) from extracellular medium [7]; Menkes ATPase, ATP7A, and Wilson ATPase ATP7B) and copper-containing enzymes (CP, SPD1, and cytochrome C oxidase, COX) were similar in the two groups of newborn rats (Table 2). The relative content of immunoreactive polypeptides CTR1 in plasma membrane-enriched fraction and SOD1 in the cytosol also remained unchanged (Fig. 1, *d, e*).

The obtained results suggest that silver ions enter the breast cells and are incorporated into CP synthesized in them [4]. In newborn rats, silver ions incorporated into CP (Ag-CP) entering with milk are specifically absorbed by the liver and accumulated there. Silver and copper ions are transported in liver cells and in the body by the same pathways. Silver reduces copper status, but does not affect copper metabolism in the liver and exists in exchangeable form.

At the next stage of experiments we studied copper metabolism in animals of the four above-specified groups. By the 6th month of life, body weight of rats

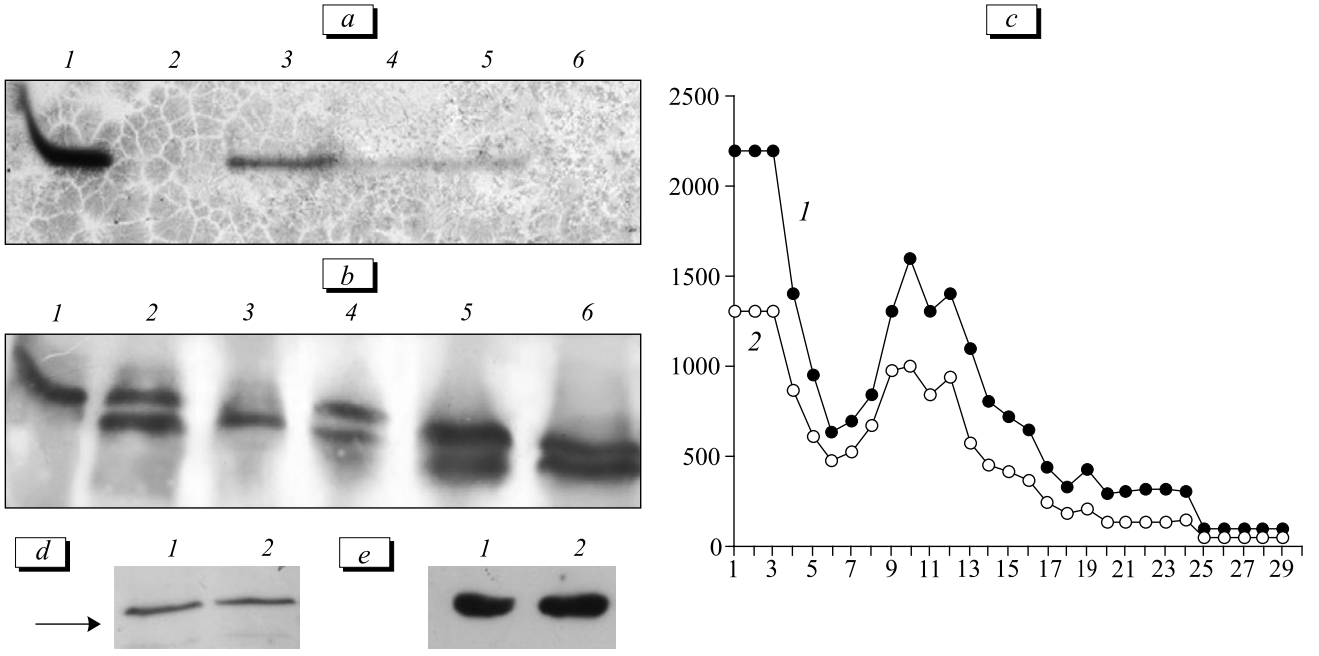

Fig. 1. Absorption, distribution, and effect of silver ions on copper metabolism in newborn rats. *a*) effect of silver ions on oxidase activity in the blood and stomach content. Samples: 1 μl blood serum and 20 μl stomach content. *b*) immunoblotting of the same samples with antibodies to rat CP; 0.1 μl blood serum and 5 μl stomach content were applied on the row. For *a*, *b*: *1*) blood serum on day 10 of lactation (control female); *2*) Ag-female; *3*) blood serum of 10-day-old control rats; *4*) Ag(10)-rats; *5*) stomach content from 10-day-old control rats; *6*) Ag(10)-rats. 7.5% PAAG after non-denaturating electrophoresis is trained with ortho-dianisidine, CP-specific chromogen. *b*) distribution of copper (*1*) and silver (*2*) ions in the liver cells of Ag(10)-rats. Abscissa: fraction number; ordinate: concentration of Ag and Cu, μg/liter. Sucrose density in fractions 9-12 corresponded to the density of mitochondria (1.2 g/cm^3). *d, e*) immunoblotting of plasma membrane and cytosol proteins with antibodies to CTR1 and SOD1, respectively. *1* and *2*: 10-day-old control and Ag(10)-rats, respectively.

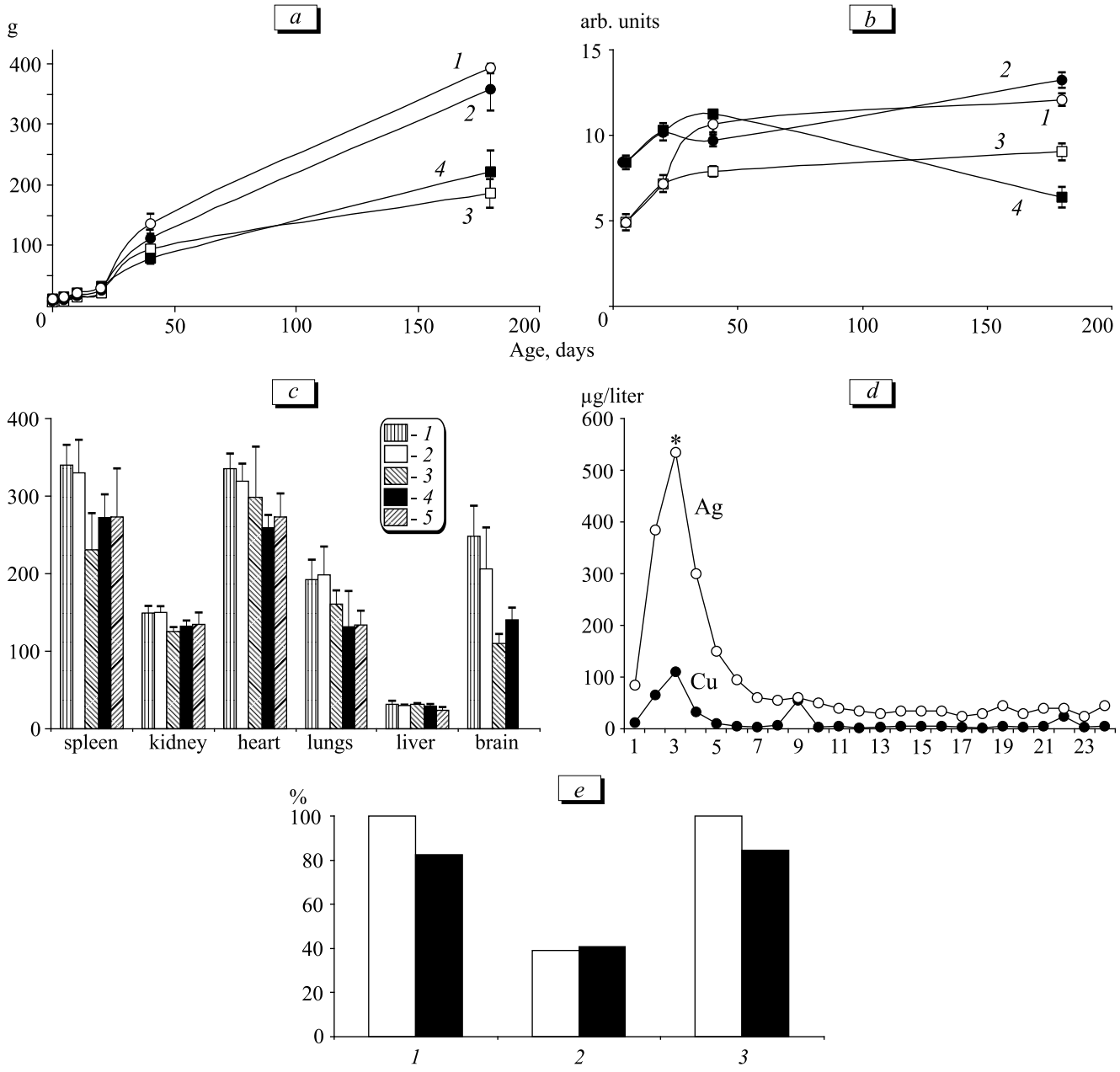

Fig. 2. Effect of silver ions on the growth and copper status in rats of groups 1-4 over 6 months of life. *a*) growth dynamics in rats of groups 1-4 (*1-4*); *b*) changes in oxidase activity. Oxidase activity was calculated using Scion Image software from the intensity of electrophoretic zones stained with ortho-dianisidine. *c*) body weight/organ weight ratio; *1-4*: groups 1-4, *5*) body weight/organ weight ratio in 2-month-old control rats. *d*) copper and silver distribution profile in blood serum. Gel-filtration of blood serum sample on a column with sephadex G75. Abscissa: fraction number. *Fraction with maximum content of CP (immunoblotting data). *e*) CP-associated copper (*1*, *2*) and silver (*3*) in the serum of control rats (*1*) and animals receiving Ag-diet for 10 days (*2*, *3*). Open bars: whole serum; dark bars: immunoprecipitate.

in groups 3 and 4 was 2-fold lower than in groups 1 and 2 (Fig. 2, *a*) and corresponded to body weight of 2-month-old rats; the delay in body weight gain was observed starting from day 10 of life. Serum oxidase activity in groups 3 and 4 was lower by ~2 times (Fig. 2, *b*) and blood copper concentration was proportional to oxidase activity (500±120 vs. 1200±210 µg/liter). Body weight/organ weight ratio in rats of groups 3-4 did not significantly differ from that in age-matched rats of groups 1-2 (Fig. 2, *c*); it remained unchanged when 2-month-old rats with the same body weight as 6-month-old Ag-rats were used as the control. The differences between groups 1-2 and 3-4 were revealed only for relative brain weight. This can be explained by the fact that brain weight in adults increases slower that body weight and weight of internal organs. This assumption is confirmed by similar absolute brain weight in animals of all groups.

TABLE 1. Distribution of Silver and Copper Ions in the Body of Controls and Ag(10)-Rats

Parameter	Lactating female		Newborn rats*	
	control	Ag-female	10-day-old rats (control)	Ag(10)-rats
Silver in the breast tissue, μg/g tissue	0.15	4.7	-	-
Silver in stomach content extracts, μg/g	-	-	1.0	35
Calcium in blood plasma, μg/liter	30	10050	10	460
Copper in stomach content extracts, μg/g	-	-	4.5	5.2
Copper in blood serum, μg/liter	3500	1500	600	650
Body weight, g	-	-	21.0±2.5 (*n*=8)	16.8±1.9 (*n*=8)
Copper in the liver, μg/g tissue	15.0	14.8	90.0	105.0
Silver in the liver, μg/g tissue	0.5	170	0.3	20
Copper in the brain, μg/g tissue	9.0	8.2	3.0	2.9
Silver in the brain, μg/g tissue	0.1	5.0	0.2	2.1
Copper in urine, μg/liter	-	-	185	160
Silver in urine, μg/liter	-	-	5	170

Note. *Means of 2 measurements are presented; in each measurement tissue, serum, or urine aliquots taken from 3 animals were mixed. –: not determined.

In the serum of group 2 rats silver was detected only when the pups were fed by Ag-female (on average 600 μg/liter). In rats of groups 3 and 4, silver concentration remained unchanged starting from the 40th day of life and to the age of 6 months: 1500 μg/liter (from 800 to 2100 μg/liter in some rats). On chromatogram of blood serum, silver was detected in a single peak corresponding by CP (immunoblotting data, Fig. 2, *d*). No low-molecular-weight fractions containing silver were detected. The data presented in Fig. 2, *e* also show that silver in the blood was present primarily in the complex with CP. Thus, serum content of silver corresponds to that of CP containing silver atoms [6].

TABLE 2. Expression of Genes Encoding Copper-Transporting Proteins and Copper-Containing Enzymes in the Liver of Control and Ag(10)-Rats

Gene	Relative content of mature transcripts, arb. units*	
	10-day-old rats (control)	Ag(10)-rats
CP-mRNA	1.3	1.4
CTR1-mRNA	0.5	0.6
ATP7A-mRNA	0.25	0.22
ATP7B-mRNA	0	0
SOD1-mRNA	2.0	1.7
COX-mRNA	0.8	0.8

Note. Measurements were performed on 3 samples of total RNA from the liver of 3 animals from each group. Mean values are presented; the range within the group did not exceed 10%. *Relative to the level of mature transcripts of β-actin.

Copper concentration in the liver in all groups increased during the first days of life, but after transition to adult type of copper metabolism this parameter sharply decreased (Fig. 3). The specific copper content was similar in all groups. The exception was copper content in the liver of 5-day-old control rats and rats fed by Ag-females (Fig. 3). However, in rats receiving silver with breast milk, copper concentration was also significantly higher that in adult rats. Copper concentration in the brain increased approximately until maturation and was similar in all groups (Fig. 3). Silver accumulation in the liver and brain continued throughout the experiment (Fig. 3). However, silver concentration in the brain was 10-fold lower than in the liver. Iron content in the liver, brain, and blood serum of group 3-4 animals did not differ from the control (data not presented).

No changes in animal behavior (mobility, food intake, relationships within the group) were revealed. No deaths or cannibalism were observed. Open-field test revealed no significant differences in psychoemotional status of animals receiving silver-enriched diet (Table

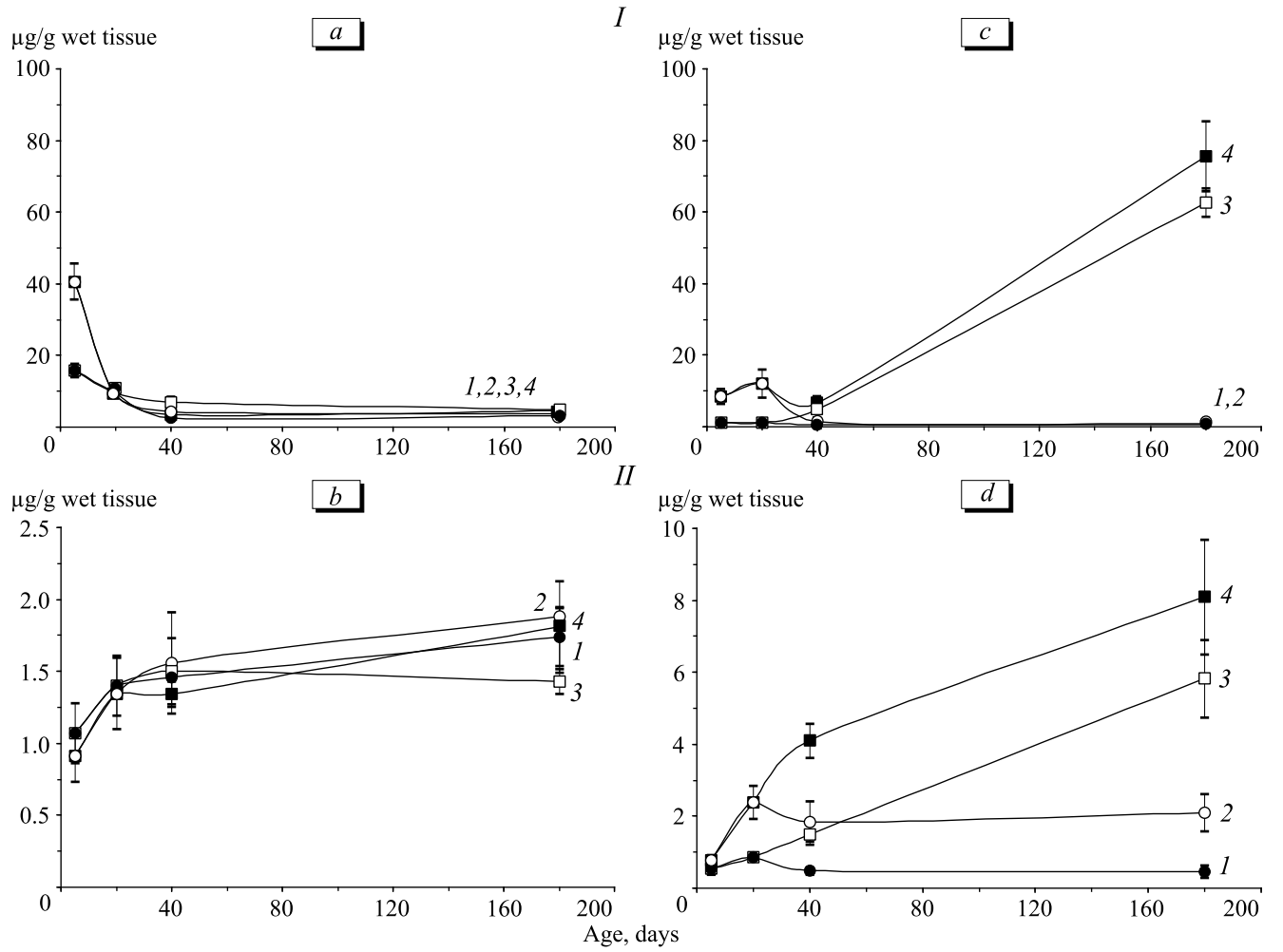

Fig. 3. Changes in the content of copper (*a*, *b*) and silver (*c*, *d*) in the liver (I) and brain (II) of growing rats of groups 1-4 (*1-4*, respectively)

3). Passive avoidance performance was not disturbed in all rats irrespective of the period of silver supplementation (Table 3).

When planning the experiments, we expected to detect signs of neurodegeneration typical of states related to oxidase CP deficiency in groups 3 and 4 [10,15]. On the contrary, copper status in these rats decreased by only 2 times and no changes in copper and iron distribution in the liver and brain as well as in their psychoemotional status and memory were revealed, probably because CP-associated copper deficiency in our experiments did not develop before tran-

TABLE 3. Quantitative Evaluation of Open-Field Behavior and Passive Avoidance Testing

Group	Locomotor activity	Exploratory activity		Emotionality		Passive avoidance
	ambulation, secotrs	number of rearings	number of explored holes	grooming	defecation	latency, sec
1 (*n*=6)	29.0±1.9	8.4±0.8	2.9±0.8	1.4±0.4	1.6±0.2	142±10
2 (*n*=5)	30.8±3.0	13.4±1.9*^{o}	4.2±0.9^{o}	1.6±0.6	1.0±0.4*	180±7
3 (*n*=11)	22.0±1.9*$^{+o}$	11.4±1.4^{o}	2.0±0.4^{+}	0.9±0.3^{o}	0	180±5
4 (*n*=7)	30.0±2.9	5.8±0.8^{+}	1.9±0.5^{+}	2.6±0.7	0	180±9

Note. $p \leq 0.05$ in comparison with: *group 1, $^{+}$group 2, ^{o}group 4.

sition to adult type of copper metabolism (Table. 1). Differentiation of neurons responsible for production of neuropeptides and neurotransmitters (processing of these substances is catalyzed by copper-containing brain-specific enzymes) is completed by this time [16]. The existence of compensatory mechanisms maintaining homeostasis of CP-associated copper under conditions of its deficiency in mammals at the early stages of their ontogeny can also be hypothesized.

We cannot explain growth retardation observed in all rats of groups 3 and 4. This phenomenon can be related to an unknown role of copper in the regulation of bone metabolism and requires further investigation, because this information can help to understand the role of copper in fundamental processes controlling bone growth.

REFERENCES

1. Ya. Buresh, O. Bureshova, and J. P. Huston, *The Techniques and Basic Experiments for the Study of Brain and Behavior* [Russian translation], Moscow (1991).
2. A. V. Vasin, S. A. Klotchenko, N. A. Platonova, *et al., Mol. Biol*., **39**, 48-60 (2005).
3. S. A. Klotchenko, N. V. Tsymbalenko, K. V. Soloviev, *et al., Dokl. Akad. Nauk*, **418**, 549-552 (2008).
4. L. V. Puchkova, T. D. Aleinikova, N. V. Tsymbalenko, *et al., Biokhimiya*, **59**, 341-348 (1994).
5. L. V. Puchkova and N. A. Platonova, *Uspekhi Sovr. Biol*., **123**, 41-58 (2003).
6. A. N. Skvortsov, E. Yu. Ilyechova, E. A. Zatulovskii, *et al., Thitologiya*, **52**, 70-76 (2010).
7. J. Bertinato, L. Cheung, R. Hoque, and L. J. Plouffe, *J. Trace Elem. Med. Biol.*, **24**, No. 3, 178-184 (2010).
8. P. Bielli and L. Calabrese, *Cell. Mol. Life Sci.*, **59**, 1433-1447 (2002).
9. E. Burstein, L. Ganesh, R. D. Dick, *et al., EMBO J.*, **23**, No. 1, 244-254 (2004).
10. E. Gaggelli, H. Kozlowski, D. Valensin, and G. Valensin, *Chem. Rev.* **106**, No. 6, 1995-2044 (2006).
11. L. S. Hurley, C. L. Keen, B. Lonnerdal, *Ciba Foundation Symp.,* **80**, 227-245 (1980).
12. S. Krupanidhi, A. Sreekumar, C. B. Sanjeevi, *Indian J. Med. Res.,* **128**, No. 4, 448-461 (2008).
13. M. C. Linder, *Mutat. Res*., **475**, Nos. 1-2, 141-152 (2001).
14. F. H. Nielson, *Trace Elements in Human and Animal Nutrition*., Vol. 2, Ed. W. Mertz, San Diego (1986), pp. 415-463.
15. T. Oide, K. Yoshida, K. Kaneko, *et al., Neuropathol. Appl. Neurobiol.*, **32**, No. 2, 170-176 (2006).
16. M. V. Ugrumov, *Neurochem. Res*., **35**, No. 6, 837-850 (2010).