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Identification of Phosphoethanolamine and Phosphoserine in the Brain of the Pond Fish *Perccottus Glehni* (Eleotridae, Perciformes, Dyb. 1877)

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Significant modifications to the free amino acid pool and various other compounds in the bodies of poikilotherms in response to decreased temperature reflect significant alterations in their mechanisms of adaptation. The literature lacks data on the contributions of such free compounds to the low-temperature adaptation of the brains of poikilotherms. Our previous studies showed that acute cold shock induced the appearance of large quantities of two ninhydrin-positive compounds of unknown nature in the brain of the eurythermal pond fish Amur sleeper. The experiments reported here show that the brain accumulates these compounds by the beginning of the winter period. They were found to be the phospholipid metabolites phosphoethanolamine and phosphoserine. The winter phosphoethanolamine pool was 94 times greater than the summer level, while phosphoserine was present only in summer. It is suggested that accumulation of phosphoethanolamine and phosphoserine is associated with adaptive modifications of membrane phospholipids at low temperatures.

Keywords: phosphoethanolamine, phosphoserine, serine, low-temperature adaptation, fish brains.

Quantitative and qualitative changes in the free amino acid composition in the bodies of poikilothermic animals, both invertebrates and vertebrates, are due to and serve as an important indicator of the processes of adaptation to low environmental temperatures and differ radically depending on the level of organization or the habitat [1–6, 19].

The accumulation of free amino acids in invertebrates is less marked than in vertebrates [17, 20]. A general classificatory sign for accumulated (so-called "protective") free amino acids in most invertebrates is provided by the fact that all are proteinogenic, and most are glucoplastic [20].

The appearance of nonprotein amino acids in the hemolymph, plasma, and organs of poikilothermic animals at particular phylogenetic levels, along with their accumulation during the seasonal low-temperature periods and their specific response to cold shock, evidently provide a marker for transfer to a higher level of evolution. For example, our data indicate that the ornithine pool in the hemolymph of higher crustaceans, the amphipod *Gammarus lacustris*, which inhabits a zone with a large difference in summer and winter temperatures, constitutes 16.5% of the total free amino acids pool in autumn, taking second place after alanine (23.3%) [4]. The literature lacks data showing that this non-proteinogenic amino acid is detected at low temperatures in the hemolymph of invertebrates at evolutionary levels below crustaceans. The clearest example of the role of non-protein amino acids in low-temperature adaptation in vertebrates is the accumulation of taurine, which is seen in the plasma and muscles of freshwater fish in the winter period [2]; after the decrease in temperature to negative values, it appears in the plasma and organs in reptiles [13, 27]; among homeothermic animals, it is seen in the blood and liver of hibernating hedgehogs during winter hibernation and artificial summer hypothermia [8]. Despite the fact that low-temperature taurine accumulation is not detected in invertebrates, including freshwater mollusks, or in worms or

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TABLE 1. Changes in Amino Acid and Phosphoethanolamine Pools in the Brain of *P. Glehni* in the Winter Period (nmol/g wet tissue)

Amino acid	July	December
Phosphoserine	n.d.	410 ± 43
Taurine	3897 ± 166	905 ± 66
Phosphoethanolamine	39 ± 15	3663 ± 137
Aspartic acid	82 ± 7	n.d.
Serine	n.d.	614 ± 37
Glutamic acid	2079 ± 140	344 ± 22
Glycine	947 ± 53	669 ± 40
alanine	896 ± 39	717 ± 66
Tyrosine	77 ± 9	n.d.
GABA	1105 ± 84	1137 ± 70
Histidine	3290 ± 283	2167 ± 94
Total NFA pool	12412 ± 524	10627 ± 226
Total EFA pool	863 ± 50	304 ± 28
Total FA pool	13275 ± 481	10931 ± 198

Notes (here and Table 2). NFA – non-essential free amino acids and phosphoethanolamine; EFA – essential free amino acids, data for individual responses not shown. Total pool (FA) – sum of the concentrations of free amino acids into the total NFA, phosphoethanolamine, and EFA pools. Cysteine, proline, arginine not detected. GABA – γ-aminobutyric acid. n.d. – not detected.

insects [20], mollusks and mussels in the littoral zone are exceptions to this: taurine is present in the organs of these animals in very large quantities [14, 26].

Studies of the responses of free amino acids in the brains of poikilotherms in hypothermic conditions, including the seasonal temperature drop, have not been reported. At the same time, our previous study [3], the first in this area, used chromatographic analysis of free amino acids and ninhydrin-positive compounds to show that acute cold exposure produced the accumulation of two ninhydrin-positive substances in the brains of pond fish, i.e., Amur snappers (*P. glehni*), which have the ability to hibernate in the winter period. The levels of these substances increased with increases in the duration of exposure to low temperature; taurine is displaced, such that the taurine pool in all other organs of vertebrate poikilotherms increases intensely as environmental temperature decreases [2, 13, 27].

The nature of these substances was unknown; additional studies were required for their identification. Thus, the aims of the present work were: 1) to determine whether seasonal temperature drops would induce these unknown substances in the brains of pond fish; 2) to identify the molecular nature of these substances; 3) to identify differences in the low-temperature modifications of amino acid pools in the brains of fish as compared with their modifications in muscle tissue.

Methods

Study system. Amur sleepers *P. glehni* were caught in Lake Tulchino (in the basin of the Oka River, 54°50' N, 37°42' E) at the end of the third ten days of June and the beginning of July and in the first 10 days of December, when the lake was covered with ice. Individuals with mean weight 28.3 ± 2.5 g were collected.

The brain and anterior part of the dorsal musculature without skin were prepared one day after the fish were caught, as the animals had to return to normal after capture-related stress. Tissues were homogenized in double-distilled water (1:9). Homogenates were centrifuged at 15000*g* for 20 min at 0°C on a K-24 centrifuge (Germany). Supernatants after centrifugation were stored at –10°C until use. After thawing, supernatants were recentrifuged at 12000 *g* for 10 min.

Assay of amino acids and ninhydrin-positive compounds. Free amino acid concentrations in brain homogenate supernatants were assayed by ion exchange liquid chromatography on a T339 automatic amino acid analyzer (Mikrotekh, Czech Republic) in a system consisting of a three-step gradient of sodium citrate buffer [25]. Post-column modification of amino acids was performed with ninhydrin and staining intensities were measured at 570 nm. A standard mix of 21 amino acids and phosphoethanolamine, which, like amino acids, stains blue with ninhydrin, was chromatographed for each series of experiments. Free amino acid contents were expressed as nmol/g wet weight.

Statistical analysis was performed in the statistical suite for Microsoft Excel 2010. Data were expressed as the mean of four parallel measurements ($n = 4$), each of which used three individuals (a total of 12 individuals) ± error of the mean (M ± SEM).

All chemical reagents were from Sigma Chemical Co. (USA).

Results

Free amino acid levels in brain. Results obtained from analysis of modifications to the pool of amino acids and the two ninhydrin-positive compounds noted in the previous

TABLE 2. Changes in Amino Acid and Phosphoethanolamine Pools in *P. Glehni* Muscle Tissue Extract in the Winter Period (nmol/g wet tissue)

Amino acid	July	December
Phosphoserine	n.d.	n.d.
Taurine	3258 ± 360	14056 ± 991
Phosphoethanolamine	n.d.	n.d.
Aspartic acid	70 ± 10	n.d.
Serine	904 ± 97	456 ± 42
Glutamic acid	971 ± 83	1504 ± 110
Proline	1237 ± 120	n.d.
Glycine	2004 ± 155	1466 ± 58
Alanine	1497 ± 132	2924 ± 328
Cystationine	535 ± 51	286 ± 30
GABA	n.d.	2405 ± 135
Tyrosine	1076 ± 88	942 ± 111
Histidine	2062 ± 122	433 ± 45
Arginine	495 ± 45	n.d.
Total NFA pool	13250 ± 1310	24715 ± 2155
Total EFA pool	4630 ± 253	2000 ± 165
Total pool	17880 ± 1020	26715 ± 904

study in the brains of *P. glehni* during the winter period are shown in Table 1. The area of the chromatogram at which the two unidentified compounds noted in previous studies of the effects of cold shock in the early autumn period [3] were located again contained two peaks of different heights in the present study. Comparison with chromatograms with the chromatogram of the standard mix showed that one of the peaks, of smaller height, could be assigned to the non-proteinogenic amino acid phosphoserine. The higher peak corresponded to phosphoethanolamine – a phosphomonoester or ester of phosphoric acid and ethanolamine.

Chromatographic analysis showed that the phosphoethanolamine pool was minimal in summer, though it showed a 94-fold increase at the beginning of the winter period to constitute one third of the total free amino acids pool (Table 1). The range of changes – from 39 ± 15 nmol/g wet weight in the summer to 3663 ± 137 nmol/g in the winter – occurred in the phosphoethanolamine level in the brain in less than half a year. It is interesting to note that an analogous reorganization occurred in the serine pool, which was virtually undetectable in the brain in the summer, but reached a level of 614 ± 37 nmol/g by the beginning of winter (Table 1). In turn, the glutamic acid pool decreased more than six-fold from summer to winter – from 2079 ± 140 to 344 ± 22 nmol/g (Table 1).

The non-proteinogenic sulfoamino acid taurine was the most abundance in the brain in summer, as compared with other free amino acids; its level was 3897 ± 166 nmol/g or about one third of the total amino acid pool. The change in the taurine pool was opposite to that of phosphoethanolamine. Thus, the taurine content in December decreased more than four-fold compared with the July level, to 905 ± 66 nmol/g ($p < 0.05$), or 8.9% of the total pool (Table 1). The glycine, alanine, and histidine pools decreased by December (particularly the histidine pool), though to a lesser extent. Aspartic acid and tyrosine were not detected in the winter period. The total pool of nonessential free amino acids and phosphoethanolamine decreased in December from the summer level, from 12412 ± 524 to 10627 ± 226 nmol/g, while the total pool of essential amino acids (not shown individually in Table 1) decreased almost three-fold. After taurine, the largest pools were, in decreasing order, histidine and glutamic acid, followed by GABA, glycine, and alanine (Table 1).

Free amino acid levels in muscle tissue are shown in Table 2. In contrast to brain, phosphoethanolamine and phosphoserine in muscle tissue were not detected either in summer or in winter.

In *P. glehni* muscle tissue (like brain), a radical low-temperature and pre-hibernation reorganization of free amino acid compartments takes place by the beginning of winter. This period was characterized by decreases in the pools of most proteinogenic amino acids, with a two-fold drop in in the total pool of essential amino acids from 4630 ± 253 to 2000 ± 165 nmol/g wet weight (Table 2). However, along with the decrease in the pools of most nonessential and all essential amino acids, the total amino acid pool in muscle tissue increased in winter, from 17880 ± 1020 to 26715 ± 904 nmol/g wet weight.

Compensation for the loss of proteinogenic amino acids in the winter period was provided by accumulation of non-proteinogenic amino acids: GABA, which was not detected in summer, increased to 2405 ± 135 nmol/g wet weight in winter. However, the greatest increase was in the taurine level, the pool of which, in contrast to its drop in the brain, increased 4.3-fold in muscle tissue to reach 14056 ± 991 nmol/g wet weight. In contrast to the three-fold drop in the brain, the glutamic acid level in muscle tissue increased significantly.

Discussion

The most important result obtained here was the separation of free amino acids and ninhydrin-positive com-

pounds forming two large peaks on chromatograms. The positions of these peaks coincided with the peaks of the unidentified substances in the previous experiment using cold shock [3]. Comparison of the chromatograms of standard solutions and the study material showed that the positions of the peaks corresponded to the positions of phosphoethanolamine and phosphoserine, which in the present experiments were included in the standard mix. Thus, the seasonal drop in temperature to winter levels, like the action of cold shock [3], stimulated the appearance of two new compounds – phosphoethanolamine and phosphoserine – in the brains of eurythermal freshwater fish (Table 1). The appearance of phosphoethanolamine and phosphoserine is a characteristic feature of the brains of fish, as these substances were not detected in muscle tissue extract (Table 2) or plasma [2] either in the summer or in the winter, or on exposure to cold [3]. Surprisingly, our literature search yielded only one report on the presence of phosphoethanolamine – in the skin of marine fish, albeit in small quantities [16].

It is also interesting that the absence of serine from the brain in summer and its high level in winter (Table 1) and the very significant increase on exposure to cold shock, as demonstrated in our previous experiments [3], are also characteristic features of the low-temperature adaptation of the brain in *P. glehni*. All three substances – phosphoethanolamine, phosphoserine, and serine – are present in bound form in the structure of membrane phospholipids and are involved in their metabolism [10, 15, 18, 29, 30]. The accumulation of phosphoethanolamine, phosphoserine, and serine probably reflects the processes of reorganization occurring in the lipid components of cytoplasmic membranes and/or the membranes of cell organelle at low temperatures.

Phosphoethanolamine is an intermediate substrate in the synthesis of phosphatidylethanolamine (PEA) and phosphatidylcholines [15, 30] and is also present in the structure of sphingomyelin [24]. At the same time, there are no published data on the presence of free phosphoethanolamine in the brains of poikilotherms; there are only two reports of its detection in the whole body in coleopterans [17, 23]. Phosphoethanolamine accumulation evidently cannot be explained in terms of phospholipase C-mediated cleavage of the bond between glycerol and the phosphate group in PEA, as this enzyme is not seen in eukaryotes in relation to this class of phospholipids [28]. It can be suggested that phosphoethanolamine accumulation is due to impaired PEA synthesis at the stage at which phosphoethanolamine interacts with cytidine triphosphate (CTP) by means of CTP:PEA-cytidylyltransferase [22]. In this situation, the PEA content in brain membranes should decrease, while the phosphoethanolamine content should increase. However, the literature contains data indicating not a decrease, but rather a significant increase (by 41%) in PEA in the winter period in the brain in the rainbow trout *Onchorhynchus mykiss* [17]. The quantity of PEA in microsomes and inner mitochondrial membranes in the goldfish *Carassius auratus* has also been shown to increase significantly at low temperatures (5°C) [12].

There is another suggestion: at low temperatures, the brains of eurythermal fish shows expression of a gene inducing the synthesis of the isoenzyme ethanolamine phosphokinase, which uses ATP to phosphorylate ethanolamine to form phosphoethanolamine at a higher rate than at normal temperatures. Excessive phosphoethanolamine concentrations may activate PEA synthesis at low temperatures.

The increase in PEA in the brain is so large that the decreases in the pools of proteinogenic amino acids is insufficient for retention of isoosmotic balance. The profound decrease in the summer taurine reserve (Table 1) may be related to this. In contrast to the specific processes occurring in the brain, taurine in muscle tissue (Table 2) and blood [2] is in high demand in winter and, despite the high summer level, increases several-fold, significantly enlarging the total amino acid pool (Table 2). However, it has to be suggested that the increase, sometimes disputed, in taurine is due not only to its function as an osmolyte [21] (sometimes contested [7]), but also to many other physiological functions known in mammals and possibly undergoing their first trials in poikilothermic animals, especially at low temperatures [9].

Thus, the studies reported here showed that by the beginning of the winter period, the same substances as seen in our previous studies on cold shock appear and accumulate in the brains of eurythermal freshwater fish. These substances were found to be phosphoethanolamine and phosphoserine – components in membrane phospholipid synthesis. In summer (end of June, beginning of July), with normal temperatures, phosphoethanolamine and phosphoserine are not seen or are present in tiny quantities (phosphoethanolamine), though they are present at large quantities at low environmental temperatures (especially phosphoethanolamine). It should be noted that the present study of the low-temperature adaptation of the brains of poikilothermic animals in the context of modifications of amino acid and phospholipid metabolite pools is the first addressing the biochemical strategy of adaptation in poikilothermic animals.

In addition, cardinal differences in low-temperature changes in amino acid pools were seen in the brains of fish as compared with muscle tissue, i.e., the behavior of taurine was opposite (decrease in brain, increase in muscle) to that of serine (increase in brain, decrease in muscle). It is entirely possible that this phenomenon reflects the redistribution of these substances between different tissues.

It can be suggested that modification of phosphoethanolamine, phosphoserine, and serine pools is due primarily to changes in brain phospholipid (perhaps including sphingomyelin) metabolism.

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