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EFFECTS OF ANOXIA ON SEROTONIN METABOLISM IN CRUCIAN CARP BRAIN

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Summary

In the brain, oxygen is required for both the synthesis and the degradation of monoamine transmitters, so monoaminergic systems can be expected to be strongly affected by anoxia. However, crucian carp (*Carassius carassius* L.) may survive anoxia for many days or even weeks. In the present study, crucian carp were exposed to anoxia for 22, 76, and 160 h at 8°C. All survived and were found to excrete ethanol at a constant rate. The brain concentrations of serotonin and its two main metabolites, 5-hydroxyindole-3-acetic acid (5-HIAA) and 5-hydroxytryptophol, were analysed after each experiment. In a preliminary experiment, it was found that the brain of the crucian carp contained about the same amount of serotonin and 5-HIAA as two species less tolerant to anoxia – the common carp and the rainbow trout. The levels of the serotonin metabolites decreased drastically (by 80–90 %) during anoxia, whereas serotonin levels were only slightly reduced (by 15 % or less). These results suggest a complete or nearly complete stop in serotonin metabolism during anoxia.

Introduction

Both the synthesis and the degradation of serotonin (Fig. 1) are oxygen-dependent. In the first step of serotonin synthesis, L-tryptophan is hydroxylated by tryptophan hydroxylase. This reaction involves a simultaneous reduction of molecular oxygen. In an analogous oxygen-dependent reaction, L-dihydroxyphenylalanine (L-dopa), the precursor of all catecholamines, is formed from L-tyrosine by tyrosine hydroxylase. Monoamine oxidase (MAO) catalyses the first step of the degradation of both serotonin and catecholamines. This reaction also involves a simultaneous reduction of molecular oxygen.

Thus, one would expect monoaminergic systems to be strongly affected by anoxia. Nevertheless, anoxia is tolerated for many days by the goldfish (*Carassius auratus* L.) and even for weeks by the crucian carp (*C. carassius* L.) (Blazka, 1958; Walker & Johansen, 1977; Van den Thillart *et al.* 1983; Piironen & Holopainen, 1986).

Previous studies on anoxic *Carassius* have mainly been focused on energy

Key words: anoxia, *Carassius carassius*, crucian carp, monoamines, serotonin.

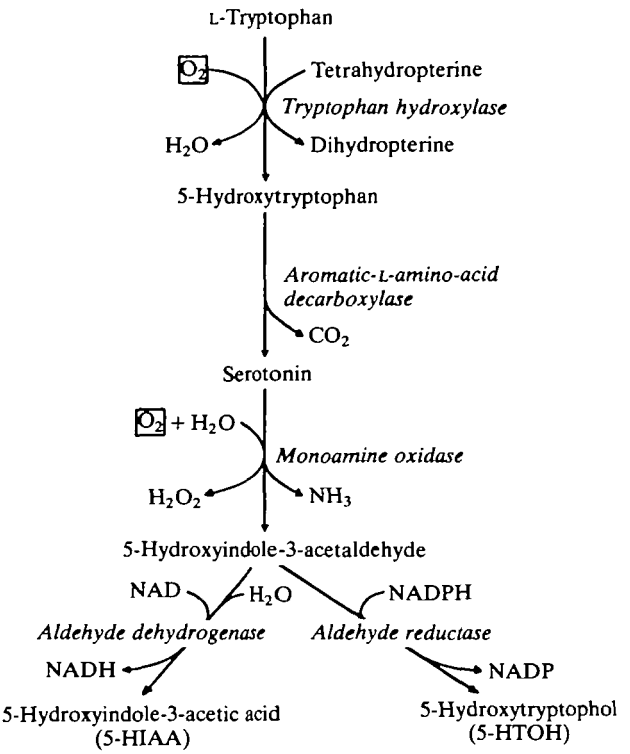

Fig. 1. Overview of serotonin metabolism. Note that both the synthesis and the degradation of serotonin involve a consumption of molecular oxygen.

metabolism. It has been revealed that both members of this genus have the ability to produce ethanol as the major glycolytic end product during anoxia (Shoubridge & Hochachka, 1980; Johnston & Bernhard, 1983). However, to my knowledge, no studies have dealt with the neurochemical effects of anoxia in *Carassius*.

The aim of the present study was to investigate the effect of anoxia on the levels of serotonin and its main metabolites in crucian carp brain.

Materials and methods

Animals

Crucian carp were caught in a small pond near Uppsala in late September. Common carp (*Cyprinus carpio* L.), weighing 233 ± 38 g, and rainbow trout (*Salmo gairdneri* Richardson), weighing 226 ± 16 g, were obtained from outdoor fish farms in central Sweden. The fish were kept indoors in tanks containing continuously exchanged Uppsala tap water (8°C). They were fed daily with commercial trout food (Ewos, Sweden). The artificial light was set on Hamburg's horizon. The experiments were carried out from January to March, after at least 3 months of acclimation to the indoor conditions.

Chemicals

Serotonin, 5-hydroxyindole-3-acetic acid (5-HIAA), 5-hydroxytryptophol (5-HTOH) and NAD were obtained from Sigma Chemicals (St Louis, MO, USA). Alcohol dehydrogenase was supplied by Boehringer (Mannheim, FRG). All other chemicals were supplied by Merck (Darmstadt, FRG).

Measurement of serotonin and its metabolites

The fish were decapitated and their brains (including bulbus olfactorius) were rapidly removed, wrapped in aluminium foil, frozen in liquid nitrogen, and kept at −80 °C. Each brain was homogenized in 4 % (w/v) ice-cold perchloric acid (PCA) containing 0·2 % EDTA, 0·05 % sodium bisulphite and 40 ng ml^{-1} epinine (deoxyepinephrine, the internal standard), using a Potter–Elvehjem homogenizer. The volume was adjusted to give a 10 % (w/v) homogenate. The supernatant obtained after centrifugation (20 000 *g* for 10 min) was frozen overnight (−20 °C) or analysed immediately.

The levels of serotonin and its metabolites in the PCA-extracts were quantified using high-performance liquid chromatography with electrochemical detection (HPLC-EC). The HPLC-EC apparatus, which was fitted with a reversed-phase column (4·0 mm × 200 mm, Nucleosil C18, 5 μm particle size), has been described in detail by Nilsson *et al.* (1987). The mobile phase consisted of 105 mmol l^{-1} citric acid, 2·5 % (v/v) methanol, 5 mg l^{-1} sodium octylsulphate and 20 mg l^{-1} EDTA (pH 2·20).

Assay of ethanol

The ethanol content of water samples was analysed spectrophotometrically, using alcohol dehydrogenase, as described by Krebs *et al.* (1969).

Experimental design

The fish were not fed during the experiments and acclimation to the experimental conditions.

Experiment 1

Five crucian carp, weighing 41 ± 8 g, were put in a 6-l glass container (originally a desiccator) in which the water (aerated Uppsala tap water) was continuously exchanged at a rate of 30 l h^{-1}. The container was kept dark in a water bath at 8 °C. The oxygen content of the water was measured continuously by circulating water between the container and a small chamber containing an oxygen electrode (WTW Oximeter OXI 191 from Wissenschaftlich Technische Werkstätten in Weilheim, FRG).

After 3 days of acclimation, the water supply was shut off and the outlet firmly closed. After 5 h 15 min, at which time the oxygen content of the water had decreased from 8·5 to 0·6 mg l^{-1} (see Fig. 2), continuous nitrogen gassing of the water was started. After 5 min, the oxygen level had decreased below the

detection limit of the electrode ($0{\cdot}1\,mg\,l^{-1}$). During the whole experiment, samples of the water were taken regularly and the ethanol content was determined. After 22 h of anoxia, the fish were taken out of the container and immediately decapitated. The brains were rapidly removed and treated as described above. After a fish had been taken out of the container, the lid was put on and, owing to the continuous nitrogen gassing, the remaining fish were prevented from coming into contact with oxygen (especially since they all hid at the bottom). In this experiment, control fish were taken directly from their storage tank. The experiment was done in January and was terminated around 15.00 h.

Experiment 2

Crucian carp, weighing 101 ± 24 g, were put in two identical 18-l plastic containers (five fish in each) with glass lids (to allow occasional observation). Aerated Uppsala tap water (8°C) was continuously supplied ($15\,l\,h^{-1}$) from the bottom of the container. The water left the container through a central hole in the lid. An oxygen electrode (same as above) was placed in this outlet and the oxygen content of the water was continuously recorded. Both containers were kept dark in a water bath at 8°C. After 3 days of acclimation in the aerated water (containing about $8{\cdot}5\,mg\,l^{-1}$ oxygen) the water supply to one of the containers (the test container) was replaced with nitrogen-gassed water (supplied at the same rate as the aerated water) containing less than $0{\cdot}1\,mg\,l^{-1}$ oxygen. After 9 h, the oxygen concentration in the test container had decreased below the detection limit of the oxygen electrode ($0{\cdot}1\,mg\,l^{-1}$). After 76 h of anoxia, the fish were decapitated and treated as described above. During the decapitation procedure, the water in the test container was continuously gassed with nitrogen to ensure that the remaining fish (which hid at the bottom) did not come into contact with oxygen. The control fish, which were decapitated at the same time, were taken from the container that had been supplied with aerated water during the whole experiment. The experiment was done in February and was terminated around 16.00 h.

Experiment 3

This experiment, which involved 15 crucian carp (weighing 86 ± 13 g), was done using the same experimental setup as experiment 2, and under the same conditions. Ten fish were put in the test container. After 160 h of anoxia in the test container, the fish in the control container were decapitated (see above) and five fish from the test container were transferred to the control container. Here they were exposed to normoxia ($8{\cdot}5\,mg\,l^{-1}$ oxygen) for 30–53 min before being killed. Meanwhile, the fish in the test container were decapitated. Thus, in this experiment, three groups of five fish were obtained: (1) fish exposed to normoxia during the whole experiment (controls); (2) fish exposed to anoxia for 160 h; (3) fish exposed to anoxia for 160 h, and then exposed to normoxia for 30–53 min. The experiment was done in March and was terminated around 11.00 h.

Results

In the three species of fish studied (crucian carp, common carp and rainbow trout), the concentrations of serotonin in the brain were similar and close to that found in the rat (Table 1). The concentrations of serotonin's acid metabolite (5-hydroxyindole-3-acetic acid, 5-HIAA) were similar in the brain of all three fish, and much lower than in rat brain (Table 1).

While the crucian carp were exposed to a reduction in oxygen concentration in experiment 1 (see Materials and methods), by turning off the water supply, there was no change in ethanol level (Fig. 2). Soon after nitrogen gassing was begun, and oxygen was virtually completely removed, ethanol production began and reached a constant rate of 11·6 nmol $min^{-1} g^{-1}$ (Fig. 2). The corresponding ethanol production rates in experiments 2 and 3 were 10·6 and 12·0 nmol $min^{-1} g^{-1}$, respectively.

During anoxia in all three experiments, the fish were physically active and

Table 1. *Brain concentration of serotonin and its acid metabolite in four vertebrates*

Species	*N*	Serotonin ($ng\,g^{-1}$)	5-HIAA ($ng\,g^{-1}$)
Crucian carp (*Carassius carassius*)	5	367 ± 54	42 ± 7
Common carp (*Cyprinus carpio*)	5	270 ± 27	55 ± 7
Rainbow trout (*Salmo gairdneri*)	5	322 ± 55	76 ± 11
Norwegian rat (*Rattus norvegicus*)	12	434 ± 43	306 ± 37

Values are mean ± S.D.
The values for the rat are from Nilsson *et al.* (1987).

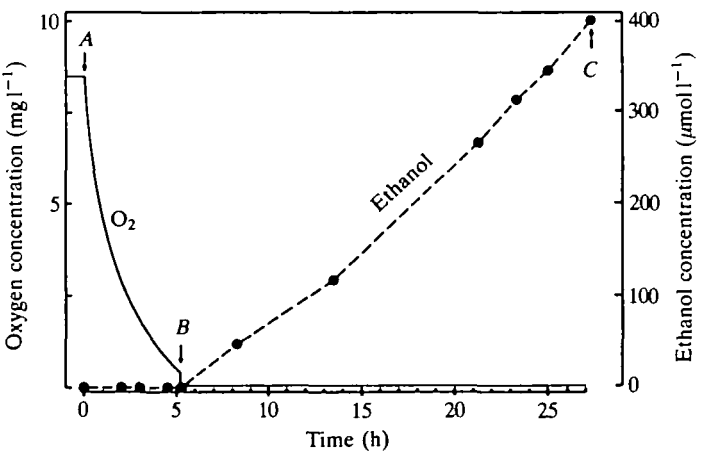

Fig. 2. The content of ethanol and oxygen in the water in experiment 1. Five crucian carp (weighing 41 ± 8 g) were kept in a 6-l container at 8°C. Arrows mark the time when the supply of aerated water was shut off (*A*), when the nitrogen gassing was started (*B*), and when the fish were decapitated (*C*). Details of the experiment are given in Materials and methods.

Table 2. *Effect of anoxia on the concentrations of serotonin and its metabolites in crucian carp brain*

	Time in anoxia (h)	Serotonin ($ng\,g^{-1}$)	5-HIAA ($ng\,g^{-1}$)	5-HTOH ($ng\,g^{-1}$)
Experiment 1	0	364 ± 18	45 ± 4	<5
	22	344 ± 64	<10***	<5
Experiment 2	0	423 ± 24	37 ± 12	4 ± 1
	76	389 ± 31*	4 ± 1***	<1***
Experiment 3	0	468 ± 97	55 ± 19	9 ± 2
	160	399 ± 53	4 ± 2***	<1***
	160 +30–53 min of normoxia	370 ± 42**	25 ± 6***	17 ± 4***

Values are mean ± S.D. from five specimens. Statistical significance was calculated using Wilcoxon rank sum test (one-tailed). * $P<0.05$; ** $P<0.01$; *** $P<0.005$.

In experiments 2 and 3, the detection limits for 5-HIAA and 5-HTOH were increased five-fold by using a longer sample loop (100 µl instead of 20 µl) in the HPLC assay.

frequently changed their positions in the chamber. They also showed continuous repetitive opercular movements. When they were caught at the end of the experiments, there seemed to be no difference between the escape reaction of anoxic crucian carp and that of controls. No fish died during the experiments.

The brain levels of serotonin and its metabolites measured after each experiment are shown in Table 2. Of the two serotonin metabolites, only 5-HIAA was above the detection limit in experiment 1. In experiments 2 and 3, the sensitivity of the HPLC-EC assay was increased five times by using a longer sample loop (100 µl instead of 20 µl), so that the alcohol metabolite of serotonin (5-hydroxytryptophol, 5-HTOH) could also be detected. The results show that there were very large drops in the levels of both serotonin metabolites when crucian carp were exposed to anoxia. The 5-HIAA level in experiment 1 (22 h anoxia) fell from 45 $ng\,g^{-1}$ to less than 10 $ng\,g^{-1}$ (i.e. below the detection limit in this experiment); in experiment 2 (76 h anoxia) the level was reduced from 37 to 4 $ng\,g^{-1}$; and in experiment 3 (160 h anoxia) a fall from 55 to 4 $ng\,g^{-1}$ was observed. The level of 5-HTOH fell from 4 $ng\,g^{-1}$ in experiment 2 and 9 $ng\,g^{-1}$ in experiment 3 to less than 1 $ng\,g^{-1}$ (below the detection limit) in the anoxia-exposed groups (Table 2).

In all three experiments, the levels of serotonin seemed to be somewhat lower in the anoxia-exposed groups than in the controls, but this effect was only statistically significant in two instances (Table 2).

In experiment 3, one group of crucian carp was exposed to 30–53 min of normoxia immediately after the anoxic period (160 h). This treatment caused the 5-HIAA level to rise up to about half (25 ± 6 $ng\,g^{-1}$) of that found in the normoxic controls (55 ± 19 $ng\,g^{-1}$), while the 5-HTOH level increased well above (17 ± 4 $ng\,g^{-1}$) that found in the normoxic controls (9 ± 2 $ng\,g^{-1}$) (Table 2). Fig. 3

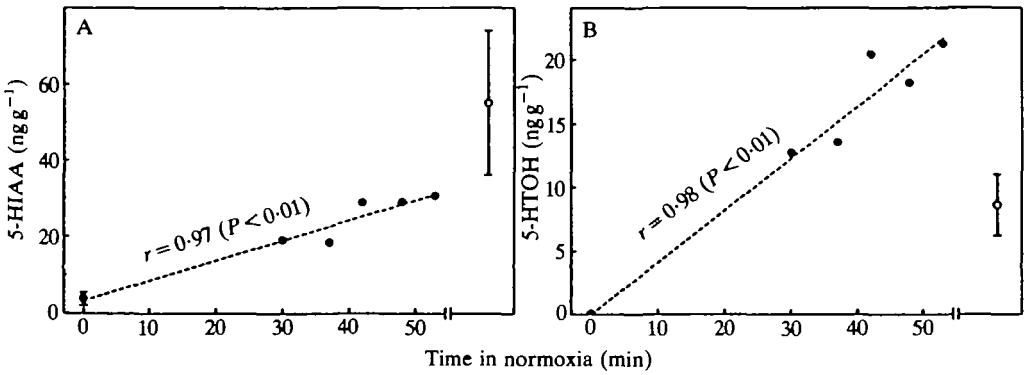

Fig. 3. Brain concentrations of (A) 5-HIAA and (B) 5-HTOH in crucian carp exposed to normoxia after 160 h of anoxia (experiment 3). Open circles show the levels of 5-HIAA and 5-HTOH in normoxic controls (mean ± S.D.).

suggests that the increases in 5-HIAA and 5-HTOH concentrations were both linear and probably started almost immediately after the crucian carp were given access to oxygen.

Discussion

Phylogenetically, monoaminergic systems are very old (Welsh, 1970). Monoamines have been detected in all vertebrates examined and also in many invertebrates. In the present study, the crucian carp brain was found to contain about the same amounts of serotonin and 5-HIAA as the brain of the common carp and that of the rainbow trout (Table 1). Thus, the results suggest that serotonin is used as a neurotransmitter in crucian carp brain to the same extent as in the brain of other fishes. Although the serotonin level in rat brain was in the same range as that in the fishes, the brain level of the main serotonin metabolite, 5-HIAA, was about six times higher in the rat. This is probably a reflection of a higher rate of serotonin metabolism in an endotherm than in ectotherms adapted to 8°C.

From histochemical studies, it is known that the organization of serotonergic systems in brain is very conservative in vertebrates (see Parent *et al.* 1984, for a review). Serotonergic systems have been extensively studied in the mammalian brain, and have been functionally linked to a variety of processes (see Bloom, 1986, for a review). There is no reason to suspect that serotonin should play a less important role in fish, although there is very little information on the exact function of serotonergic systems in the brain of lower vertebrates.

As mentioned in the Introduction, both the synthesis and the degradation of monoamines require oxygen, suggesting that anoxia would strongly affect monoaminergic systems. For example, Hedner & Lundborg (1979) found that the brain

activities of tyrosine hydroxylase and tryptophan hydroxylase decreased to around 50% of the control values in young rats exposed to hypoxia (6% oxygen) for 30 min.

There are two main metabolites of serotonin, since the aldehydic intermediate, formed by the MAO-catalysed oxidation of serotonin, is either further oxidized by NAD-dependent aldehyde dehydrogenase to form an acid, 5-HIAA, or reduced by NADPH-dependent aldehyde reductase to form an alcohol, 5-HTOH (Fig. 1). In the present study, anoxia resulted in a large decrease (at least 80–90%) in the levels of both these metabolites (Table 2). This suggests a complete or nearly complete stop in the breakdown of serotonin. The small amount of 5-HIAA found after anoxia might be a remainder from the normoxic period. It is also possible that some 5-HIAA could have been produced during the anoxia owing to traces of oxygen in the water or to minor oxygen stores in the fish.

Since anoxia had only slight effects on the level of serotonin itself, even after 160 h (Table 2), the results suggest that serotonin synthesis had stopped owing to the lack of oxygen. If synthesis had continued during anoxia, then one would expect the serotonin level to have risen owing to the inhibited breakdown. However, because serotonin synthesis involves the reduction of molecular oxygen, it would seem to be an impossibility during anoxia.

There is much more reason to expect the serotonin level to decrease during anoxia. It is unlikely that the crucian carp could make unlimited re-use of the same serotonin molecules. During anoxia, more and more of the serotonin pool will probably become unavailable for presynaptic uptake, owing to postsynaptic uptake, uptake by glial cells, diffusion out of the serotonergic brain areas (or even out of the brain) or deactivation by more or less unspecific mechanisms. The results show a small decrease in the serotonin level, but it is surprising that this decrease was not more than 15%, even after nearly 1 week of anoxia. This suggests that there is almost no oxygen-independent degradation of serotonin in the crucian carp. It is, of course, likely that much of the serotonin found after the anoxic events is present outside synaptic vesicles and of no use for synaptic transmission. Thus, a depletion of the serotonergic (and probably also the catecholaminergic) transmitter systems could be a serious problem for crucian carp exposed to prolonged anoxia.

However, there are other studies suggesting that the brain of efficient ectothermic anaerobes is especially well adapted to anoxic conditions with regard to membrane properties (see Hochachka, 1986, for a review) and gamma-aminobutyric acid metabolism (Lutz *et al.* 1985). Thus, one may speculate on the possibility that the crucian carp has developed particularly effective mechanisms for the uptake and storage of monoamines.

There is no comparable study on the effects of anoxia on monoamine levels in an anoxia-intolerant species. However, there are numerous studies on the effects of short-term hypoxia (from a few minutes to 24 h) on monoamine levels in the mammalian brain. For example, Prioux-Guyonneau *et al.* (1982) found a 30% decrease in hypothalamus serotonin level in rats exposed to hypobaric hypoxia

(corresponding to an altitude of 7000 m) for 24 h. This field of research, which contains some seemingly discrepant findings, has been reviewed by Gibson (1985).

In experiment 3, one group of crucian carp was exposed to normoxia for 30–53 min, after having been anoxic for nearly 1 week. The results showed that the renewed contact with oxygen caused a rapid increase in the levels of both serotonin metabolites, suggesting an immediate start of serotonin metabolism (Fig. 3). The rise in the 5-HIAA level was rather modest compared with the increase in the 5-HTOH level (reaching twice that in the normoxic controls). This suggests that the cells were still in a reduced state after 50 min of normoxia, thereby favouring serotonin breakdown *via* NADPH-dependent aldehyde reductase. The NAD-demanding breakdown, catalysed by aldehyde dehydrogenase, apparently dominated in the normoxic controls.

The present study dealt with one of the monoamines in brain. However, monoamines (notably epinephrine and norepinephrine) also play an important role as hormones in vertebrates. Anoxia is also likely to have profound effects on their synthesis and breakdown, since central and peripheral monoamine metabolism seem to be based on the same oxygen-dependent reactions.

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