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LONG-TERM ANOXIA IN CRUCIAN CARP: CHANGES IN THE LEVELS OF AMINO ACID AND MONOAMINE NEUROTRANSMITTERS IN THE BRAIN, CATECHOLAMINES IN CHROMAFFIN TISSUE, AND LIVER GLYCOGEN

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Summary

Crucian carp (*Carassius carassius* L.), which are extremely anoxia-tolerant, were exposed to 17 days of anoxia at 8°C. One group of fish was transferred to normoxic water for 1–8 h immediately after the anoxic period.

All the eight amino acids measured in brain (including four putative neurotransmitters) were more or less strongly affected by anoxia. Gamma-aminobutyric acid (GABA) displayed a nearly fivefold increase during anoxia. It is hypothesized that the increased level of this inhibitory transmitter, maybe in combination with the decrease seen in excitatory amino acids (glutamate and aspartate), causes a lowered brain activity and, hence, is a key factor behind the decrease in physical activity and systemic energy metabolism seen in anoxic *Carassius*.

The brain levels of serotonin, dopamine and norepinephrine were remarkably well preserved after anoxia (although their synthesis is oxygen-dependent), suggesting adaptive mechanisms. However, anoxia reduced the norepinephrine level in kidney (chromaffin tissue) by 92 % and, in contrast to previous results on shorter anoxic periods (3–7 days), the peripheral catecholamine store showed little sign of recovery during the subsequent normoxia.

Anoxia was found to deplete the liver glycogen store severely, and the few fish that died after 15–17 days of anoxia contained no detectable liver glycogen.

Introduction

As a rule, vertebrates can only survive short periods of anoxia. The brain is probably the most anoxia-intolerant organ, and among the brain functions that are strongly affected by anoxia are energy production and the metabolism of several neurotransmitters.

Nevertheless, a few fishes and some turtles readily tolerate long periods of anoxia and, hence, clearly deviate from the general rule. By studying these

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animals, we hope to gain a better understanding of the problems that anoxia causes in the vertebrate brain.

The crucian carp (*Carassius carassius*) is one of the most anoxia-tolerant vertebrates known. Both in nature and in the laboratory, crucian carp have been found to survive many days or, at low temperatures, even several weeks of anoxia (Blazka, 1958; Piironen and Holopainen, 1986). Its extreme anoxia-tolerance enables the crucian carp to be the sole piscine inhabitant of many small North European ponds that are covered with ice for several months during the winter.

A major problem for the anoxic vertebrate brain is to maintain an energy production sufficient to preserve the ion gradients, i.e. to meet the ATP consumption of the Na^+/K^+ pumps (Hansen, 1985). The species of the cyprinid fish genus *Carassius* (the crucian carp and the goldfish) have, together with the related bitterling (*Rhodeus sericeus amarus* Bloch), found a solution to this problem which appears to be unique among vertebrates. They produce ethanol as the major end-product of anaerobic glycolysis (Shoubridge and Hochachka, 1980; Mourik *et al.* 1982; Johnston and Bernard, 1983; Van den Thillart *et al.* 1983; Nilsson, 1988; Wissing and Zebe, 1988). Ethanol is excreted into the ambient water, thus avoiding a build-up of the metabolic end-product. Moreover, to maintain glycolytic energy production for prolonged periods, the genus *Carassius* keeps the largest liver glycogen store found in vertebrates, 25–30 % of the liver wet mass being glycogen (Hochachka and Somero, 1984; Hyvärinen *et al.* 1985).

Research on anoxia-tolerant vertebrates has concentrated on energy metabolism, and very little is known about the effects of anoxia on neurotransmitter systems in the brain of these animals. Still, all catecholamines (dopamine, norepinephrine and epinephrine) demand molecular oxygen for their synthesis and for the important part of their degradation that is catalysed by monoamine oxidase (e.g. Nagatsu, 1973; Kirshner, 1975; Kaufman, 1985). Also, the synthesis of serotonin has an absolute demand for molecular oxygen (e.g. Kaufman, 1985), and the only route for serotonin degradation seems to be the oxygen-utilizing deamination catalysed by monoamine oxidase (e.g. Korf, 1985).

Moreover, the metabolism of amino acid transmitters [glutamate, aspartate, gamma-aminobutyric acid (GABA) and glycine] is intimately coupled to the tricarboxylic acid cycle (McGeer and McGeer, 1989; Gibson, 1985), and is therefore readily affected by a decrease in oxygen availability.

Consequently, the extensive literature on the effects of hypoxia and anoxia on anoxia-intolerant vertebrates (mainly on mammals) shows that the metabolism of many neurotransmitters is strongly affected by the short periods of hypoxia or anoxia that these animals can survive (see Gibson, 1985, for a review).

In a recent study on the crucian carp, it was found that 1–7 days of anoxia at 8 °C hardly affects the brain level of serotonin (a 15 % decrease or less), although the serotonin metabolites virtually vanish from the brain, clearly suggesting a stop in serotonin metabolism (Nilsson, 1989*a*). A subsequent study (Nilsson, 1989*b*) showed that the levels of dopamine and norepinephrine in crucian carp brain also remain fairly constant (a 17 % fall or less) for 1 week of anoxia at 8 °C, while the

catecholamines stored in peripheral chromaffin tissue display more prominent decreases (22–60%).

With regard to the effect of anoxia on amino acids in the brain of anoxia-tolerant vertebrates, two studies have been carried out on turtle (*Pseudemys scripta* Schoepff) (Lutz *et al.* 1985; Hitzig *et al.* 1985). Both these studies suggested that the GABA level increased during 2–4 h in anoxia at 20–25 °C.

The aim of the present study on the crucian carp was to find out how a prolonged exposure to anoxia affects the brain levels of several putative neurotransmitters and related substances: serotonin and its two metabolites 5-hydroxyindole-3-acetic acid (5-HIAA) and 5-hydroxytryptophol (5-HTOH), dopamine and its metabolites homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine, and eight brain amino acids (glutamate, aspartate, GABA, glycine, glutamine, taurine, alanine and serine). Changes in the main glycogen store in the liver and the catecholamine content (norepinephrine and epinephrine) in the peripheral chromaffin tissue were also studied.

Materials and methods

Animals

Crucian carp were caught in a small pond near Uppsala in October. The fish were stored indoors in tanks containing 400 l of continuously exchanged aerated Uppsala tap water held at 8 °C. They were fed daily with commercial trout food (Ewos, Sweden). The artificial photoperiod corresponded to Hamburg's latitude and longitude. The experiments were carried out in March. No animals died before the experiments.

Chemicals

Monoamines, monoamine metabolites, amino acids, glycogen (Type III from rabbit liver) and *o*-phthaldialdehyde reagent solution (complete) were obtained from Sigma Chemicals (St Louis, MO, USA). All other chemicals were supplied by Merck (Darmstadt, FRG).

Experimental design

Crucian carp, weighing 83±24 g, were put in two identical 18 l dark plastic containers (20 fish in each) with glass lids (to allow occasional observation). The glass lids were shaded to 90% of their area with black plastic bags, allowing the fish to detect the light/dark cycle. Aerated Uppsala tap water (8 °C) was continuously supplied from the bottom of the containers at a rate of 15 l h^{-1}. The water left the containers through a central hole in the lid. An oxygen electrode (WTW Oximeter OXI 191 from Wissenschaftlich Technische Werkstätten in Weilheim, FRG) was placed in this outlet and the oxygen content of the water was continuously recorded. Both containers were kept in a water bath at 8 °C. After 24 h of acclimation in the aerated water (containing 8.5 mg l^{-1} oxygen) the water

supply to one of the containers (here denoted the anoxic container) was replaced by nitrogen-gassed water (supplied at the same rate as the aerated water) containing less than $0.1\,mg\,l^{-1}$ oxygen. Consequently, the oxygen level fell from 8.5 to $0.5\,mg\,l^{-1}$ during the following 2h. After 3h, the oxygen concentration reached $0.2\,mg\,l^{-1}$, and after 4h (at 15:00h), it decreased below the detection limit of the oxygen electrode ($0.1\,mg\,l^{-1}$). This time was regarded as the onset of anoxia.

The fish were not fed during the experiment and preceding 24h of acclimation. There were several reasons for this. First, anoxic fish are unlikely to eat, owing to the decreased physical activity (see below). Second, if anoxic fish were to eat, their food intake would probably be much less than that of normoxic controls. Thus, feeding would introduce an uncontrollable variable. Third, the presence of undigested and digested food in the small experimental container (which could not be cleaned during the 18-day experiment) would have had a negative effect on the water quality. Finally, previous experiments on long-term anoxia in crucian carp (Piironen and Holopainen, 1986) have been carried out on starved fish. Nevertheless, to study possible effects of starvation, an additional control group (fed fish from the storage tank) was introduced.

Seventeen days later, after 402–403h of anoxia in the anoxic container (between 09:00 and 10:00h), seven fish in the control (normoxic) container were decapitated (see below) and the remaining 13 fish were removed. Eleven fish from the anoxic container were then transferred to the control container. These were hereby exposed to normoxia ($8.5\,mg\,l^{-1}$ oxygen) for 1–8h before decapitation. After 403–403.5h of anoxia (at 10:00–10:30h), the fish remaining in the anoxic container were decapitated. During the decapitation and tissue-sampling procedure, the water in the anoxic container was continuously and vigorously nitrogen-gassed to make sure that the remaining fish (which all hid at the bottom) did not come into contact with oxygen. The nitrogen gassing created a pocket of nitrogen between the water surface and the lid (which was only removed for a few seconds each time). On the same day (at 15:00h), a group of five fish, weighing 88 ± 27g, was taken directly from the storage container, immediately decapitated, and analysed just like the experimental fish.

Of the 20 fish exposed to anoxia, one died after 15 days of anoxia, two died after 16 days of anoxia and one died less than 1h before the anoxic fish were to be decapitated. Of the first three dead fish, only the livers were sampled (at the end of the experiment), but all parameters were measured on the last one. In order not to disturb the fish during anoxia, the dead fish were not removed before the experiment was terminated.

Thus, five groups of fish were obtained. (1) Fish exposed to normoxia during the whole experiment (i.e. the controls) ($N=7$). (2) Fish exposed to anoxia for 403h (i.e. about 17 days) ($N=5$). (3) Fish exposed to anoxia for 403h, and then exposed to normoxia for 1–8h ($N=11$). (4) Fish taken directly from the storage tank and, hence, fed during the whole experiment ($N=5$). (5) The four fish that died during the last 48h of the experiment.

Tissue sampling

The fish were decapitated (within 10 s after being netted) and their brains, kidneys and livers were rapidly removed (brain within 1 min and kidney within 2 min), frozen in liquid nitrogen, and kept at −80 °C. Each brain and kidney was homogenized in 4 % (mass/vol) ice-cold perchloric acid (PCA) containing 0.2 % EDTA, 0.05 % sodium bisulphite and 40 ng ml^{-1} epinine (deoxyepinephrine, the internal standard used in the analyses of monoamines and monoamine metabolites), using a Potter–Elvehjem homogenizer. The volume was adjusted to give a 10 % (mass/vol) homogenate. The supernatants obtained after centrifugation (20 000 *g* for 10 min) were frozen at −80 °C overnight (monoamines) or for 1 week (amino acids).

Although the peripheral catecholamine-producing cells are concentrated in the head part of the kidney (the pronephros), the whole organ was taken for catecholamine analysis to increase the speed and reproducibility of the sampling.

Determination of monoamines and their metabolites

The amounts of monoamines and monoamine metabolites present in 100 μl samples of the supernatants obtained after centrifugation (see above) were quantified using reversed-phase ion-pair high-performance liquid chromatography (HPLC) with electrochemical detection. The HPLC system consisted of a 6000 A solvent delivery system and a U6K injector (both from Waters Associates Inc., Milford, MA, USA), a reversed-phase column (4.6 mm×125 mm, Nucleosil 120, C18, 3 μm, from Macherey-Nagel, Düren, FRG) kept at 30 °C, and an LC-3 electrochemical detector with a glassy carbon working electrode, which was set at +750 mV *versus* a Ag/AgCl reference electrode (all from Bioanalytical Systems, West Lafayette, IN, USA). The flow rate was 1.3 ml min^{-1}.

For the assay of norepinephrine, epinephrine and HVA, the mobile phase consisted of 100 mmol l^{-1} NaH_2PO_4, 9 % (vol/vol) methanol, 0.63 mmol l^{-1} sodium octylsulphate, and 0.2 mmol l^{-1} EDTA, pH 3.6. The performance of this mobile phase has been described in detail by Nilsson (1990).

For the assay of 5-HTOH, the mobile phase consisted of 105 mmol l^{-1} citric acid, 2.5 % (vol/vol) methanol, 20 μmol l^{-1} sodium octylsulphate and 0.2 mmol l^{-1} EDTA, pH 2.20.

Dopamine, DOPAC, serotonin, 5-HIAA and epinine (the internal standard) were measured with both mobile phases.

As in a previous study (Nilsson, 1990), no free DOPAC was found in crucian carp brain and, in an effort to detect sulpho-conjugates of DOPAC in crucian carp brain, samples of the centrifuged PCA extracts were made 1 mol l^{-1} with respect to PCA and then held at 95 °C for 5 min, as described by Mefford *et al.* (1983), whereupon they were alumina-extracted (Nilsson *et al.* 1987) to clean up and concentrate the samples prior to the HPLC analysis. However, this procedure gave no indication of the presence of DOPAC in this species, although I have successfully used this method to measure conjugated DOPAC in the brain of

another fish species (arctic char, *Salvelinus alpinus* L.; G. E. Nilsson, unpublished results).

The monoamine concentrations are given as ng per g tissue (wet mass).

Determination of amino acids in brain

The HPLC apparatus used for the assay of monoamines (see above) was also utilized for the assay of amino acids. However, instead of the electrochemical detector, an RF-535 fluorescence detector (Shimadzu Corporation, Kyoto, Japan) was used. Excitation was set at 335 nm and emission at 455 nm. 5 μl of the supernatants obtained after PCA extraction and centrifugation (see above) were mixed with 5 μl of 4 % (mass/vol) PCA containing 0.4 μg of alpha-aminobutyric acid (AABA, the internal standard) and then rapidly mixed with 90 μl of the complete *o*-phthaldialdehyde reagent solution (Sigma P0532) at 25 °C. After exactly 1 min, 25 μl of this mixture was injected onto the HPLC column (4 mm×75 mm, Nucleosil 120, C18, 3 μm, from Macherey-Nagel, Düren, FRG) which was held at 30 °C. The flow rate was 1 ml min^{-1} and the mobile phase consisted of 60 mmol l^{-1} K_2HPO_4, 30 mmol l^{-1} citric acid, 0.36 mmol l^{-1} EDTA and 35 % methanol (pH 6.3). The mobile phase was a slight modification of that described by Lindgren and Andén (1985). In the present system, aspartate, glutamate, serine, glutamine, glycine, taurine, alanine and GABA eluted within 10 min (Fig. 1), while 14 min was needed for AABA. Amino acid concentrations are given as μg per g brain (wet mass).

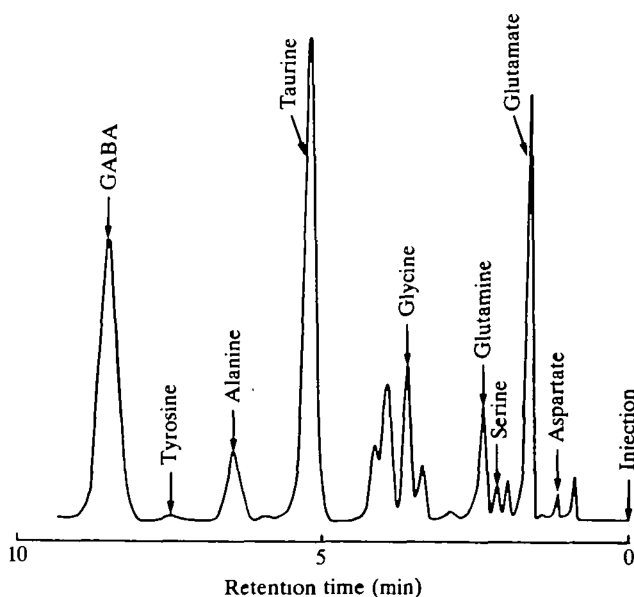

Fig. 1. Chromatogram showing the performance of the HPLC system used for the analysis of amino acids in crucian carp brain. This example shows the separation of amino acids in a perchloroacetic acid extract obtained from the brain of a crucian carp held in anoxia for 17 days (403 h). Peaks were identified using amino acid standards.

Determination of the liver glycogen content

The glycogen contents of samples of liver were measured by the method described by Siu *et al.* (1970). The detection limit was about 5 mg glycogen per g liver (wet mass).

Statistics

Data are presented as means ±S.D. The two-tailed Wilcoxon rank sum test was used in comparisons between experimental groups after significant differences had been found with Kruskal–Wallis analysis of variance by ranks. In addition, the effects of exposure to normoxia were evaluated with least-squares linear regression, whereafter Pearson's correlation coefficients and the corresponding P values were calculated.

Results

Behavioural changes during anoxia

Although no systematic (quantitative) behavioural study was conducted, some general observations were made. Anoxic crucian carp voluntarily changed their positions in the experimental container, performed opercular movements (perhaps to facilitate ethanol excretion), and showed no signs of torpor. However, they moved around much less than during normoxia. After 2–3 days of anoxia, the fish gathered close together immediately below the glass lid, and each fish left its position only occasionally (probably less than once every half hour). This behaviour was maintained for the rest of the anoxic period, and was not seen among the normoxic controls. Nevertheless, when they were caught at the end of the experiment, both anoxic and normoxic crucian carp displayed the usual moderate flight reactions that characterize this relatively calm species.

The liver glycogen store

Fig. 2A–D shows that 17 days (403 h) of anoxia severely depleted the liver glycogen store in crucian carp. There was no difference between the liver glycogen concentration of the crucian carp taken directly from the storage tank (275±33 mg g^{-1}, Fig. 2A) and that of the controls (273±57 mg g^{-1}, Fig. 2B) which had been starved for 18 days. In contrast, after 17 days of anoxia (Fig. 2C), only 62±42 mg glycogen per g liver remained. The 16 anoxic fish presented in Fig. 2C include those exposed to 1–8 h of normoxia after the anoxic period, since this treatment had no significant effect on the liver glycogen level (48±27 mg g^{-1}, range 14–86 mg g^{-1}, in the anoxia group, and 69±47 mg g^{-1}, range 8–148 mg g^{-1}, in those exposed to subsequent normoxia). In fact, linear regression analysis of the glycogen level *versus* time in normoxia (0–8 h) gave a horizontal line (r=0.021, N=16). Three of the crucian carp exposed to anoxia showed an almost total liver glycogen depletion (with livers containing 8, 8 and 14 mg glycogen g^{-1}). No liver glycogen could be detected (i.e. less than 5 mg g^{-1}) in the four crucian carp that died during the last 2 days of anoxia (Fig. 2D).

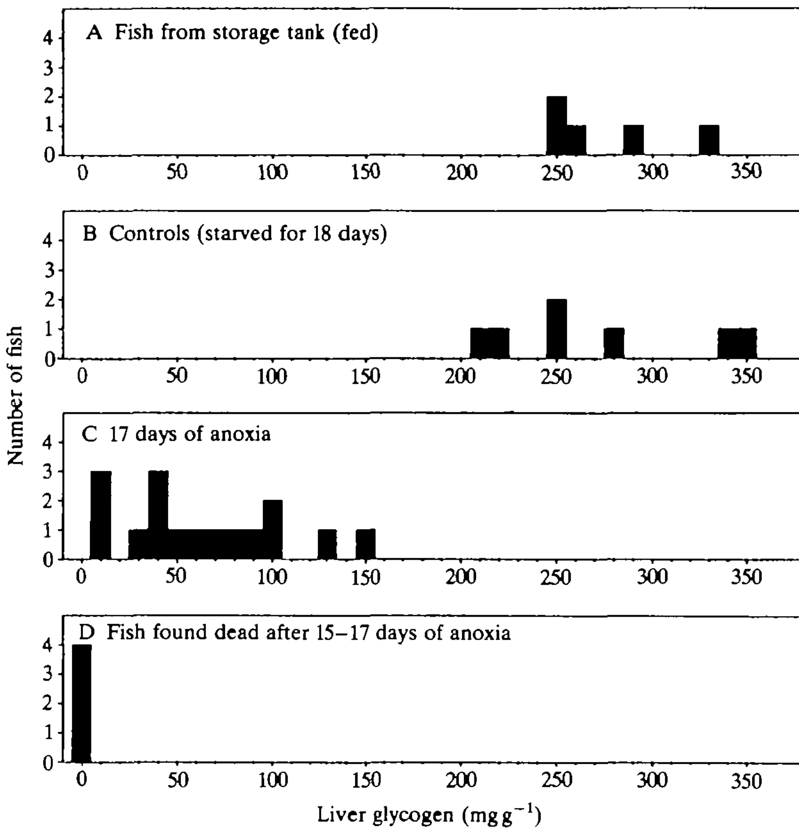

Fig. 2. (A–D) The effect of 17 days (403 h) of anoxia on the concentration of glycogen in crucian carp liver. C includes fish that were exposed to 1–8 h of normoxia after 17 days of anoxia (N=11) as well as those killed immediately after anoxia (N=5).

Brain serotonin

The effects of 17 days (403 h) of anoxia and a subsequent period of 1–8 h of normoxia on serotonin and its metabolites (5-HIAA and 5-HTOH) are shown in Fig. 3. The results show that there was a 24 % decrease in brain serotonin after 17 days of anoxia, and that this serotonin loss was regained during 1–8 h of normoxia.

With regard to 5-HIAA (the acid metabolite of serotonin), 17 days of anoxia had a dramatic effect, since no 5-HIAA could be detected in anoxic fish. Thus, after anoxia, the 5-HIAA level had fallen from 13.0±2.5 ng g^{-1} to less than 1.0 ng g^{-1} (the detection limit). During the subsequent normoxia, 5-HIAA showed a rapid increase and was well above the control values after 3.5–8 h of normoxia (26.7±8.4 ng g^{-1}, P<0.01). The 5-HIAA level was significantly lower (38 %, P<0.05) in the controls than in fish taken directly from the storage tank.

5-HTOH (the alcohol metabolite of serotonin) also fell dramatically during 17 days of anoxia, from 4.4±1.0 ng g^{-1} in the controls to less than 1.0 ng g^{-1} (the

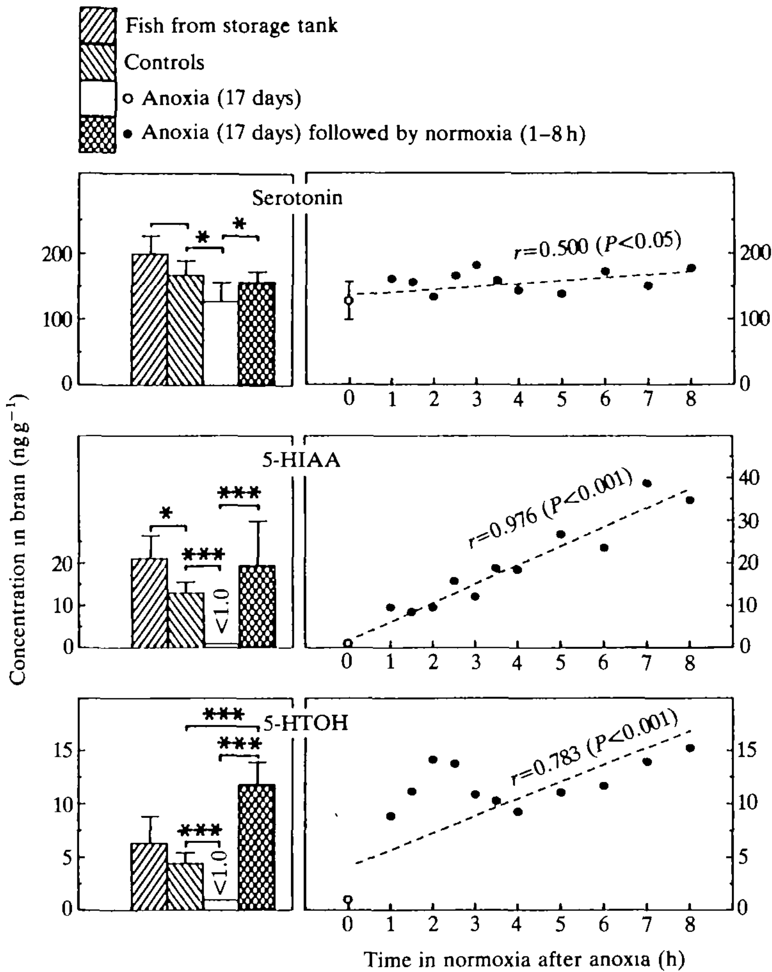

Fig. 3. The effects of 17 days (403 h) of anoxia and a subsequent period (1–8 h) of normoxia on the levels of serotonin and its metabolites, 5-hydroxyindole-3-acetic acid (5-HIAA) and 5-hydroxytryptophol (5-HTOH), in crucian carp brain. Values are mean and S.D. An analysis of variance was first made (Kruskal–Wallis test). If a significant difference ($P<0.05$) was found, Wilcoxon rank-sum test (two-tailed) was used for comparisons between the experimental groups, i.e. controls ($N=7$), anoxia ($N=5$) and anoxia followed by 1–8 h of normoxia ($N=11$), and for a comparison between the experimental controls and fish from the storage tank ($N=5$). The result of the Wilcoxon rank-sum test is shown if $P<0.05$ (* $P<0.05$; ** $P<0.01$; *** $P<0.001$).

detection limit) in anoxic fish. The 5-HTOH level increased rapidly during the subsequent anoxia and was above the control values after only 1 h of normoxia. There was a rapid increase during the first 2 h of normoxia. This period seemed to be followed by a decrease and finally a steady increase in the 5-HTOH level between 4 and 8 h.

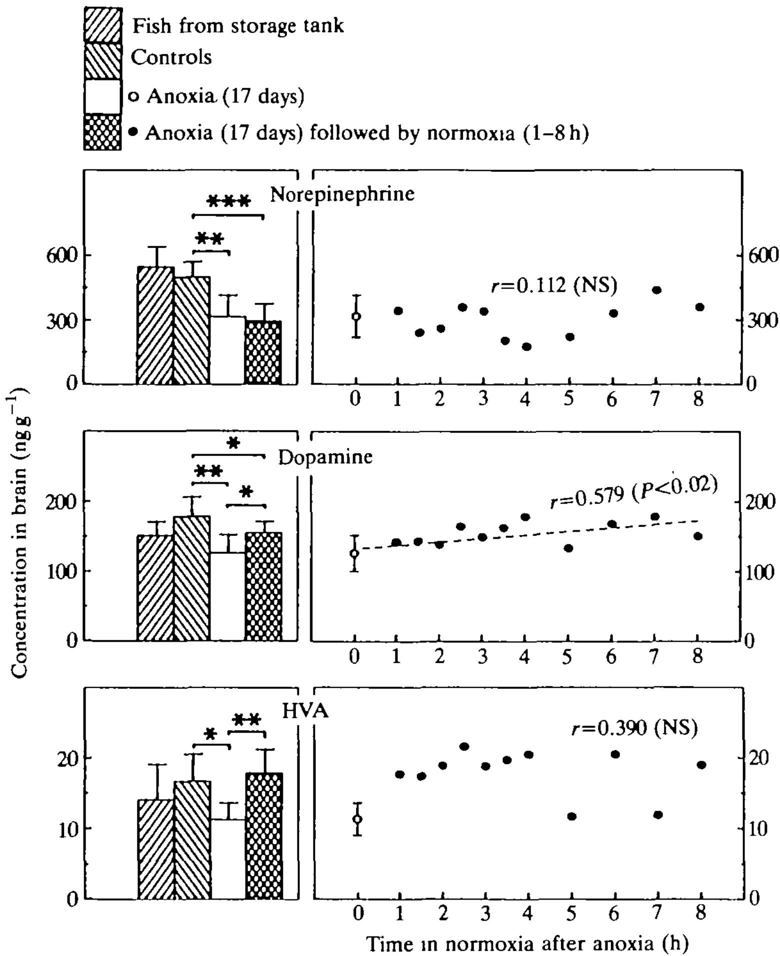

Fig. 4. The effects of 17 days (403 h) of anoxia and a subsequent period (1–8 h) of normoxia on the levels of norepinephrine, dopamine and homovanillic acid (HVA) in crucian carp brain. Values are mean and S.D. For details of statistical evaluations, see legend to Fig. 3. NS, not significant.

Brain catecholamines

Fig. 4 shows the effect of 17 days (403 h) of anoxia and a subsequent period of 1–8 h of normoxia on the brain levels of norepinephrine and dopamine as well as that of HVA, which appears to be the main dopamine metabolite in crucian carp brain. DOPAC, another major dopamine metabolite in many vertebrates, was below the detection limit (about 2 ng g^{-1}) in crucian carp brain (see also Materials and methods).

There was a 36 % decrease in brain norepinephrine after 17 days of anoxia. In contrast to dopamine (see below) and serotonin, the brain norepinephrine level showed no sign of recovery during the subsequent 1–8 h of normoxia.

The dopamine level decreased significantly (by 29 %) during anoxia. However,

as for serotonin, it showed a steady increase back to the control level during the 1–8 h of normoxia that followed the anoxic period.

HVA decreased by 32 % during anoxia. However, the results suggested that HVA reached the control level very soon (within 1 h or less) after the fish were allowed contact with normoxic water.

The experimental situation *per se* seemed to have no effect on the norepinephrine, dopamine or HVA levels, as judged by the lack of any significant differences between the controls and the fish taken directly from the storage tank.

Kidney catecholamines

The effects of anoxia and subsequent normoxia on the levels of norepinephrine and epinephrine in the peripheral chromaffin tissue situated in the kidney are shown in Fig. 5. During anoxia, kidney norepinephrine decreased from 164±95 ng g^{-1} (range 49–282 ng g^{-1}) to 12.5±15.8 ng g^{-1} (range 3–44 ng g^{-1}). During the subsequent 1–8 h of normoxia, norepinephrine showed a small, but nevertheless significant, increase and reached a level of 35.2±16.6 ng g^{-1} after 4–8 h of normoxia ($P<0.05$ compared to the anoxia group; $P<0.01$ compared to the controls).

The response of kidney epinephrine appeared to be similar to that of

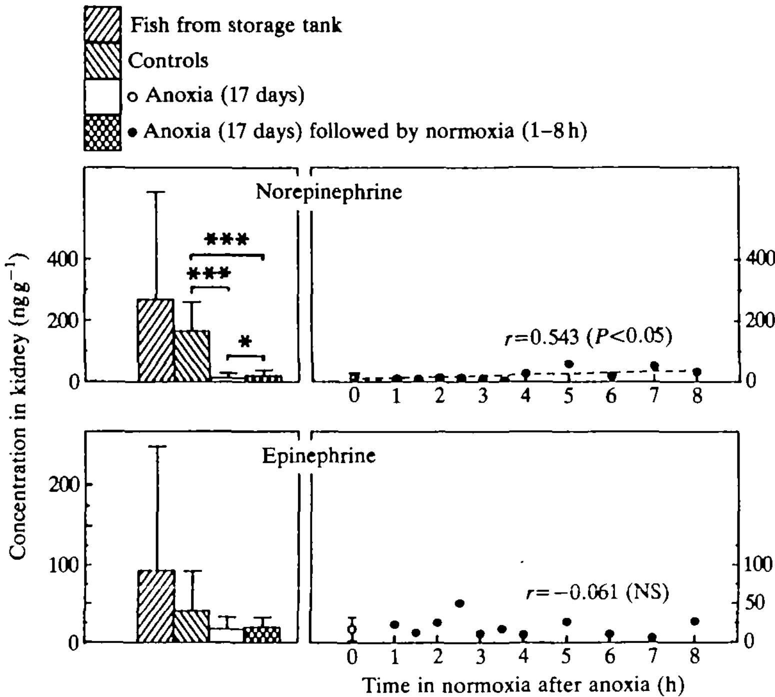

Fig. 5. The effects of 17 days (403 h) of anoxia and a subsequent period (1–8 h) of normoxia on the levels of norepinephrine and epinephrine in crucian carp kidney. Values are mean and S.D. For details of statistical evaluations, see legend to Fig. 3. NS, not significant.

norepinephrine, but the epinephrine values showed a very high variance and no significant changes could be detected.

Brain amino acids

Fig. 6 shows the effects of 17 days (403 h) of anoxia and a subsequent period of 1–8 h of normoxia on the brain levels of two putative excitatory amino acid neurotransmitters, glutamate and aspartate, and two putative inhibitory amino acid neurotransmitters, GABA and glycine.

Both glutamate and aspartate levels were found to decrease profoundly during anoxia (by 49 % and 67 %, respectively). During the subsequent 1–8 h of normoxia, both these amino acids showed rather steady increases, although glutamate returned only about half-way back to the control level.

The effects on GABA and glycine were the other way around. Thus, GABA showed a very large increase from $134\pm10\,\mu g\,g^{-1}$ in the controls to $612\pm147\,\mu g\,g^{-1}$ after anoxia. The exposure to normoxia for 1–8 h caused the GABA level to fall to about $300\,\mu g\,g^{-1}$. Glycine also showed a large increase during anoxia, from 48.8 ± 4.1 to $97.1\pm11.1\,\mu g\,g^{-1}$. However, the subsequent 1–8 h of normoxia had no significant effect on the glycine level, although there was a slight decrease.

Fig. 7 shows the effects of anoxia and subsequent normoxia on four other amino acids occurring at high concentrations in crucian carp brain: glutamine, taurine, alanine and serine.

Glutamine behaved much like glutamate and aspartate, displaying a large (70 %) decrease after anoxia and a fairly constant increase during the following 1–8 h of normoxia.

Taurine showed a comparatively moderate decrease (20 %) during anoxia. Some of this fall in taurine seemed to be regained during the subsequent normoxia.

Alanine displayed the most dramatic increase (from 17.8 ± 3.9 to $156\pm42\,\mu g\,g^{-1}$) seen in any of the amino acids studied. The alanine level showed a distinct decrease during the succeeding normoxia, and it had almost decreased to the control level after 8 h.

The behaviour of serine differed from that of the other amino acids. Thus, while anoxia had no significant effect on the serine level, the subsequent 1–8 h of normoxia resulted in a highly significant increase above the control level.

Fish that died during anoxia

Fish were considered dead when equilibrium had been lost and no fin and opercular movements were seen. Of the 20 fish exposed to anoxia, four died. One died after 15 days and two after 16 days of anoxia. Since the container was observed only once a day (in order not to disturb the fish), the exact time of death for these three fish was not recorded. However, a fourth fish died on the morning the experiment was terminated. This individual had lost its equilibrium when it was first observed at 9:00 h. By 09:30 h, no more fin or opercular movements were seen, and when it was decapitated at 10:15 h, its heart had stopped beating. Of the

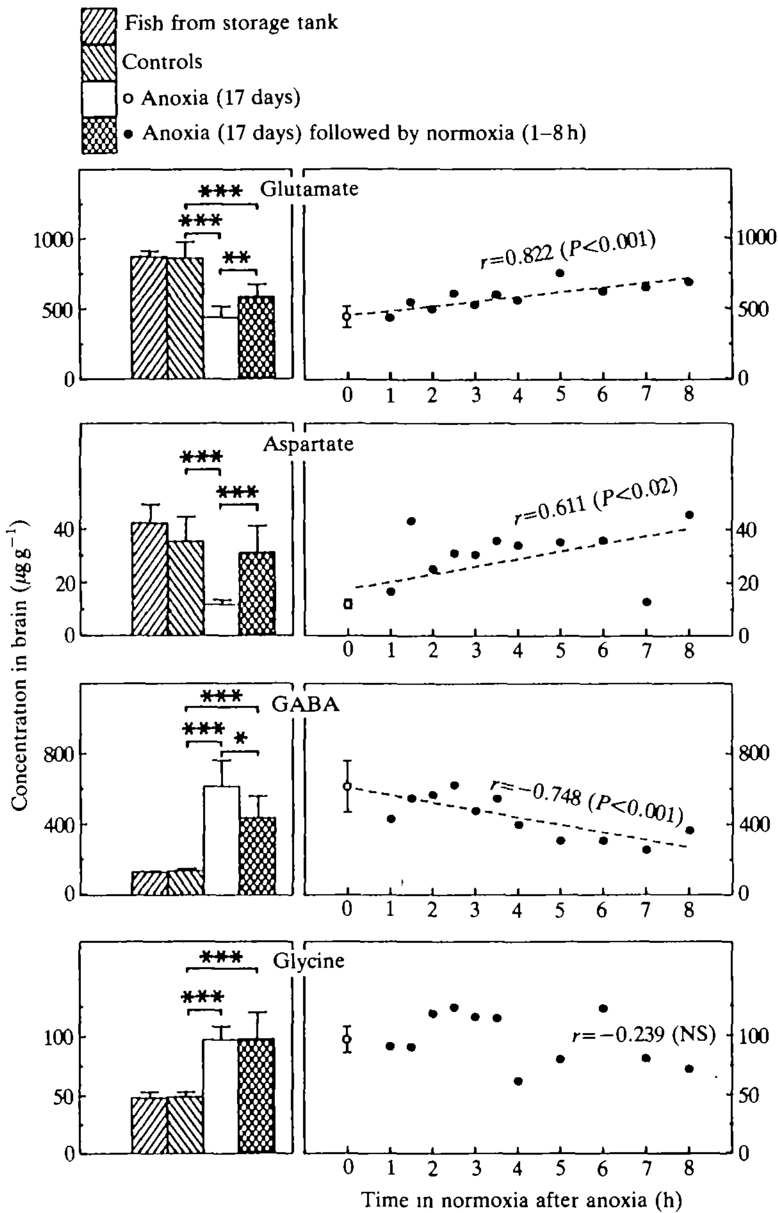

Fig. 6. The effects of 17 days (403 h) of anoxia and a subsequent period (1–8 h) of normoxia on the levels of four putative amino acid neurotransmitters in crucian carp brain. Values are mean and S.D. For details of statistical evaluations, see legend to Fig. 3. NS, not significant.

first three dead fish, only the livers were sampled (at the end of the experiment), while all the variables were measured in the fish that died on day 17. Although the first three dead fish had been 'stored' under anoxic conditions at 8°C, it seemed

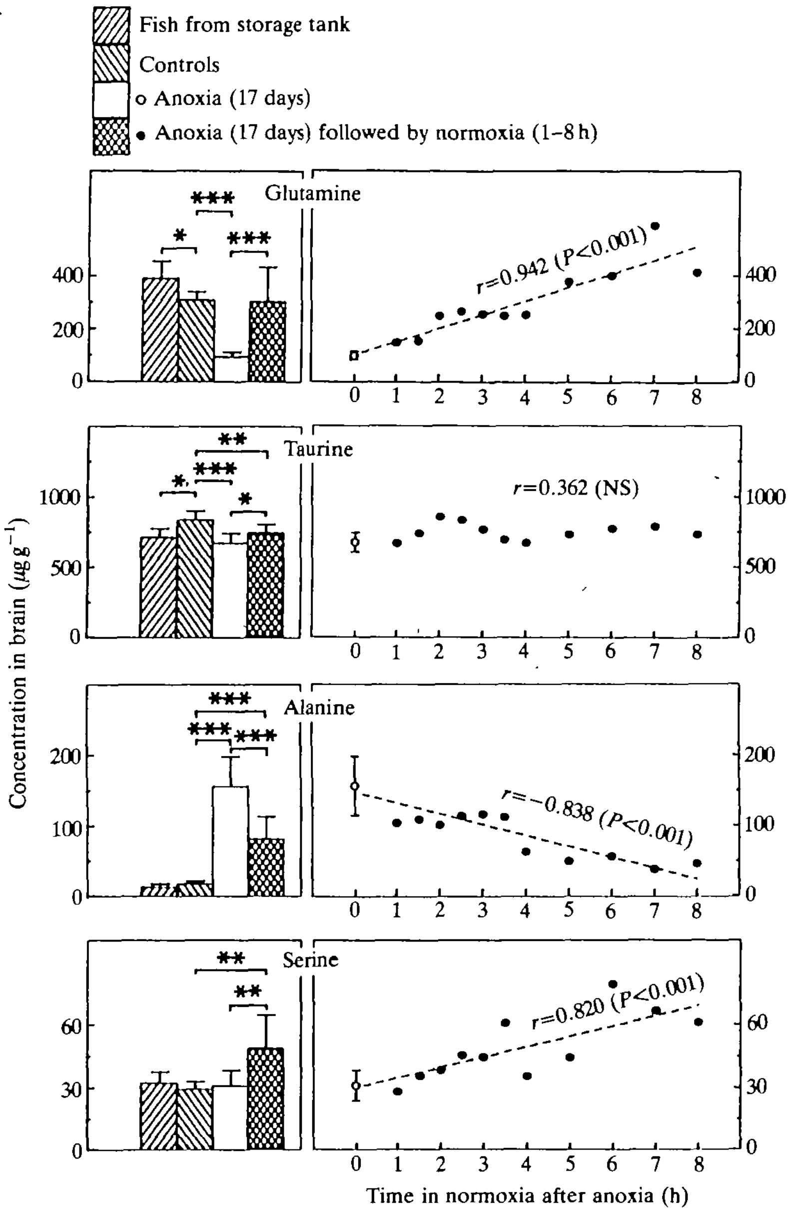

Fig. 7. The effects of 17 days (403 h) of anoxia and a subsequent period (1–8 h) of normoxia on the levels of glutamine, taurine, alanine and serine in crucian carp brain. Values are mean and S.D. For details of statistical evaluations, see legend to Fig. 3. NS, not significant.

meaningless to measure monoamines and amino acids in these specimens because of the high risk of *post-mortem* alterations (Korf, 1981; Tews *et al.* 1963).

No obvious pathological changes were seen in any of the dead individuals. When the fish that died during anoxia were compared with the anoxic survivors,

the only striking difference seen was the absence of detectable liver glycogen in dead fish (Fig. 2). In the single dead fish in which the concentrations of monoamines (brain and kidney) and amino acids were measured, the values were within the range of those of the survivors, with two possible exceptions. The HVA level in the dead specimen was slightly below the range in the survivors (7.9 compared with 9.6–15.3 ng g^{-1}), and the glycine level was slightly higher in the dead specimen (130 compared with 74.7–118 $\mu g\, g^{-1}$ in survivors). Both these differences could very well be due to chance.

Discussion

Remarks on the behaviour during anoxia

The activity of the crucian carp slowed down markedly during anoxia. This will save energy and will probably also help to maintain the functional status of other systems that are depleted during anoxia. The decreased physical activity seen in anoxic crucian carp agrees with the decrease (by 70 %) in overall energy consumption (heat production) found in anoxic goldfish by previous authors (Van Waversveld *et al.* 1989).

It is tempting to hypothesize that the gathering of anoxic crucian carp below the glass lid of the experimental chamber reflects a behaviour that this fish might use in nature in order to utilize oxygen that reaches the water through micropores and cracks in the ice.

The liver glycogen store

The present results confirm previous observations showing that the species in the genus *Carassius* (the crucian carp and the goldfish) possess exceptionally large glycogen stores (Hochachka and Somero, 1984; Hyvärinen *et al.* 1985). The liver glycogen concentration measured in the controls (27 % of the liver mass) is very similar to that measured by Hyvärinen *et al.* (1985) in March (25 %). The same authors also found that the crucian carp liver constitutes 10–15 % of the body mass and that muscle (which makes up 40–50 % of the body mass) only contains about 2–4 % glycogen. Hence, the liver is the main store of glycogen in the crucian carp. Furthermore, experiments on goldfish have shown that brain and heart glycogen stores are rapidly depleted during anoxia (McDougal *et al.* 1968; Merrick, 1954).

During the present conditions, 15–17 days in anoxia at 8°C was apparently enough to deplete the liver glycogen store totally in four out of 20 crucian carp, and to deplete it severely in most of the 16 survivors. There is little reason to doubt that lack of glycogen was the direct cause of death in these four fish. Van Waversveld *et al.* (1989) showed that glycolysis is the major route for energy production during anoxia, and that other sources of energy (protein and fat) are of minor or no quantitative importance for anoxic *Carassius* (goldfish). In fact, the same authors found that anoxic goldfish actually produce fat, probably by running β-oxidation backwards to make some tricarboxylic acid cycle activity possible during anoxia. However, they studied goldfish held in anoxia at 20°C, and this

feature is probably temperature-dependent, since there is very little sign of tricarboxylic acid cycle activity in anoxic goldfish held at 4°C (Shoubridge and Hochachka, 1981).

In contrast to the present results, a previous study on anoxic goldfish at 20°C (Van den Thillart *et al.* 1980) suggested that the glycogen store only decreases by 50–70 % during lethal periods of anoxia (12–15 h for goldfish at 20°C). Thus, it is possible that the depletion of glycogen reserves is the limiting factor for anoxia-tolerance at low temperatures (8°C in the present study), while the failure of some other system becomes important at higher temperatures.

It should be emphasized that 18 days of starvation *per se* had no significant effect on the liver glycogen store. This is in agreement with previous results, which suggest that *Carassius* (goldfish) preferentially oxidize fat reserves during normoxic starvation, whereas the rate of carbohydrate consumption increases 100-fold during anoxia (see Van Waversveld *et al.* 1989, for a review).

Brain serotonin

Both the synthesis and the degradation of serotonin 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. Monoamine oxidase (MAO) catalyses the first step of the degradation of serotonin, in which an aldehydic intermediate is formed. This reaction also involves a simultaneous reduction of molecular oxygen. The aldehydic intermediate is then rapidly 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 (see Tipton *et al.* 1977, for a review).

As in the previous study on the effect of 1–7 days of anoxia on serotonin and its metabolites (Nilsson, 1989*a*), the present results suggested a stop in serotonin metabolism during anoxia, as judged by the complete absence of detectable levels of 5-HIAA and 5-HTOH (Fig. 3). The previous study showed that this fall in metabolite level occurred during the first 22 h.

The turnover time (as calculated from the *in vivo* synthesis rate) for serotonin in mammals is about 1–2 h (Korf, 1985). In contrast, I recently obtained results suggesting that the serotonin turnover time in different brain regions of normoxic crucian carp held at 8°C varies between 1 and 3 days (G. E. Nilsson, unpublished results). However, the present results showed that there was only a 24 % decrease in the brain serotonin level after 17 days in anoxia. The ability to maintain the serotonin level during anoxia is remarkable even if one assumes that the serotonin turnover rate decreases by about 70 % (like energy consumption) during anoxia. Since anoxia will abolish both catabolism and synthesis of serotonin, the result may be explained by a very high degree of recycling of serotonin molecules. We do not yet know how this is done.

However, if serotonin leaves the brain only after being metabolized, there is no reason to expect a large fall in the serotonin level. Hence, much of the serotonin found in brain after prolonged anoxia could be present in compartments where it

would normally (in normoxia) be metabolized, i.e. outside the synaptic vesicles. In these compartments it will be of no use for synaptic transmission. In fact, the large and rapid increases seen in the levels of the serotonin metabolites after anoxia (Fig. 3) suggest that a substantial amount of serotonin was present outside synaptic vesicles and therefore readily available for metabolism during the renewed contact with oxygen. However, since nothing is known about the kinetics of the elimination of serotonin metabolites from crucian carp brain, or of the ability of this fish to increase the rate of serotonin synthesis when necessary, it is not possible to speculate about the size of the extravesicular serotonin pool without further experiments.

In a previous study on serotonin in anoxic crucian carp (Nilsson, 1989*a*), the effects of 30–53 min of subsequent normoxia were studied. During this period, a very rapid production of 5-HTOH was seen, suggesting that the brain is in a reduced state (i.e. unusually high levels of NADH and NADPH compared with NAD^+ and $NADP^+$) within the first hour of normoxia. The present results suggest that this rapid 5-HTOH production and, hence, the reduced state, is maintained during the first 2.5 h of normoxia after anoxia (Fig. 3).

The level of 5-HIAA was significantly ($P<0.05$) lower in experimental controls than in fed fish taken directly from the storage tank, suggesting that the experimental situation *per se* may decrease serotonergic activity. It is not possible to say whether this difference is related to the 18 days of starvation, a changed social situation (20 fish in 18 l of water compared with 60 fish in 400 l), or pure chance.

Brain catecholamines

Catecholamine synthesis resembles serotonin synthesis and has the same absolute requirement for molecular oxygen. In the first step of the synthesis of all catecholamines, L-tyrosine is hydroxylated by tyrosine hydroxylase to form L-Dopa. The oxygen atom that is added to tyrosine in this reaction is directly derived from molecular oxygen (Kaufman, 1985; Kirshner, 1975; Nagatsu, 1973). The synthesis of norepinephrine from dopamine, which is catalysed by dopamine-β-hydroxylase, has a direct requirement for molecular oxygen (Kirshner, 1975; Nagatsu, 1973). With regard to catabolism, the oxidative deamination of catecholamines that is catalysed by monoamine oxidase demands molecular oxygen (Nagatsu, 1973; Yoshino *et al.* 1984). However, in contrast to the absolute oxygen demand of serotonin catabolism, catecholamine inactivation could probably proceed in the absence of oxygen, *via* methylation catalysed by catechol-*O*-methyl-transferase (COMT) or by sulphate or glucuronide conjugation (Nagatsu, 1973). Furthermore, catecholamines might leak out of brain. There are results suggesting that *Carassius* lacks a blood–brain barrier for norepinephrine (Busacker and Chavin, 1977). Hence, there are plenty of reasons for expecting large decreases in catecholamine levels during anoxia. In spite of this, previous results on the effects of 3–7 days of anoxia on catecholamine levels in crucian carp

brain (Nilsson, 1989*b*) only indicated minor decreases after 7 days in anoxia (a 17 % fall in norepinephrine and a nonsignificant 9 % fall in dopamine).

Also, in the present study, the falls in norepinephrine and dopamine levels caused by anoxia (Fig. 4) were surprisingly small (only 36 % and 29 %, respectively). In combination with the results of a previous study (Nilsson, 1989*b*), the present results suggest a rather constant decrease in the levels of these catecholamines during anoxia.

Norepinephrine showed no sign of recovery during the subsequent 8 h of normoxia. Since norepinephrine is synthesized from dopamine by DBH inside the synaptic vesicles, the most obvious explanation for this lack of norepinephrine recovery is that much of the dopamine found after anoxia is present outside synaptic vesicles and cannot be used for norepinephrine synthesis. However, it is also possible that the brain noradrenergic system is impaired by prolonged anoxia in the crucian carp. Gromova *et al.* (1988) obtained results suggesting that the noradrenergic, but not the dopaminergic, system is damaged by prenatal anoxia in rats. Moreover, the present results on kidney norepinephrine indicated damage by anoxia (see below). A failure of the noradrenergic system during anoxia could be very unfortunate since this system exerts an important inhibitory effect on brain (much like GABA, see below), and recent results suggest that norepinephrine may have a prominent role in counteracting brain damage during anoxia (Magnusson *et al.* 1988; Gustafson *et al.* 1989).

In contrast to norepinephrine, levels of both dopamine and its metabolite HVA increased significantly during normoxia (Fig. 4). However, the decrease in HVA during the preceding anoxia was unexpectedly small compared with the disappearance of serotonin metabolites. This might reflect a very low rate of HVA elimination from the brain and/or a very slow turnover rate of dopamine. In the mammalian brain, the dopamine turnover time is usually 3–5 h, which is about three times longer than that of serotonin (Korf, 1985). If this is also the case in the crucian carp brain, where normoxic serotonin turnover times of 1–3 days have been estimated (G. E. Nilsson, unpublished results, see above), then the turnover time of dopamine might be around a week in normoxia. Hence, decreased brain activity during anoxia could even make it possible for the dopamine pool to outlast 17 days of anoxia.

Kidney catecholamines

In the crucian carp, as in most fish, the head kidney is analogous to the mammalian adrenal gland and, hence, contains the chromaffin cells that synthesize and store the catecholamines that are to be released into the bloodstream as stress hormones (Chavin and Young, 1970; Fänge and Hanson, 1973; Matty, 1985). Catecholamine metabolism in kidney and in brain follow the same oxygen-dependent routes (described above). However, the chromaffin cells have a very remote chance of re-using the same catecholamine molecules since these are distributed all over the body and not, as in brain, released into thin synaptic clefts. Hence, one would expect anoxia to cause a drastic decrease in kidney catechol-

amine levels. This was the case in the present study, where kidney norepinephrine fell by 92 %, an almost total depletion, and the results indicated a similar drop in epinephrine. In the previous study on catecholamines in anoxic crucian carp (Nilsson, 1989*b*), 7 days of anoxia caused a 34 % decrease in kidney norepinephrine.

During the 1–8 h of normoxia that succeeded the 17 days of anoxia, the level of norepinephrine showed a very slow rate of recovery. This is in sharp contrast to the previous results (Nilsson, 1989*b*), which showed that all the norepinephrine lost during 7 days of anoxia was regained within 40 min of normoxia. Hence, it is conceivable that the very long anoxic period employed in the present experiment damaged the norepinephrine synthesizing system in chromaffin tissue. The same slow (or absent) norepinephrine recovery was also seen in brain (see above). One possible explanation for both these results is that dopamine-β-hydroxylase is impaired by prolonged anoxia. This remains to be studied experimentally.

Methodological considerations regarding catecholamines

No anaesthesia was used in the present study since it is possible that centrally acting anaesthetics will affect brain neurotransmitter systems. Furthermore, adding an anaesthetic to the water would have created a very large variability in the time of anaesthesia since the same container was sampled repeatedly (for 8 h for the fish exposed to normoxia after anoxia). However, the short period of stress that the fish may have experienced before decapitation is unlikely to affect the catecholamine concentrations measured in the present study. First, it should be emphasized that brain norepinephrine is an inhibitory neurotransmitter and not a stress hormone (Bradford, 1986; Cooper *et al.* 1986). It has been shown that the sampling procedure (including electric shock 15 min prior to sampling) has no significant effect on monoamine levels in rainbow trout brain (Sloley *et al.* 1986). In fish where blood catecholamine levels have been measured during physical stress (rainbow trout and cod), elevations from undisturbed levels (2–8 $ng\,ml^{-1}$) to about 10–30 $ng\,ml^{-1}$ (norepinephrine) or 40–100 $ng\,ml^{-1}$ (epinephrine) are seen (Nakano and Tomlinson, 1967; Nilsson *et al.* 1976). In the present experiments, no epinephrine could be detected in crucian carp brain, and a blood concentration of 30 $ng\,ml^{-1}$ norepinephrine is only 5 % of the brain concentration and would not significantly affect the brain norepinephrine levels measured, especially since the light colour of the brain shows that the blood volume only constitutes a small fraction of the total brain volume.

Also, the kidney catecholamine content is unlikely to have been affected by the short period of stress that may have been imposed on the fish immediately before decapitation. It has been found (in rainbow trout) that even 2 h of physical disturbance does not affect the amount of catecholamines present in the kidney (Nakano and Tomlinson, 1967).

Brain amino acids

The brain levels of all eight amino acids displayed more or less dramatic changes

during 17 days of anoxia and/or during the subsequent 1–8 h of normoxia. In mammals, the role of GABA as an inhibitory neurotransmitter and the roles of aspartate and glutamate as excitatory neurotransmitters are well established (McGeer and McGeer, 1989; Bradford, 1986; Cooper *et al.* 1986). Available data on amino acid transmitters in *Carassius* reveal no major differences compared with mammals (Villani *et al.* 1982; Poli *et al.* 1984, 1985; Bissoli *et al.* 1985; Yazulla *et al.* 1985; Cha *et al.* 1986; Henley and Oswald, 1988). Hence, in the discussion below, it is assumed that the synaptic effects of these transmitters are essentially the same in crucian carp and mammals.

The increase in brain GABA (nearly fivefold, Fig. 6) during anoxia is probably an effect of the oxygen-dependence, i.e. dependence on tricarboxylic acid cycle activity, of GABA degradation (McGeer and McGeer, 1989). This increase could be very important for preserving the crucian carp brain during anoxic episodes. It seems likely that the massive elevation of GABA will result in an increased concentration of this inhibitory transmitter at receptor sites and, hence, cause a decrease in the synaptic and electrical activity. This would save neurotransmitters as well as energy, ion movements caused by electric activity being a main energy consumer in brain (Hawkins, 1985). In fact, synaptic activity has been found to mediate nerve cell death during anoxia (Rothman, 1983).

Increasing evidence suggests that excitatory amino acids are important mediators of anoxic brain damage (Benveniste *et al.* 1984; Rothman, 1985; Choi, 1988; Somjen, 1988). Therefore, the decrease seen in the brain levels of excitatory amino acids (glutamate and aspartate, Fig. 6) may play an important role in counteracting neuronal injury in anoxic crucian carp.

Consequently, it is a distinct possibility that the decline in physical activity seen in anoxic crucian carp is caused by the massive elevation of GABA in brain, maybe in combination with decreased levels of excitatory amino acids. Interestingly enough, benzodiazepine sedatives such as diazepam, which seem to act through a facilitation of GABAergic transmission (Cooper *et al.* 1986), have been found to exert a potent protective action on anoxia-sensitive neurones in mammalian hippocampus (Lust *et al.* 1988).

More moderately increased levels of GABA in combination with lowered levels of aspartate (Tews *et al.* 1963; Norberg and Siesjö, 1975) or aspartate and glutamate (Duffy *et al.* 1972; Erecinska *et al.* 1984) have previously been seen in the brain of anoxic or ischaemic mammals. Similar results have also been obtained in two studies in anoxia-tolerant turtle (*Pseudemys scripta*). One of these studies found a 60 % increase in GABA, a 50 % fall in glutamine and a possible decrease in glutamate (by 20 %) in the brain of turtle exposed to 2 h of anoxia at 20°C (Hitzig *et al.* 1985). The other study showed a decrease in glutamate and an increase in GABA after 4 h of anoxia (Lutz *et al.* 1985).

GABA should be thoroughly examined as a possible factor behind the benign decrease in brain activity displayed by anoxia-resistant animals in response to decreased oxygen availability. However, it is not possible to draw any conclusions before we know that the increased tissue level of GABA corresponds to an

increased transmitter pool and an increased GABA concentration at receptor sites.

The lowered level of glutamate after anoxia is probably intimately connected to the decrease seen in the glutamine level (Fig. 7), since glutamine seems to be a major precursor of glutamate in brain (Bradford, 1986). Glutamate may also be derived from α-ketoglutarate. Thus, the fall in glutamate level could be directly related to a decreased tricarboxylic acid cycle activity. However, glutamate breakdown is also dependent on tricarboxylic acid cycle activity and there is no obvious effect of anoxia on glutamate metabolism. In fact, glutamate levels have been found to increase in the liver of goldfish held in anoxia for 12 h at 20 °C (Van Waarde *et al.* 1982), while unchanged glutamate levels were seen in muscle and blood.

The most straightforward explanation of the decreased levels of glutamate (and the communicating pool of glutamine) is related to the fact that glutamate is also the immediate precursor of GABA. Hence, if the synthesis and degradation of glutamate are very slow or run at equal rates during anoxia, then the irreversible conversion of glutamate to GABA, *via* glutamate decarboxylase, would be enough to explain the decrease in glutamate (and glutamine) levels, as well as the increase in GABA concentration [bearing in mind that GABA degradation probably comes to a halt during anoxia (see above)]. The fact that the GABA level increased during anoxia actually proves that the glutamate decarboxylase reaction proceeds during anoxia. It is interesting to note that the increase in GABA during anoxia, $4.64 \pm 1.43\,\mathrm{mmol\,l^{-1}}$, is very similar to the decreases in glutamate and glutamine taken together, $4.36 \pm 0.58\,\mathrm{mmol\,l^{-1}}$ ($4.54 \pm 0.60\,\mathrm{mmol\,l^{-1}}$ if the decrease in aspartate is also included in this figure).

Aspartate and glutamate readily undergo interconversion, catalysed by aspartate aminotransferase, and the decrease in aspartate may thereby be directly related to the decrease in glutamate. However, Van Waarde *et al.* (1982) found decreased levels of aspartate (by 73–85 %) together with unaltered or increased levels of glutamate in four tissues (red muscle, white muscle, liver and blood) of anoxic goldfish, suggesting a more complex situation.

The nearly 10-fold increase in brain alanine level observed in anoxic crucian carp (Fig. 7) should be commented upon. Alanine is unlikely to be a neurotransmitter (McGeer and McGeer, 1989), although it occurs at relatively high levels in brain. The accumulation of alanine is probably related to the fact that it is an end-product of anaerobic energy metabolism in many animals and its level is often found to increase during anoxia (Van Waarde *et al.* 1982; Hochachka and Somero, 1984). A large increase in alanine concentration has previously been found in several tissues (including brain) of anoxic *Carassius* (Van den Thillart, 1982; Van Waarde *et al.* 1982; Johnston and Bernard, 1983; Shoubridge and Hochachka, 1983) as well as in the brain of mammals (Tews *et al.* 1963; Erecinska *et al.* 1984). Alanine is formed from pyruvate *via* a transamination that may be coupled with the formation of α-ketoglutarate from glutamate or oxaloacetate from aspartate (Martin *et al.* 1985). Hence, the increase in alanine might be related to the

decrease seen here in glutamate and/or aspartate. However, the general assumption is that the reaction serves much the same purpose as the formation of lactate, since α-ketoglutarate may be reduced back to glutamate (by glutamate dehydrogenase) during a simultaneous oxidation of NADH to NAD (which is in short supply during anoxia). Thus, the formation of alanine would cause no net decrease in glutamate.

One might argue that the increase seen in the glycine level (Fig. 6) could help to decrease brain activity during anoxia, especially in the lower brain areas and spinal cord, where it has a long-established role as an inhibitory neurotransmitter (McGeer and McGeer, 1989). However, unlike GABA, glycine has many metabolic functions in brain, being an important constituent of proteins. Thus, it is not possible to know to what extent the increased brain level of glycine affects its neurotransmitter function. Moreover, in mammals, recent evidence strongly suggests that glycine is an important allosteric activator of the NMDA receptor for glutamate, and thus is acting as an excitatory factor in higher brain areas (Foster and Kemp, 1989). In connection with glycine, it should be mentioned that serine, the level of which remained unaltered after anoxia but increased during the subsequent normoxia (Fig. 7), is the immediate precursor of glycine. Serine has some glycine-like actions, but it is probably not a neurotransmitter (McGeer and McGeer, 1989).

Finally, the behaviour of taurine during anoxia should be commented upon. The changes seen in the taurine level were all comparatively small and this remarkable stability during and after anoxia (or ischaemia) has also been observed in mammals (Lehmann *et al.* 1988). Taurine occurs at very high levels in the vertebrate brain, and the present results show that the crucian carp is no exception. However, a neurotransmitter role for taurine remains doubtful (Hanretta and Lombardini, 1987; McGeer and McGeer, 1989).

Concluding remarks

Although the crucian carp is an extremely anoxia-tolerant vertebrate (16 of 20 individuals survived 17 days of anoxia at the present conditions), its internal milieu remains far from unaltered during anoxia. All the seven putative neurotransmitters studied displayed significant changes in their concentrations during anoxia. Most of these changes seemed to be benign (judged by the recovery seen during a renewed contact with oxygen), with the possible exception of norepinephrine in brain and chromaffin tissue.

The most prominent change in transmitter levels seen during anoxia was the massive (nearly fivefold) increase in brain GABA, suggesting a role for this inhibitory transmitter in decreasing brain activity during anoxia. A decrease in brain activity would explain the decrease seen in physical activity and, thus, maybe also the decrease in a systemic energy consumption observed by others (Van Waversveld *et al.* 1989).

The importance of a lowered energy consumption as a strategy for surviving anoxia has often been emphasized (Hochachka, 1986; Hochachka and Guppy,

1987; Rosenthal *et al.* 1988). That the depletion of energy stores is a limiting factor during anoxia has been clearly demonstrated in the present study.

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