J. exp. Biol. **104**, 73–78 (1983)
Printed in Great Britain

UTILIZATION OF THE ETHANOL PATHWAY IN CARP FOLLOWING EXPOSURE TO ANOXIA

BY IAN A. JOHNSTON AND LYNNE M. BERNARD

Department of Physiology, University of St. Andrews, St. Andrews, Fife, Scotland, Great Britain

(*Received 14 July 1982—Accepted 23 November 1982*)

SUMMARY

1. Crucian carp (*Carassius carassius* L.) and Common carp (*Cyprinus carpio* L.) were subjected to 2 h progressive hypoxia followed by up to 6 h anoxia in closed respirometers at 15 °C.

2. The concentrations of glycogen, glucose, phosphoryl creatine, alanine, succinate, lactate, ethanol and ammonia were determined in whole Crucian carp following exposure to both hypoxia and anoxia.

3. Ethanol and lactate were the main anaerobic end products. Glycogen utilization during anoxia amounted to 2 mmol glucose equivalents·kg wet weight^{-1} h^{-1}. Around 85 % of the ethanol produced (2·8 mmol kg^{-1} h^{-1}) was excreted. Lactate accumulation (0·7 mmol kg^{-1} h^{-1}) was only sufficient to account for 18·5 % of the glycogen stores utilized. Ammonia production (0·2 mmol·kg body wt^{-1} h^{-1}) was independent of the environmental oxygen availability.

4. Ethanol concentrations (μmol·g wet wt^{-1}) were 7 in red myotomal muscle, 4 in white myotomal muscle, and 2 in liver after 6 h anoxia.

5. In contrast to Crucian carp, Common carp (*Cyprinus carpio* L.) did not produce ethanol at 15 °C even after near lethal exposure to anoxia (5–6 h).

INTRODUCTION

Goldfish (*Carassius auratus* L.) have a remarkable tolerance of anoxia. Survival time is highly temperature dependent, ranging from 16 h at 20 °C to several weeks at 0 °C (Van Den Thillart, 1977; Walker & Johansen, 1977). Anoxia for 12 h has little effect on adenylate energy charge in the swimming muscles but causes a significant decline in the charge in the liver (Van Den Thillart, Kesbeke & Van Waarde, 1980; Van Den Thillart, Van Waarde, Dobbe & Kesbeke, 1982). Under these conditions lactate accumulation in the whole carcass was found to be less than half that expected from measurements of glycogen breakdown (Van Den Thillart, 1977). Evidence for some unusual pathway of anaerobic metabolism was provided by measurements of CO_2 production during anoxia (Hochachka, 1961; Van Den Thillart, 1977). Recently, in experiments in which goldfish were made anoxic by carbon monoxide poisoning, Shoubridge & Hochachka (1980) discovered that CO_2 excretion is linked to the production of a novel anaerobic end product, ethanol.

Key words: Crucian carp, Common carp, anoxia, anaerobiosis, hypoxia.

In the present study we have investigated the ability of Crucian carp (*Carassius carassius* L.) and Common carp (*Cyprinus carpio* L.) to produce ethanol during anoxia.

MATERIALS AND METHODS

The fish were maintained in aerated freshwater aquaria at 15 °C. They were fed daily on trout pellets, supplemented with occasional meals of chopped pigs' liver. No food was given for 2 days prior to experiments. Fish were transferred in pairs to open respirometers (1 l capacity, opaque boxes) containing aerated water at least 24 h before experiments. The boxes were immersed in the holding tanks so as to maintain the temperature at 15 °C. At the start of the experiments the boxes were sealed and the respiration of the fish was allowed to reduce the water PO_2 over 3 h. Routine oxygen consumption was calculated from serial measurements of water PO_2 obtained through a sampling port in the side of the respirometer. PO_2 was measured using a Radiometer PMH 72 Oxygen Analyser. After 3 h the PO_2 of the water was reduced from 121 mmHg to around 10 mmHg. At this point N_2 gas was bubbled through the respirometer until the water PO_2 reached zero (~ 10 min).

The concentrations of metabolites in whole Crucian carp were determined prior to the onset of hypoxia (Sample 1, 20 fish, length $8{\cdot}4 \pm 0{\cdot}2$ cm, body weight $13{\cdot}9 \pm 0{\cdot}7$ g), at the attainment of zero PO_2 following progressive hypoxia (Sample B, 12 fish, length $9{\cdot}3 \pm 0{\cdot}4$ cm, body weight $14{\cdot}8 \pm 2{\cdot}0$ g), and following 6 h anoxia (Sample C, 20 fish, length $9{\cdot}1 \pm 0{\cdot}3$ cm, body weight $14{\cdot}5 \pm 1{\cdot}5$ g). Half the fish were killed by stunning and half by injecting concentrated MS222 [Tricaine methanosulphonate (Sigma)] into the respirometer to a final concentration of 200 ppm. One of the fish in each respirometer box was diced into small pieces (15 s) and dropped into boiling 30 % KOH. Following 15 min digestion, glycogen was precipitated from the extract and the concentration of glucose equivalents determined by an anthrone method (Johnston & Moon, 1980). The other fish was homogenized directly in 6 volumes of ice-cold 6 % perchloric acid using a Waring blender (5 × 15 s with cooling). The perchloric acid extracts were centrifuged at 15 000 ***g*** for 5 min and aliquots neutralized with 2·2 M-K_2CO_3 containing methyl orange indicator. Samples were stored at −35 °C until analysis. The perchloric acid extraction method used has been shown to give better preservation of phosphorylated intermediates than freezing the tissue prior to extraction (Van Den Thillart *et al.* 1980).

The following metabolites were determined in duplicate from the neutralized extracts: glucose (Bergmeyer & Bernt, 1965), lactate (Hohorst, 1965), alanine (Pfleiderer, 1965), succinate (Michal, Beutler, Lang & Gunter, 1976) and phosphoryl creatine (Walesby & Johnston, 1980). In addition, ammonia and ethanol concentrations were determined in both whole fish and water samples using commercial kits obtained from Sigma Chemicals Ltd. and Boehringer Mannheim (Poole), respectively. No difference was found in metabolite concentrations between fish killed by stunning or anaesthesia and so the results were combined.

In a separate series of experiments samples of liver, superficial red and deep white myotomal muscles were rapidly excised from anaesthetized Crucian carp (16 fish, mean bodyweight 9·3 g) and freeze-clamped using liquid nitrogen cooled brass ton

[redacted]rchloric extracts were prepared from the tissues of fish prior to hypoxia (Sample A) and after 6 h anoxia (Sample C). All metabolite concentrations were expressed as μmol·g wet wt tissue^{-1}.

A group of eight Common carp (*Cyprinus carpio* L.) (mean ± S.E.; length 9·5 ± 0·4 cm, bodyweight 15·7 ± 1·9 g) were also subject to 6 h anoxia in respirometer boxes. The experimental protocol was identical to that already described for Crucian carp. Serial water samples were taken and analysed for ethanol.

Statistical analysis was carried out using two-way analysis of variance.

RESULTS

Experiments with Crucian carp

Routine oxygen consumption of 14 g Crucian carp was 69·7 ± 2·8 ml·kg^{-1} h^{-1} at STP (Fig. 1). This is equivalent to an ATP production from aerobic metabolism of 18·7 mmol ATP kg^{-1} h^{-1} assuming a P/O ratio of 3. When the respirometers were sealed $\dot{V}O_2$ decreased in parallel to the fall in water PO_2 (Fig. 1). Hypoxia (sample B) resulted in significant increases in glucose, alanine and succinate concentrations ($P < 0{\cdot}01$) and a small, but not statistically significant, rise in lactate ($P > 0{\cdot}05$) (Table 1). Glycogen and phosphoryl creatine stores were not significantly different between hypoxia and control samples (Table 1).

All 44 Crucian carp studied were able to withstand 6 h anoxia without any apparent ill-effects. Following 6 h anoxia glycogen stores were reduced by 11·7 μmol glucose equivalents·g^{-1} and phosphoryl creatine concentrations by 1·9 μmol g^{-1} compared to hypoxia values (Table 2, $P < 0{\cdot}01$). The concentrations of metabolites in the carcasses of control, hypoxic and anoxic samples are shown in Table 1. The net lactate accumulation after 6 h anoxia was 4·3 μmol·g wet wt^{-1} (Table 1). The total ethanol

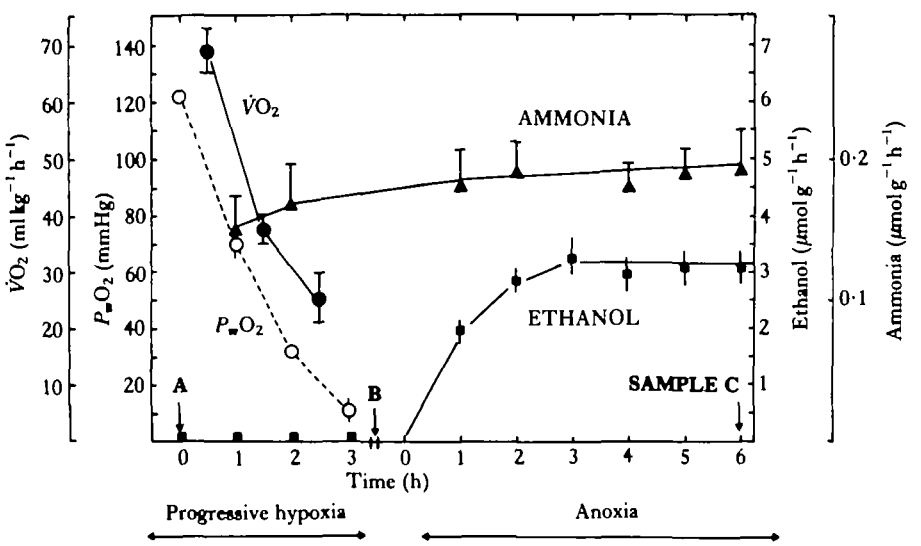

Fig. 1. Routine oxygen consumption (●), and ammonia (▲) and ethanol (■) excretion rates in Crucian carp exposed to hypoxic and anoxic conditions in closed respirometers. Values represent mean ± S.E. for 12 fish. The points at which fish were sampled (A–C) for whole-body metabolite analyses are also indicated.

Table 1. *Metabolite concentrations in whole fish*

Metabolite (μmol·g wet wt fish^{-1})	Sample points (see Fig. 1) A Aerated water	B Initial anoxia sample	C Following 6 h anoxia
Glycogen	40·5 ± 5·4	36·2 ± 4·9	24·5 ± 3·5^{++}
Glucose	1·17 ± 0·13	3·16 ± 0·63	2·80 ± 0·16
Phosphoryl creatine	3·56 ± 0·15	3·20 ± 0·32	1·32 ± 0·14^{++}
Ammonia	2·36 ± 0·32	2·37 ± 0·45	2·11 ± 0·11
Alanine	1·58 ± 0·29	2·36 ± 0·34	3·30 ± 0·42
Succinate	0·39 ± 0·053	1·77 ± 0·35	0·90 ± 0·19
Lactate	3·48 ± 0·24	4·02 ± 0·38	8·36 ± 0·23^{++}
Ethanol	–	0·45 ± 0·074	3·13 ± 0·34^{+++}

Values represent mean ± S.E. for 10 (A,C) or 6 (B) fish.
Statistically significant differences between the initial and final anoxic samples at the $P<0·01$ (++) and $P<0·001$ (+++) levels are shown in the right hand column.

Table 2. *Lactate and ethanol concentrations in red and white myotomal muscle and liver*

Tissue	Sample A Aerated water	Sample B Following hypoxia and 6 h anoxia
Lactate (μmol·g wet wt tissue^{-1})		
Red muscle	3·07 ± 0·46	11·89 ± 0·75
White muscle	3·12 ± 0·24	12·54 ± 1·50
Liver	0·99 ± 0·27	5·54 ± 0·43
Ethanol (μmol·g wet wt tissue^{-1})		
Red muscle	–	7·11 ± 0·40
White muscle	–	3·93 ± 0·64
Liver	–	2·23 ± 0·24

Values represent mean ± S.E. of 10 fish for sample A and 6 fish for sample B.

concentration in control fish was below the detection limit of the assay technique. Anoxia resulted in an increase in tissue ethanol concentration to 2·7 μmol·g wet wt^{-1} ($P<0·01$) (Table 1). Ethanol is freely diffusable across the gills and accumulated in the water (Fig. 1). Ethanol production was only observed after the water PO_2 fell to zero and maximal excretion rates were not established until after 2 h anoxia (Fig. 1). Total ethanol production following 6 h anoxia was 19·3 μmol·g body wt^{-1} (tissue + water concentrations).

The concentrations of ethanol in the red muscle were more than twice those for the other tissues analysed (Table 2).

Little or no significant changes were observed in the whole body concentrations of alanine or succinate, two important end products produced by certain invertebrates under anoxic conditions (Table 1).

Ammonia excretion (0·2 μmol·g^{-1} h^{-1}) was found to be almost independent of environmental oxygen tensions (Fig. 1).

Experiments with Common carp

A total of six out of eight Common carp died after 5–6 h exposure to zero PO_2, suggesting that this is near the limit of their anoxic tolerance at this temperature. In contrast to Crucian carp, Common carp (*Cyprinus carpio*) did not produce any ethanol even after lethal exposure to anoxia.

DISCUSSION

The major anaerobic end-product produced by Crucian carp during anoxia is ethanol, thus confirming the results of Shoubridge & Hochachka (1980) on goldfish. In our experiments tissue ethanol accumulation was 0·45 μmol g^{-1} h^{-1} and the average excretion rate was 2·8 μmol g^{-1} h^{-1} (Table 1; Fig. 1). This is equivalent to the utilization of 1·67 μmol glycosyl-units g^{-1} h^{-1} or 86 % of the glycogen breakdown during anoxia (Table 1). In Crucian carp 85 % of the ethanol produced over 6 h appeared in the water (17·2 μmol g^{-1}). In contrast lactate was not excreted to any appreciable extent under these conditions (Shoubridge & Hochachka, 1980). The net lactate accumulation after 6 h anoxia was 4·3 μmol g^{-1}, which is sufficient to account for 18·5 % of the glycogen breakdown (Table 1). In CO-poisoned goldfish, Shoubridge & Hochachka (1980) found that a proportion of ^{14}C-lactate injected into anoxic goldfish could be recovered in the ethanol fraction thus providing evidence for a conversion of lactate to ethanol.

Mourik, Raeven, Steur & Addink (1982) have shown that the pyruvate dehydrogenase complex of goldfish mitochondria is able to decarboxylate pyruvate to form acetaldehyde under anaerobic conditions. Acetaldehyde is subsequently reduced to ethanol in the cytoplasm by alcohol dehydrogenase (Van Den Thillart, 1982). In Crucian carp the highest accumulation of ethanol occurred in the red muscle (Table 2). This parallels the relative activities of alcohol dehydrogenase determined for goldfish tissue which were red muscle > white muscle > liver (Van Den Thillart, 1982). The alcohol dehydrogenase reaction in goldfish is strongly biased in the direction of ethanol production and it is unlikely to be coupled to ATP production (Van Den Thillart, 1982; Mourik *et al.* 1982). The utilization of the ethanol pathway allows redox balance to be maintained and glycolysis to proceed without the accumulation of an acid end-product.

If glycolysis is the only pathway available for generating ATP then the net ATP turnover during anoxia (calculated from phosphoryl creatine and glycogen breakdown) is 4·2 mmol ATP kg^{-1} h^{-1}. This represents 22 % of the energy expenditure during routine activity in aerated water. Anderson (1975) found that heat production by anoxic goldfish was reduced by 80 % at 18 °C. It seems likely that a reduction in metabolic rate is important in extending anoxic tolerance, particularly at low temperatures.

Rates of ammonia excretion were found to be relatively independent of the PO_2 of the water (Fig. 1). Very similar results have been obtained for anoxic goldfish (Van Den Thillart & Kesbeke, 1978). The origin of anaerobic ammonia production is obscure. It is probably derived in part from the deamination of the adenylate pool and

possibly from certain free amino acids (Van Den Thillart & Kesbeke, 1978; Van D[redacted] Thillart, 1982).

In contrast, to Crucian carp, common carp do not appear to utilize the ethanol pathway during anoxia. The anoxic tolerance of Crucian carp is significantly greater than that of Common carp. It would appear that the utilization of the ethanol pathway, although wasteful of carbon, extends the survival of *Carassius* to outside the usual range found for vertebrates.

We are grateful to the Natural Environment Research Council for support.

REFERENCES

ANDERSON, J. (1975). Anaerobic resistance of *Carassius auratus* (L). Ph.D. thesis, Australian National University.

BERGMEYER, H. Y. & BERNT, E. (1965). D-Glucose: determination with glucose oxidase and peroxidase. In *Methods of Enzymatic Analyses*, (ed. H. V. Bergmeyer), pp. 123–130. New York: Academic Press.

HOCHACHKA, P. W. (1961). Glucose and acetate metabolism in fish. *Can. J. Biochem. Physiol.* **39**, 1937–1941.

HOHORST, H. J. (1965). L(+) lactate determination with lactate dehydrogenase and DPN^+. In *Methods of Enzymatic Analyses*, (ed. H. V. Bergmeyer), pp. 266–270. New York: Academic Press.

JOHNSTON, I. A. & MOON, T. W. (1980). Exercise training in skeletal muscle of brook trout (*Salvelinus fontinalis*). *J. exp. Biol.* **87**, 177–194.

MICHAL, G., BEUTLER, H-O., LANG, G. & GUNTER, U. (1976). Succinate determination in foodstuffs. *Z. Anat. Chem.* **279**, 137–138.

MOURIK, J., RAEVEN, P., STEUR, K. & ADDINK, A. O. T. (1982). Anaerobic metabolism of red skeletal muscle of goldfish, *Carassius auratus* (L). Mitochondrial produced acetaldehyde as anaerobic electron acceptor. *FEBS Letters* **137**, 111–114.

PFLEIDERER, G. (1965). L-Alanine. Determination with glutamate-pyruvate transaminase and lactate dehydrogenase. In *Methods of Enzymatic Analyses*, (ed. H. V. Bergmeyer), pp. 378–380. New York: Academic Press.

SHOUBRIDGE, E. A. & HOCHACHKA, P. W. (1980). Ethanol end product of vertebrate anaerobic metabolism. *Science, N.Y.* **209**, 307–308.

VAN DEN THILLART, G. (1977). Influence of oxygen availability on the energy metabolism of goldfish, *Carassius auratus* (L.). Ph.D. thesis, University of Leiden.

VAN DEN THILLART, G. (1982). Adaptations of fish energy metabolism to hypoxia and anoxia. *Molecular Physiology* **2**, 49–61.

VAN DEN THILLART, G. & KESBEKE, F. (1978). Anaerobic production of carbon dioxide and ammonia by goldfish, *Carassius auratus* (L.). *Comp. Biochem. Physiol.* **59**A, 393–400.

VAN DEN THILLART, G., KESBEKE, F. & VAN WAARDE, A. (1980). Anaerobic energy metabolism of goldfish, *Carassius auratus* (L.). Influence of hypoxia and anoxia on phosphorylated compounds and glycogen. *J. comp. Physiol.* **136**, 45–52.

VAN DEN THILLART, G., VAN WAARDE, A., DOBBE, F. & KESBEKE, F. (1982). Anaerobic metabolism of goldfish, *Carassius auratus* (L.). Effects of anoxia on the measured and calculated NAD^+/NADH ratios in muscle and liver. *J. comp. Physiol.* **146**, 41–49.

WALESBY, N. J. & JOHNSTON, I. A. (1980). Temperature acclimation in brook trout muscle: Adenine nucleotide concentrations, phosphorylation state and adenylate energy charge. *J. comp. Physiol.* **139**, 127–133.

WALKER, R. M. & JOHANSEN, P. H. (1977). Anaerobic metabolism in goldfish *Carassius auratus*. *Can. J. Zool.* **55**, 609–619.