Phosphorylation state of red and white muscle in tilapia during graded hypoxia: an in vivo ^{31}P-NMR study

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Van Ginneken, V. J. T., G. E. E. J. M. Van Den Thillart, H. J. Muller, S. Van Deursen, M. Onderwater, J. Visée, V. Hopmans, G. Van Vliet, and K. Nicolay. Phosphorylation state of red and white muscle in tilapia during graded hypoxia: an in vivo ^{31}P-NMR study. *Am. J. Physiol.* 277 (*Regulatory Integrative Comp. Physiol.* 46): R1501–R1512, 1999.—The aim of this study was to measure the energetic consequences of hypoxia in different types of skeletal muscle within a single tilapia species ($n = 5$). To that aim, 81.0 MHz ^{31}P-nuclear magnetic resonance (NMR) spectra were collected, alternately, from three surface coils placed adjacent to the tissues of interest (dorsal white muscle, ventral white muscle, and lateral red muscle) during a graded hypoxia load over 6 h followed by a 5-h recovery period. The fish were contained in a flow cell, enabling us full control of the oxygen content of the bathing medium. The intracellular pH and the concentrations of ATP, phosphocreatine (PCr), and P_i were determined from the NMR spectra. For normoxia, biochemical differences for [γ-ATP], [PCr], and [sugar phosphates] (SP) were observed between all three locations, especially between the red and white muscle. During hypoxia stress, loss of phosphorylated compounds (PCr+P_i+SP) was observed at all locations but was the most severe in red muscle. When the aerobic (respirometry) and anaerobic (^{31}P-NMR) ATP production via an energy balance are compared, flexible metabolic depression is demonstrated during anaerobioses. It is concluded that control of the aerobic and anaerobic component of metabolism during metabolic depression is independent of each other.

in vivo ^{31}P-nuclear magnetic resonance; intracellular pH; metabolic depression

THE MYOTOME OF MOST TELEOST fish is composed of distinct regions of red and white muscle. Red muscle (RM) has a well-developed blood supply, a high myoglobin and mitochondria content, high concentrations of lipids and cytochromes, and high activities of respiratory and citric acid cycle enzymes. RM has an active aerobic metabolism, using both carbohydrates and lipids as substrates (12, 16, 17). The bulk of the muscle tissue in fish, however, consists of white muscle, which depends mainly on anaerobic glycolysis for its energy supply. White muscle has a much higher glycolytic potential and a higher buffer capacity than RM (12, 16, 17). Biochemical differences between those two tissues were observed for amino acids (38), phosphorylated compounds (30, 47), and enzyme characteristics (3, 48). Due to the clear differences between red and white muscle, a different response to a stressor, such as a graded hypoxia load, might be expected in these two types of tissue.

^{31}P-nuclear magnetic resonance (NMR) is widely used to study the biochemical effects of environmental changes on animal tissues in vivo (34). The effects of hypoxia and anoxia on white muscle tissue have been investigated in unanesthetized fish in a specially developed flow-through cell (31). Spectra were obtained with good resolution and high signal-to-noise ratio. These were indicative of a high phosphocreatine (PCr)-to-P_i ratio (>25–40) and neutral tissue pH (7.1–7.3) under control conditions. Exposure to anoxia resulted clearly in the activation of anaerobic processes, in a decline of high-energy phosphates, and in metabolic acidosis (36, 49). Two important conclusions were drawn from these studies: *1*) the decline of [PCr] appeared to be coupled to acidosis (35, 49) and *2*) there were characteristic differences between species (i.e., goldfish showed a strongly reduced acidosis compared with carp, whereas tilapia exhibited an intermediate response). It was concluded that these differences were related to the occurrence of metabolic depression, which can be defined as a depression of the metabolic rate below the standard metabolic rate (SMR). This metabolic depression was observed in goldfish, but not in carp (43).

There is a marked difference between anoxia and hypoxia. Under hypoxic conditions, aerobic and anaerobic energy production may work simultaneously. In former studies, NMR experiments were performed with the same setup measuring with a surface coil. In these studies only dorsal white muscle (DWM) was measured under controlled conditions in which oxygen levels were declining stepwise. These studies showed no major differences between species: goldfish, tilapia, and carp (44). All three of these species demonstrated rapidly declining energy stores and high glycolytic activities at levels <10% air saturation. In addition, there was no indication of metabolic depression.

So far, in vivo NMR studies have not differentiated between the different locations and different muscle types within the same animal. Metabolic activity in an animal tissue may be related to its location and its use. Lateral RM is used continuously for slow swimming and steady-state movements, whereas dorsal white musculature is primarily used above certain swimming speeds. Therefore, under hypoxic conditions, the meta-

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bolic activity of RM is expected to be much higher than that of white muscle. For these reasons, we hypothesize that stabilization of the energy state of RM, under hypoxic conditions, is more important for the animal than that of white muscle. So, in this study, we aimed for simultaneous measurement of the response to hypoxic stress in white muscle and RM. Two different regions of white muscle were compared to test the homogeneity of the response within the same tissue type. To assess the tissue-specific response, three separate ^{31}P-NMR surface coils were placed at appropriate locations. To avoid significant interference between the coils, we used fish that were larger than those used in previous studies. Although in earlier studies we worked with 60- to 80-g fish in an 8-cm bore magnet (31, 36, 37, 44, 45, 49), in this study we used fish of 400 g in a 40-cm bore magnet. The data indicate that, for normoxia, biochemical differences existed between the red and white muscle, with respect to the concentrations of γ-ATP, PCr, and sugar phosphates (SP). Furthermore, during hypoxia stress, loss of phosphorylated compounds ($PCr+P_i+SP$) was observed at all locations, but was the most severe in RM. An energy balance was drawn from NMR data and compared with aerobic oxygen consumption. Comparison of the anaerobic response [PCr depletion and drop in intracellular pH (pH_i)] with the aerobic response (oxygen consumption) showed that the metabolic rate dropped below the standard metabolic rate (SMR), a process called metabolic depression.

MATERIAL AND METHODS

Animals and Handling

The experiments were performed with Mozambique tilapia (*Oreochromis mossambicus* Peters) purchased from Nijmegen University, the Netherlands. The mean weight of the animals used in the ^{31}P-NMR experiment was 400 ± 74 g (n = 5), whereas the mean weight of the animals used in parallel respirometry studies was 457 ± 95 g (n = 7). The animals were kept in the laboratory in local tap water at 20°C for at least 6 mo. They were fed daily with Trouvit pellets (Trouw, Putten, The Netherlands), and acclimatized to a 14:10-h light-dark cycle. Before the experiment, the fish were starved in a 150-liter glass tank for 24 h. On the day of the experiment, the fish were anesthetized with 3-aminobenzoate ethyl ester methanesulphonate (MS-222, Sigma, St. Louis, MO) at a final concentration of 100 parts per million (ppm). The fish were placed in a Perspex flow-through cell with three windows for the placement of three surface coils. The fish were immobilized with an inflatable plastic bag filled with water, and pressed with their left body sides against the flat window of the flow-through cell. A tube was then placed in the mouths of the fish, and the gills were irrigated with a constant water flow of 600 ml/min. The experimental setup was a modification of that used by van Ginneken et al. (44). The animals awoke within 5 min, and, as could be deduced from the NMR spectra, stayed quiet in the darkness of the magnet. During the first 30 min, a slight increase in the P_i concentration, combined with a slight reduction of the pH_i, was observed. Both parameters normalized again within 1 h. The high PCr-to-P_i ratio (>25–40) and neutral tissue pH (7.1–7.3) indicated that the steady-state situation in skeletal muscle during the initial normoxic conditions corresponded to a high-energy status.

NMR Measurements

^{31}P-NMR studies were performed at 81 MHz on a SIS (Palo Alto, CA) 200/400 in vivo NMR spectrometer interfaced to a 40-cm horizontal 4.7-Tesla Oxford magnet. Three different radio frequency (RF) coils were used for signal acquisition, alternating from coil to coil in a cyclic protocol (see below). The resonance frequency of each coil was brought close to the ^{31}P frequency by placing appropriate tuning capacitors close to the coil. The loading conditions during the in vivo experiments were simulated with an isotonic saline solution. The placement of the coils, their diameter, and the number of turns were as follows: RM, diameter 1.4 cm, two turns; ventral white muscle (VWM), diameter 2.5 cm, two turns; and DWM, diameter 4.0 cm, single turn. They were fixed to the flow cell at positions corresponding to 6.5 cm between the first and the second coil, 4.75 cm between the first and the third coil, and 7 cm between the second and the third coil (Fig. 1). In pilot experiments, it was observed that RM of tilapia of this size (~400 g) had a diameter of ~6 mm. Because the radius of the RM coil was 7 mm, it can be concluded that the tissue sampled by the RM coil consisted of a minimum of 86% of RM. After the surface coils had been properly placed, the flow cell was positioned in the magnet such that the largest coil was in the isocenter of the magnet. Thereafter, the coils were individually tuned to the ^{31}P Lamor frequency using three separate tuning/matching circuits. Interference between the coils was minimized by short circuiting at the RF shield of the magnet by placing end caps on the coils that were not used. Extensive tests demonstrated that coupling between the coils was negligible. After being tuned and matched to the ^{31}P frequency, the magnetic field was optimized for each coil by a pulsation on the H-1 frequency and the use of the free-induction decay (FID) signal from water for shimming. Water line widths were typically between 32 and 64 Hz. Next, the ^{31}P-NMR protocol was started. For each coil, excitation was with a 1-ms adiabatic half-passage pulse with offset at the PCr peak. The optimal pulse power for the adiabatic regimen was optimized once and subsequently kept constant for each experiment. The rationale for using adiabatic half-passage pulses for signal excitation was that the tip angle is exactly 90° throughout the sensitive volume of the coil. A poten-

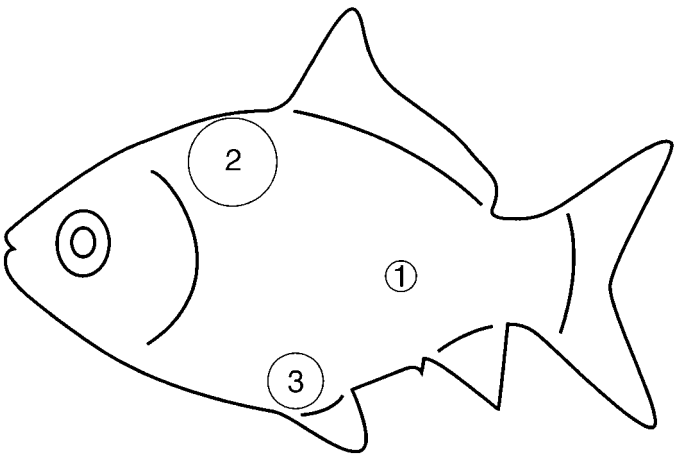

Fig. 1. Schematic drawing of position of surface coils on fish. Coils had diameters of 1.4 [red muscle (RM), 2 turns], 2.5 [ventral white muscle (VWM), 2 turns] and 4.0 cm [dorsal white muscle (DWM), 1 turn] and were fixed to flow cell at positions corresponding to 6.5 cm between first and second coil, 4.75 cm between first and third coil, and 7 cm between second and third coil. For simplicity, flow cell and remaining devices are not indicated.

tial disadvantage of adiabatic RF pulses is that their off-resonance performance is less good than that of hard pulses. A consequence of this may be, for example, that the signal intensity of the β- to the γ-peak of ATP is below one. Nevertheless, we considered the use of adiabatic pulses advantageous because of their uniform excitation profile, in spatial terms, and their immunity to power variations.

Control experiments on an in vitro sample containing PCr and ATP in a 4:1 molar ratio were performed to assess the reliability of the three-coil setup in terms of the PCr-to-ATP ratio determined from the spectra. The solution was contained in a plastic bag that was placed in the flow cell. The coils were positioned as in the above in vivo studies. It appeared that similar metabolite ratios were measured with each coil, both when present simultaneously and when tested individually with only one coil present. Importantly, the ratio of γ-ATP to PCr was identical when adiabatic pulses were used in the above setup and when hard pulses were used with a small vial placed centrally in a homogeneous solenoidal ^{31}P coil. The mean line broadening in five experiments was 59.4 Hz for the first coil, 54.8 Hz for the second coil, and 56.8 Hz for the third coil. For each FID, 4,096 complex data points were accumulated for an acquisition time of 0.41 s and spectral width of 5,000 Hz. A repetition time of 10 s was used. For *coil 2* (DWM), a series of nine spectra (24 scans each) was collected, whereas for *coils* 1 (RM) and *3* (VWM), 36 scans were averaged for a single FID for each experimental condition.

The pH_i was estimated from the difference in chemical shift between PCr and P_i. The pH measurements were calibrated using several model solutions, as described previously (36). The pH_i was calculated using the formula

$$pH_i = 6.72 + \log [(\sigma - 3.27)/(5.69 - \sigma)]$$

where σ corresponds to the chemical shift between PCr and P_i.

ATP concentrations in extracts (see below) were measured with the enzymatic assay of Lamprecht and Trautschold (19). The mean ATP concentration was 5.03 ± 0.57 μmol/g in white muscle and 3.60 ± 0.40 μmol/g in RM. [Total creatine] was measured according to the methods of Ennor and Stocken (10). A [total creatine] of 32.26 ± 1.38 μmol/g wet wt was found in white muscle and 23.80 ± 1.19 μmol/g wet wt in RM.

[PCr] for white muscle and RM was estimated from the ratio of the relative resonance intensities (RRIs) of PCr and ATP, starting from a normoxic RRI for ATP. This corresponds to a tissue concentration of 5.03 μmol/g wet wt in white muscle and 3.60 μmol/g wet wt in RM.

Protocol

The fish used in the in vivo ^{31}P-NMR experiments were exposed, for a period of 60 min, to normoxic conditions [100% air saturation (AS)]. This was followed by a stepwise decrease in oxygen content to 40, 30, 20, 10, 5, and 3% AS for a period of 60 min each. Hypoxia was followed by a variable period of anoxia [method described by van Ginneken et al. (44)]. Thereafter, the animals were exposed to a recovery period of 5 h at 100% AS. Surface *coils 1*, *2*, and *3* were placed on the caudal part of the lateral RM, the rostral part of the epaxial white muscle, and the VWM next to the gill, respectively (Fig. 1). Over each 60-min period, 11 spectra were collected in the following sequence: 9 spectra from *coil 2* (24 scans each), 1 spectrum from *coil 1* (36 scans), and 1 spectrum from *coil 3* (36 scans). During anoxia, because of the limited period, the measurements were made with *coil 2* only. The anoxia exposure length was based on the degree of exhaustion of the PCr stores. When the PCr peak was depleted until its peak height equalled the γ-ATP peak, reoxygenation was activated. Pilot experiments revealed that mortality occurred only when PCr levels were reduced even further. In the experimental group, no mortality was observed during or after the experiments.

Oxygen Registration

Two types of respirometric studies were performed: *1*) directly in the flow-through cell in the NMR apparatus with an immobilized fish (see Table 1) and *2*) in parallel experiments in a respirometer with free-swimming fish to determine the SMR and percentage of metabolic depression during a graded hypoxia load (see Table 2).

Respirometry in the flow-through cell. The oxygen content of the medium was measured with a registration system [described by van Ginneken et al. (44)] that consisted of a computer-driven rotating valve (Bürckert type 332-E-B-G¼-220/50-F-024), which alternately directed the waterflow from the inlet or outlet of the flow-through cell over an oxygen electrode (Radiometer Copenhagen E5046 with thermostatted cell D616). The oxygen detection system was a Radiometer Denmark Digital Oxygen analyzer PHM 72 with a P_{O_2} module PHA 932. The oxygen electrode was calibrated in a 10% sodium sulfite solution (zero) and at 100% in air-saturated water. In the reference position, normoxic or hypoxic water from the gas equilibration cylinders flowed directly over the electrode. In the measurement position, the water first passed through the flow-through cell with fish and then over the electrode. The selected time intervals for the two different valve positions were 10 min in the reference position and 50 min in the measurement position.

The oxygen consumption was calculated as

$$\dot{V}_{O_2} = v\,(C_{ref} - C_{meas})\ \text{mg}\ O_2 \cdot h^{-1}$$

where v is the flow through the flow-through cell (600 ml/min) and C_{ref} and C_{meas} are the oxygen concentrations of the water flowing into and out of the flow-through cell. The analog signal from the oxygen electrode values was digitized and registered online by a personal computer. Oxygen data were read into a spreadsheet program (QuatroPro) and converted to oxygen consumption rates.

Metabolic rate measurements in a respirometer. Seven fish were placed individually in a flow-through respirometer of 20 liters at normal oxygen levels (80% AS; Ref. 33). The supply of water to the respirometer was regulated by an EIL O_2 monitor/controller type 9401. The controller activates a solenoid valve when the P_{O_2} value falls below a set point. Air saturated water flows from a storage tank into the respirometer chamber until the preset level is reached. The flow is measured by a digital flowmeter, which is coupled to a counter and data logger (33). Temperature in the experimental set up was kept at 20 ± 0.1°C.

Sampling Procedure and Tissue Preparation

Tissue samples were taken in parallel experiments to measure ATP and creatine levels. Fish were acclimatized to the experimental set up at 20°C for 2 days under normoxic conditions to reduce handling stress. Fish (anesthetized with 200 ppm MS-222) were killed by decapitation. Tissue samples of white muscle (within 15 s) and RM (within 30 s) were removed and freeze-clamped between aluminum tongs cooled in liquid nitrogen. The freeze-clamped muscle samples were stored in liquid nitrogen until analysis. Tissue extraction was carried out according to van den Thillart and colleagues (29, 33). Frozen tissue was powdered [in a porcelain tissue grinder

(Retsch, type RMO), cooled with liquid nitrogen] together with a 4.0-ml vol of perchloric acid (8% vol/vol) in ethanol (40% vol/vol) containing 4 mM NaF and 10 mM EDTA. The powder was stored for 10 min at −20°C in a centrifuge tube. Thereafter, the powder was further homogenized on ice with a high-speed mixer (Salm and Kip BV, type X 1020, Dottingen, Germany). The homogenate was stored for 30 min on ice and was then centrifuged (Sorvall RC-5B) for 20 min at 30,000 *g*. The supernatant was neutralized to pH 7.0 with 3 M potassium carbonate in 0.5 M triethanolamine. Finally, the extracts were again centrifuged (20 min at 30,000 *g*), aliquotted into Eppendorf tubes, and stored at −180°C until use.

The method used for determining the buffer capacity of tilapia red and white muscle was slightly modified after Castellini and Somero (4). Muscle was excised from tilapia after anesthesia with 200 ppm MS-222. The muscle was frozen in 0.5-g parts and powdered in liquid nitrogen together with 10 ml 0.9% NaCl (4.00 meq H_2SO_4/l). The powder was transferred into a 50-ml titration vial and rinsed with 2 ml H_2O. The titration vial was temperature controlled at 20°C. The initial pH of RM was 4.5 ± 0.3 and of white muscle 5.1 ± 0.2. The tissue homogenate was titrated with NaOH (10.00 meq/l) with a Metrohm titrator in steps of 0.200 ml. The pH was read 20 s after addition, which was sufficient to reach a stable pH. The buffer capacity was calculated from the pH increase between 6 and 6.8, which gave, in that part, a linear relationship. For acidification and titration, titrisol grade solutions of 1.000 eq/l were used (Merck).

Method for Estimation of Percentage of Metabolic Depression

Estimation of the percentage of metabolic depression can be inferred by comparing the SMR (see Table 2) with the estimated anaerobic and aerobic ATP production (6). In Teleostei, muscle tissue is, quantitatively, the major metabolically active tissue. It determines, to a great extent, the metabolic rate. For fish, the somatic index of muscle tissue was estimated to be 42%, whereas the remaining metabolically active tissue was estimated to be 21.3% [liver 1.3%, blood 5%, remaining metabolic active tissue (i.e., heart, brain, and gut) 15% (6, 39)]. Because muscle tissue mainly determines the metabolic rate, an energy budget can be estimated on the basis of ^{31}P-NMR data and whole body oxygen consumption. Estimation of the anaerobic ATP production is based on the changes in the most important energy-rich compounds, such as PCr and ATP, and on lactic acid production. However, because the [ATP] was unchanged during the stepwise hypoxia load, only the changes in PCr were considered. Anaerobic lactic acid production, derived from NMR data, was based on the method described by van den Thillart and van Waarde (35). This is based on the formula of Kushmerick and Meyer (18)

$$\Delta\text{lactate } (\mu\text{mol/g}) = a(\Delta\text{pH}) + b(\Delta\text{PCr})$$

where a is the buffering capacity of the muscle, corresponding to 28.1 ± 3.4 for RM and 40.0 ± 1.4 meq·g^{-1}·pH unit^{-1} for white muscle (see RESULTS), and b is the stochiometric coefficient of the PCr hydrolysis reaction, which is pH dependent and corresponds to a tissue buffer capacity of 0.42 eq/mol over this pH range (14). From the buffer capacity for fish muscle, and via the ^{31}P-NMR measured ΔpH between the different hypoxia levels, the acid production can be calculated in milliequivalents per kilogram. From the total acid production and the buffer capacity, the total lactic acid production can be calculated. For the anaerobic ΔATP, 1 mol of lactate corresponds to 1.5 mol ATP (6). The PCr, used as a buffer for stabilization of the ATP pool, is measured via ^{31}P-NMR. One mole of PCr is equivalent to 1 mol of ATP (6). Because we measured three different tissues, the somatic indexes between the different tissues (RM, DWM, and VWM) are estimated to be 10, 70, and 20%, respectively, whereas the relative weight of muscle tissue in fish corresponds to 42% (39). The level of high-energy phosphates in the remaining tissues is a minor part of the energy budget, and estimation during a similar graded hypoxia load is based on HPLC measurements performed earlier, resulting in an anaerobic ATP production at 30, 20, 10, 5, and 3% of, respectively, 0.47, 0.36, 0.79, 1.27, and 0.78 μmol ATP·g^{-1}·h^{-1} (39). The combination of muscle tissue and remaining tissue gives a total anaerobic ATP production, as revealed in Table 6. Because the oxygen consumption was measured, it was possible to calculate the aerobic ATP production. This is based on the assumption that 32 mg O_2 correspond to 6 mmol ATP (26). The sum of the aerobic and anaerobic ATP production gives the total ATP production.

Statistics

For each muscle type, the values for the different parameters (PCr, SP, P_i, pH_i, ATP) during the graded hypoxic levels and recovery were compared with the initial 60-min normoxic period. Statistics were performed in SAS (statistical analyzing software). We applied a one-way ANOVA, comparing hypoxia and recovery with normoxia. $P \leq 0.05$ was considered statistically significant. Normality of the data and homogeneity of variances were checked by Kolmogorov-Smirnov and F_{max} tests. For each experiment, the mean value of the metabolites over the corresponding interval was calculated. Experimental data are presented as the means ± SD.

RESULTS

The mean oxygen consumption under control conditions and during a graded hypoxia load and reoxygenation is given in Table 1. The oxygen consumption fell from 16.63 mg·100 g^{-1}·h^{-1}at normoxia to 2.13 mg·100 g^{-1}·h^{-1} at 3% AS. Because the standard metabolic rate for tilapia is ~9.78 mg O_2·100 g^{-1}·h^{-1} (Table 2), the animals were apparently below this level at 20, 10, 5,

Table 1. *Oxygen consumption of an immobilized Mozambique tilapia measured during ^{31}P-NMR experiment in flow-through cell during graded hypoxia and reoxygenation*

Condition	Oxygen Consumption, mg·100 g^{-1}·h^{-1}	%Metabolic Depression
N	16.63 ± 1.66	170.0
40% AS	10.41 ± 1.34	106.4
SMR	9.78	100
30% AS	9.66 ± 0.47	98.8
20% AS	5.78 ± 0.84*	59.1*
10% AS	2.63 ± 0.59*	26.9*
5% AS	4.13 ± 0.25*	42.2*
3% AS	2.13 ± 0.09*	21.8*
R1	8.50 ± 2.16	86.9
R2	17.01 ± 0.99	173.9
R3	No data	No data
R4	No data	No data
R5	No data	No data

Values are means ± SD of 5 animals. SMR, standard metabolic rate (see Table 2). AS, air saturation; N, normoxia; R1–R5, recovery periods. *Significant difference from normoxia with $P \leq 0.05$.

Table 2. *Oxygen consumption of a free-swimming Mozambique tilapia measured in a respirometer in parallel experiments during graded hypoxia and reoxygenation*

Condition	Oxygen Consumption, mg·100 g^{-1}·h^{-1}	%Metabolic Depression
N	9.78 ± 2.80	100 (=SMR)
60% AS	9.00 ± 2.89	94.67 ± 26.90
40% AS	8.68 ± 2.73	90.43 ± 20.02
20% AS	9.25 ± 3.40	96.02 ± 24.00
10% AS	9.29 ± 3.41	95.13 ± 20.43
5% AS	7.46 ± 2.50	77.07 ± 14.85
3% AS	6.21 ± 2.58*	64.18 ± 17.58*
R1	11.76 ± 3.91	119.48 ± 14.74

Values are means ± SD of 7 animals. The SMR is determined 2 days after introducing animals to the respirometer. *Significant difference from normoxia with $P \leq 0.05$.

and 3% AS. The animals must have compensated for this by anaerobic processes or by depressing their overall metabolic rate by ~78%.

In Fig. 2, a series of ^{31}P-NMR spectra from a typical experiment for the lateral RM (Fig. 2*A*), the DWM (Fig. 2*B*), and the VWM (Fig. 2*C*) are shown. The patterns are rather similar: unchanged ATP levels; a drop of PCr during hypoxia, with a concomitant rise of P_i; and a rise of SP. Some differences are obvious: the PCr peak in the DWM is higher than in the RM. Furthermore, the initial [P_i] in the RM does not approach the high levels observed in dorsal and VWM.

Data obtained from five complete series, each with three coils and measured under 13 conditions, are presented in Tables 3, 4, and 5. The 13 conditions are indicated as: normoxia; hypoxia at 40, 30, 20, 10, 5, and 3% AS; anoxia; and recovery over five consecutive periods (R1, R2, R3, R4, and R5). Each condition lasted 60 min, except for the anoxia period, which was variable (see above).

Table 3 shows the content of PCr and ATP at the three different locations under the different conditions tested. Enzymatic measurements showed that ATP amounted to 3.60 and 5.03 μmol/g in RM and DWM, respectively. The ATP levels remained constant during hypoxia. In contrast, major changes occurred with the PCr levels. The values of PCr during normoxia were 23.80 mM in the RM, 32.26 mM in the DWM, and 23.77 mM in the VWM. During the 3% AS period, the PCr levels were reduced to 44.8, 50.4, and 56.5% of control in RM, DWM, and VWM, respectively. In RM and DWM, the decline was significantly different, but not in VWM. During anoxia, the PCr level in the DWM fell to 36%. During the first 2 h of recovery, PCr resynthesis proceeded relatively fast in all three tissues.

Table 4 shows the content of SP and P_i in the three tissues. Initial normoxic SP values were 0.85 in RM, 1.53 in DWM, and 1.32 mM in VWM. During the graded hypoxia load, a value of 2.66 mM was reached in RM during 3% AS, whereas this value was 4.02 in DWM and 3.18 mM in VWM. Interestingly, the SP level in the DWM was significantly lower at 40, 30, and 20% AS. During anoxia, the SP rose in DWM to 4.58 mM. During

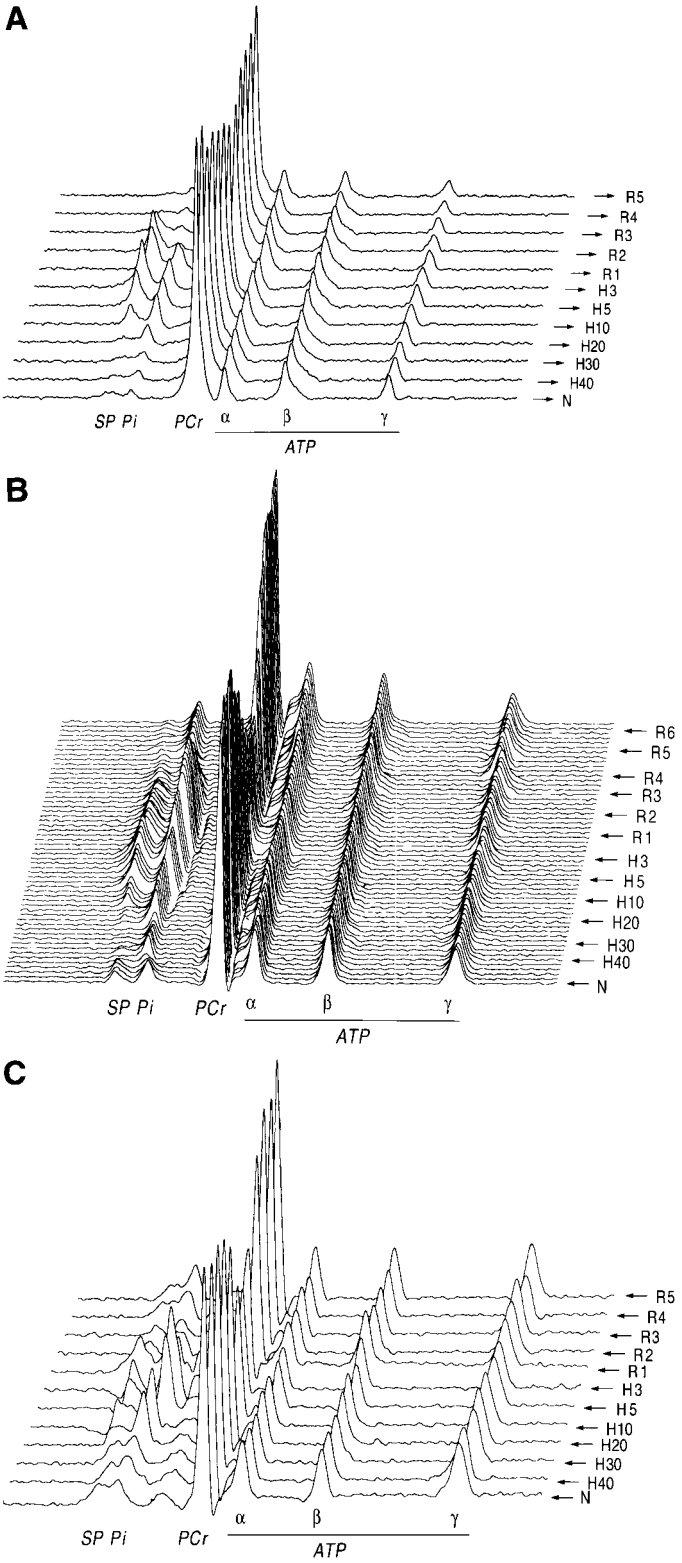

Fig. 2. Stacked plots of ^{31}P-nuclear magnetic resonance (NMR) spectra of a typical experiment with tilapia during normoxia (N), hypoxia (H), and recovery (R) for RM (*A*), DWM (*B*), and VWM (*C*). Normoxia, hypoxia, and anoxia (A) and part of recovery period are depicted. Peaks from sugar phosphates (SP), P_i, creatine phosphate (PCr), and γ-, α-, and β-phosphate atoms of ATP are visible. Data are from same individual.

Table 3. *PCr and γ-ATP levels of red muscle and dorsal and ventral epaxial white muscle of Mozambique tilapia during graded hypoxia, anoxia, and recovery*

Condition	PCr, μmol/g			γ-ATP, μmol/g		
	Red muscle	Dorsal white muscle	Ventral white muscle	Red muscle	Dorsal white muscle	Ventral white muscle
Normoxia						
N control	23.80 ± 5.08	32.26 ± 9.33	23.77 ± 5.99	3.60 ± 0.0	5.03 ± 0.0	5.03 ± 0.0
Hypoxia						
40%	25.10 ± 2.22	33.41 ± 11.63	24.55 ± 6.71	3.67 ± 0.19	5.68 ± 1.88	4.77 ± 0.68
30%	24.01 ± 3.54	31.76 ± 9.86	22.14 ± 6.55	3.44 ± 0.27	5.66 ± 1.95	4.79 ± 0.67
20%	20.89 ± 1.29	32.05 ± 12.92	20.44 ± 6.14	3.30 ± 0.41	5.40 ± 1.89	5.04 ± 0.86
10%	15.49 ± 3.10*	25.47 ± 10.59	15.89 ± 3.83	3.86 ± 1.13	5.51 ± 1.86	5.13 ± 1.09
5%	13.16 ± 3.97*	21.85 ± 8.57	14.85 ± 5.07	3.43 ± 1.08	5.57 ± 1.35	5.12 ± 1.33
3%	10.68 ± 5.60*	16.27 ± 4.03*	13.44 ± 5.11	3.72 ± 0.81	5.47 ± 2.02	4.79 ± 1.20
Anoxia	No data	11.68 ± 3.71*	No data	No data	5.38 ± 1.65	No data
Recovery						
1	12.95 ± 5.14*	12.26 ± 5.60*	17.08 ± 7.23	3.25 ± 0.23	5.12 ± 1.72	5.68 ± 0.68
2	16.07 ± 3.31*	17.93 ± 3.42*	20.30 ± 6.62	2.83 ± 0.23	5.09 ± 1.54	5.13 ± 1.08
3	19.26 ± 2.01*	22.99 ± 7.41	22.02 ± 7.28	2.99 ± 0.23	5.29 ± 1.81	4.86 ± 1.56
4	21.84 ± 3.53	24.05 ± 4.19	23.26 ± 6.74	3.15 ± 0.13	5.25 ± 1.53	5.62 ± 1.07
5	20.83 ± 1.50	28.94 ± 9.96	24.95 ± 6.70	3.04 ± 0.08	5.28 ± 2.14	5.63 ± 1.59

Values are means ± SD of 5 animals. PCr, phosphocreatine. *Significant difference from normoxia with $P \leq 0.05$.

the 5-h recovery period, the SPs were reduced to levels below the initial normoxic values in all three tissues.

P_i values in resting normoxic animals were the lowest in RM (0.87 mM), whereas in DWM and VWM these were 2.37 and 3.0 mM, respectively (Table 4). During hypoxia, P_i values increased in RM, DWM, and VWM to 8.32, 6.21, and 9.08 mM, respectively. During anoxia, the P_i increased further in DWM to 8.43 mM. The highest value of 11.73 mM was reached after 1 h recovery in the DWM. Recovery was fast. Within 3 h, control values were reached in DWM and VWM. In RM, no further recovery of P_i occurred after 3 h.

Table 5 shows the sum of PCr, SP, and P_i, as well as the pH_i, of the three muscle tissues during hypoxia and recovery. The sum of PCr, SP, and P_i appeared relatively stable in DWM and VWM, although there was a tendency toward a decline during hypoxia and an increase to normoxic values during recovery. During anoxia, the sum was significantly different in the DWM. Remarkably, the sum declined in the RM during exposure to 20 and 10% AS and did not decrease further during hypoxia, nor did it increase again during 5 h recovery. This resulted in a significant semipermanent loss of 18% of the sum of PCr, SP, and P_i in RM compared with the normoxic situation.

During normoxia, the pH_i of RM, DWM, and VWM was 7.24, 7.16, and 7.42, respectively (Table 5). During hypoxia, there is a tendency to acidosis in all tissues, which was significant in RM at 5 and 3% AS and in DWM during anoxia. In the DWM, the pH decreased further during anoxia and reached its lowest level after 2 h of recovery. In the RM, the pattern was similar to that of the DWM; the pH fell from 7.2 at normoxia to 6.8 at 3% AS. The lowest pH value of 6.6, however, occurred

Table 4. *Sugar phosphates and P_i levels of red muscle, dorsal, and ventral epaxial white muscle of Mozambique tilapia during graded hypoxia, anoxia, and recovery*

Condition	Sugar Phosphates, μmol/g			P_i, μmol/g		
	Red muscle	Dorsal white muscle	Ventral white muscle	Red muscle	Dorsal white muscle	Ventral white muscle
Normoxia						
N control	0.85 ± 0.88	1.53 ± 0.89	1.32 ± 1.13	0.87 ± 0.30	2.37 ± 1.82	3.00 ± 1.33
Hypoxia						
40%	0.47 ± 0.65	0.80 ± 0.77	1.10 ± 0.84	1.39 ± 0.62	1.41 ± 0.99	3.71 ± 3.24
30%	0.39 ± 0.45	0.55 ± 0.75	1.61 ± 0.52	1.52 ± 0.89	2.10 ± 1.25	4.34 ± 3.63
20%	0.77 ± 0.70	0.69 ± 0.90	1.38 ± 1.71	2.36 ± 0.87*	3.49 ± 1.53	5.67 ± 2.12*
10%	1.96 ± 0.85	1.47 ± 0.81	2.51 ± 0.58	4.84 ± 2.23*	4.77 ± 1.76	7.97 ± 4.49*
5%	1.82 ± 1.48	3.12 ± 0.81*	2.82 ± 1.65*	6.42 ± 2.22*	6.03 ± 2.01*	8.16 ± 4.77*
3%	2.66 ± 2.53	4.02 ± 2.57*	3.18 ± 2.14*	8.32 ± 3.35*	6.21 ± 4.59	9.08 ± 5.48*
Anoxia	No data	4.58 ± 0.20*	No data	No data	8.43 ± 3.99*	No data
Recovery						
1	1.84 ± 1.58	3.60 ± 1.43*	3.66 ± 1.04*	6.27 ± 6.28	11.73 ± 3.53*	4.80 ± 2.55
2	2.02 ± 1.66	3.09 ± 1.28	2.90 ± 1.52	2.27 ± 2.01	6.52 ± 3.67	3.31 ± 1.93
3	1.51 ± 0.66	2.40 ± 1.94	1.81 ± 1.73	1.42 ± 1.22	2.60 ± 1.76	2.27 ± 2.16
4	0.57 ± 0.49	2.05 ± 1.24	2.02 ± 1.13	1.47 ± 1.27	2.01 ± 1.04	2.17 ± 1.35
5	0.21 ± 0.29	1.09 ± 0.71	1.03 ± 0.99	1.63 ± 1.05	2.56 ± 1.18	2.62 ± 0.83

Values are means ± SD of 5 animals. *Significant difference from normoxia with $P \leq 0.05$.

Table 5. *Sum of phosphorylated compounds: Σ(PCr + P_i + SP) and pH_i of red muscle, dorsal, and ventral epaxial white muscle of Mozambique tilapia during graded hypoxia, anoxia, and recovery*

	PCr + P_i + SP, μmol/g			pH_i, μmol/g		
Condition	Red muscle	Dorsal white muscle	Ventral white muscle	Red muscle	Dorsal white muscle	Ventral white muscle
Normoxia						
N control	27.83 ± 1.76	37.83 ± 8.80	30.30 ± 6.64	7.24 ± 0.26	7.16 ± 0.26	7.42 ± 0.04
Hypoxia						
40%	27.26 ± 1.57	37.70 ± 11.42	31.40 ± 10.66	7.24 ± 0.10	7.30 ± 0.16	7.44 ± 0.10
30%	26.89 ± 3.53	36.46 ± 10.10	30.72 ± 8.65	7.32 ± 0.14	7.29 ± 0.09	7.37 ± 0.15
20%	24.38 ± 1.76*	35.21 ± 11.95	28.71 ± 7.93	7.09 ± 0.22	7.26 ± 0.08	7.21 ± 0.08
10%	23.26 ± 2.04*	33.59 ± 12.07	27.52 ± 4.19	7.03 ± 0.10	7.17 ± 0.08	7.05 ± 0.05
5%	21.85 ± 2.54*	32.73 ± 9.68	28.10 ± 7.02	6.89 ± 0.19*	7.03 ± 0.07	6.92 ± 0.09
3%	22.32 ± 2.84*	29.07 ± 6.97	27.87 ± 9.11	6.82 ± 0.17*	6.90 ± 0.09	6.85 ± 0.10
Anoxia	No data	27.66 ± 2.16*	No data	No data	6.85 ± 0.11*	No data
Recovery						
1	22.45 ± 2.31*	29.80 ± 7.67	26.15 ± 10.24	6.63 ± 0.25*	6.70 ± 0.11*	6.93 ± 0.20
2	20.86 ± 2.94*	29.59 ± 5.28	28.06 ± 9.10	6.72 ± 0.40	6.63 ± 0.13*	7.07 ± 0.25
3	22.52 ± 1.28*	29.99 ± 6.89	28.36 ± 10.65	6.96 ± 0.41	6.73 ± 0.12*	7.16 ± 0.18
4	24.17 ± 3.80	29.43 ± 3.29	28.59 ± 9.28	7.12 ± 0.29	7.12 ± 0.16	7.25 ± 0.20
5	22.84 ± 1.74*	34.52 ± 10.56	29.76 ± 8.73	7.18 ± 0.31	7.25 ± 0.12	7.27 ± 0.23

Values are means ± SD of 5 animals. *Significant difference from normoxia with $P \leq 0.05$.

after 1 h of recovery. Recovery of pH_i in RM, DWM, and VWM took ~5 h.

Table 6 shows the anaerobic ATP production calculated per hypoxia condition for all three muscle types. In the calculation we used in this study, measured buffer capacity for RM was 28.1 ± 3.4 meq·g^{-1}·pH $unit^{-1}$ and for white muscle was 40.0 ± 1.4 meq·g^{-1}·pH $unit^{-1}$.

Estimation of the percentage of metabolic depression is given in Table 7. It can be seen that the total ATP turnover fell below the SMR at 3% AS, which is compensated by a reduction of the metabolic rate until 58% of the SMR.

DISCUSSION

Oxygen Consumption

The normoxic oxygen consumption rate of the Mozambique tilapia in this ^{31}P-NMR study corresponded to 16.63 mg $O_2 \cdot 100 \cdot g^{-1} \cdot h^{-1}$. In a parallel respirometric study, a standard metabolic rate of 9.78 mg O_2·

Table 6. *Anaerobic ATP production in dorsal, ventral white muscle, and red muscle*

Condition	Acid Production, meq/kg	Buffer Capacity, meq/l	Total Lactic Acid Produciton, μmol/g	ΔATP Based on [PCr] Usage, μmol·g^{-1}·h^{-1}	ΔATP Anaerobic, μmol·g^{-1}·h^{-1}
Dorsal white muscle					
Normoxia	40·0 = 0	0.42·0 = 0	0	0	0
Hyp-40%	40·−0.14 = −5.6	0.42·−1.15 = −0.483	−6.083	0	−6.083
Hyp-30%	40·0.01 = 0.4	0.42·1.65 = 0.693	1.093	1.65	3.2895
Hyp-20%	40·0.03 = 1.2	0.42·0 = 0	1.2	0	1.8
Hyp-10%	40·0.09 = 3.6	0.42·6.58 = 2.7636	6.3636	6.58	16.1254
Hyp-5%	40·0.14 = 5.6	0.42·3.62 = 1.5204	7.1204	3.62	14.3006
Hyp-3%	40·0.13 = 5.2	0.42·5.58 = 2.3436	7.5436	5.58	16.8954
Ventral white muscle					
Normoxia	40·0 = 0	0.42·0 = 0	0	0	0
Hyp-40%	40·−0.02 = −0.8	0.42·−0.78 = −0.3276	−1.1276	0	−1.1276
Hyp-30%	40·0.07 = 2.8	0.42·2.41 = 1.0122	3.8122	2.41	8.1283
Hyp-20%	40·0.16 = 6.4	0.42·1.70 = 0.714	7.114	1.70	12.371
Hyp-10%	40·0.16 = 6.4	0.42·4.55 = 1.911	8.311	4.55	17.0165
Hyp-5%	40·0.13 = 5.2	0.42·1.04 = 0.4368	5.6368	1.04	9.4952
Hyp-3%	40·0.07 = 2.8	0.42·1.41 = 0.5922	3.3922	1.41	6.4983
Red muscle					
Normoxia	28.1·0 = 0	0.42·0 = 0	0	0	0
Hyp-40%	28.1·0 = 0	0.42·−1.3 = −0.546	−0.546	0	−0.546
Hyp-30%	28.1·−0.08 = −2.248	0.42·1.09 = 0.4578	−1.7902	1.09	−0.7002
Hyp-20%	28.1·0.23 = 6.463	0.42·3.12 = 1.3104	7.7734	3.12	14.7801
Hyp-10%	28.1·0.06 = 1.686	0.42·5.40 = 2.268	3.8238	5.40	11.1357
Hyp-5%	28.1·0.14 = 3.934	0.42·2.33 = 0.9786	4.9126	2.33	9.6989
Hyp-3%	28.1·0.07 = 1.967	0.42·2.48 = 1.0416	3.0086	2.48	6.9929

Lactate production and buffer capacity are based on formula of Kushmerick and Meyer (18). Acid production is buffer capacity (28.1 for red muscle, 40 meq·g^{-1}·pH $unit^{-1}$ for white muscle) multiplied by change in intracellular pH (pH_i) between different hypoxic conditions (see Table 5). Buffer capacity is the stochiometric coefficient for the PCr hydrolysis reaction (0.42 eq/mol) multiplied by change in [PCr] between different hypoxic conditions (see Table 3).

Table 7. *Aerobic versus anaerobic energy production in Tilapia (Oreochromis mossambicus Peters) exposed to a graded hypoxia load*

Condition	Aerobic ATP	Anaerobic ATP in Muscle Tissue	Anaerobic ATP in Remaining Tissue	Total ATP
Normoxia	31.18	0	0	31.18 (170.0%)
Hypoxia				
40%	19.52	−1.90	Not detected	17.62 (96.1%)
30%	18.11	1.62	0.47	20.2 (110.2%)
20%	10.84	2.18	0.36	13.38 (73.0%)
10%	4.93	6.64	0.79	12.36 (67.4%)
5%	7.74	5.41	1.27	14.42 (78.6%)
3%	3.99	5.81	0.78	10.58 (57.7%)

Values are means ± SD in $\mu mol \cdot g^{-1} \cdot h^{-1}$ (%SMR in parentheses). Metabolic depression is calculated based on an SMR of 9.78 mg $O_2 \cdot 100\ g^{-1} \cdot h$ (see Table 2), which corresponds to 18.33 $\mu mol\ ATP \cdot g^{-1} \cdot h^{-1}$.

$100\ g^{-1} \cdot h^{-1}$ for tilapia at 20°C was reported (Table 2). Thus the results presented here indicate that the measured O_2 consumption of the fish in the NMR flow cell is ~1.70 times the SMR. Even under resting conditions, some changes in activity level can be expected due to muscle tension and small movements. Hypoxia exposure depresses the activity level of free-swimming fish and thus reduces the mean oxygen consumption level. Only at very low oxygen levels does the oxygen consumption fall (sometimes) below the SMR (28). This is likely due to a reduction of muscle tension and blood flow. Although the oxygen consumption in this study is sampled at relatively long intervals, and therefore activity peaks cannot be recognized, a similar pattern is obvious. First, we see a decline in the metabolic rate at 20% AS, but then the oxygen consumption decreases further to even 21.8% of the SMR at 3% AS. Evidence for metabolic depression in tilapia has been described before (40, 41, 49). To prove that an animal uses the survival strategy of metabolic depression, it is necessary to show that the reduction in aerobic energy production is only partly compensated for by anaerobic processes. In this study, we cannot quantify the total anaerobic energy production, but a semiquantitative approach is possible (see Table 6).

SPs

In tilapia, SPs rise to much higher levels under hypoxia than in other fish species, such as common carp and goldfish (44). This indicates, in tilapia, that glycogenolysis, and thus glycogenphosphorylase, is relatively insensitive to feedback inhibition by glucose 6-phosphate. This shifts the control of the glycolytic rate mainly to phosphofructokinase.

It is evident from Fig. 2 and Table 4 that the SPs rise in the early phase of hypoxia, before the onset of metabolic acidosis. During anoxia, when the glycolytic rate is high, the SP level does not decrease. This suggests a sufficient capacity of the glycogenphosphorylase reaction.

PCr-to-Cr Ratio

The PCr level appears to differ markedly between the different sample points for RM, DWM, and VWM, respectively, 23.8, 32.3, and 23.8 mM. With in vivo ^{31}P-NMR methods, PCr levels of 24.4 mM have been observed in epaxial white muscle of unanesthetized goldfish (37), whereas in another ^{31}P-NMR study with tilapia, PCr values between 15.8 and 21.0 mM were reported for the DWM (45). The higher values for the DWM in this study are likely related to the weight of the animal. Larger fish usually have higher glycolytic potentials than smaller ones (3).

In general, if we compare enzymatic determinations in perchloric acid extracts (obtained via freeze-clamping) with in vivo ^{31}P-NMR measurements, a discrepancy can be observed for the [PCr]-to-[PCr+Cr] ratio (amount of phosphorylated total creatine). With traditional freeze-clamping, it is observed that only a small fraction of the total Cr is phosphorylated. In dogfish muscle, 19% phosphorylation was observed (36.6 µmol Cr/g vs. 8.8 µmol PCr/g; Ref. 2). In white muscle of goldfish, phosphorylation percentage was 27% (21.8 µmol Cr/g vs. 8.3 µmol PCr; Ref. 30). In white muscle of rainbow trout, 45% phosphorylation was measured (25.7 µmol Cr/g vs. 20.8 µmol PCr; Ref. 9). In another study with white muscle of rainbow trout, 39% phosphorylation was measured (27.5 µmol Cr/g vs. 17.5 µmol PCr; Ref. 7).

Only in ^{31}P-NMR studies were very high levels of phosphorylation observed in intact unanesthetized goldfish, 95.3% (1.2 µmol Cr/g vs. 24.4 µmol PCr; Ref. 37). From the results presented in this paper, we calculated the percentage of phosphorylation in DWM as 100% (0 µmol Cr/g vs. 32.26 µmol PCr); in VWM, 73.7% (8.5 µmol Cr/g vs. 23.77 µmol PCr), and in RM, 92.4% (1.96 µmol Cr/g vs. 23.80 µmol PCr). The discrepancy between PCr-to-Cr ratios obtained by in vivo ^{31}P-NMR and conventional freeze-clamping techniques is likely due to handling and artifacts developed during tissue excision and tissue extraction (37, 49).

P_i

The $\Sigma(PCr+P_i+SP)$ showed a drop in red and white muscle during reoxygenation. This decreased phosphate content may be explained by washout of P_i or by deposition of calcium phosphate in mitochondria, which are NMR invisible (24). The former, however, seems more likely, because the phosphate loss is more prominent in the better perfused RM. Furthermore, it should be mentioned that the possibility of a mitochondrial accumulation of phosphate in primarily RM is also increased due to a probably higher mitochondrial density in red than in white muscle.

The observed P_i values for tilapia white muscle were between 2.4 and 3.0 mM and in agreement with a previous study (44). P_i has several functions in the cell. First, it may act as a buffer (22); second, it may act as a substrate in glucogenolysis; and, third, it may act as a metabolic regulator (44). A qualification for a metabolic regulator is that the concentration of the compound

must be in the range of the Michaelis-Menten constant (K_m). In vitro measurement of P_i with isolated mitochondria have shown a K_m of 1 mM. In this study, the observed P_i during normoxia (0.9–3.0 mM) is higher than the K_m of 1 mM. This indicates that P_i does not function as a metabolic regulator. This observation corroborates that of the ^{31}P-NMR study of Chance et al. (5), in which the [P_i] of isolated mitochondria (state 4) were >1 mM. Only in the liver [where the creatine kinase (CK) equilibrium reaction is not present] is there some evidence that P_i is a regulator of oxidative phosphorylation (27).

pH_i

The pH_i is the lowest in RM. pH_i is 6.63, whereas it is 6.70 in DWM and 6.85 in VWM. This suggests that RM accumulates more lactic acid than white muscle during anaerobioses. Another possibility is that RM has a much lower buffer capacity than, for example, white muscle. This is confirmed by our data where a buffer capacity of RM was recorded of 28.1 ± 3.4 meq·g^{-1}·pH unit^{-1} and for white muscle of 40.0 ± 1.4 meq.g^{-1}·pH unit^{-1}. With respect to recovery, a faster recovery is observed in RM during reoxygenation. This can probably be explained by lactic acid recycling, which can occur in RM (15). Lactate dehydrogenase is bifunctional in RM, in contrast to white muscle, which essentially can only convert pyruvate to lactic acid (15). Also, in another ^{31}P-NMR study, it was demonstrated that after ischemia, the pH_i in RM dropped from 7.5 to 5.8 after excision. This drop was only from 7.3 to 6.4 in white muscle (37).

With respect to the reported pH_i values in muscle in this study, it should be noted that the existence of an active H^+ transport across the sacrolemma (8, 45) may exist. This probably may lead to an underestimation of the anaerobic metabolism.

A characteristic of oxidative phosphorylation is that no net protons are formed (14, 22). Consequently, the acidosis observed in this study during hypoxia and the period of recovery is the net result of *1*) the flux through the CK reaction, *2*) ion exchange between intracellular and extracellular compartments, *3*) net hydrolysis of adenine nucelotides (14), and *4*) the glycogen/glucose-to-lactic acid conversion. The drop in pH_i during the first hour of reoxygenation can probably be explained by the first mechanism, the flux through the CK reaction. In DWM, the pH_i dropped from 6.85 at anoxia to 6.70 at the first hour of reoxygenation and even to 6.63 during the second hour of reoxygenation. Most likely, the observed acidosis can be ascribed to resynthesis of PCr during the initial reoxygenation period. Resynthesis of PCr is an acidic process, which can be observed from the CK reaction (35). Also, in the muscle contraction study of Meyer et al. (20), recovery acidosis has been described. In that study, a significant decline from pH 6.9 to 6.6 was observed after stimulation of the rat gastrocnemius muscle. The second process, acidosis due to ion exchange, is not likely to happen in muscle tissue. It may work the other way around, eventually, by the Na^+/H^+ exchanger, which can be driven by the Na^+ gradient over the plasma membrane. An active acid extrusion mechanism has been described in cardiac muscle and seems to rely primarily on both Na^+/H^+ exchange and Na^+-HCO_3^- cotransport (8, 45). The third process, net hydrolysis of adenine nucleotides is unimportant for acid-base balance in this case, because the ATP concentration remains constant. The fourth process, glycogen/glucose-to-lactic acid conversion, however, becomes important when anaerobic metabolism is activated. It is possible to estimate the total amount of lactate produced in muscle tissue from the [PCr] decline and the buffer capacity (35). Thus we find for DWM, VWM, and RM calculated lactate values of, respectively, 17.24, 27.14, and 17.18 mM over the total hypoxia protocol (see Table 6). However, because the hypoxic conditions lasted many hours and the RM is well perfused, the lactate actually produced in the different tissues may have been much higher.

From two former ^{31}P-NMR studies, an anoxia study (49) and a hypoxia study (44), we find similar values for the lowest pH that can be observed during anaerobiosis in fish muscle. During conditions of anoxia, pH values were 6.9, 6.7, and 6.7 for goldfish, tilapia, and carp, respectively (49). However, during severe hypoxia of 3% AS in white muscle, values of 6.9, 6.8, and 6.8 were reached for these three species. In this study, the lowest pH in DWM was 6.63 during the second hour of reoxygenation, whereas the lowest value during anoxia was 6.85.

High-Energy Phosphates

The concentrations of PCr and ATP in white muscle and RM show striking differences. In general, [PCr] and [ATP] are lower in RM. This was also observed with ^{31}P-NMR techniques in excised goldfish muscle tissue (37) and with HPLC techniques on freeze-clamped tissue of common carp and rainbow trout (47). In ischemic white goldfish muscle at $t = 0$, a relative resonance frequency for PCr and ATP was observed to be 2.28 and 0.53, respectively. For PCr and ATP in the corresponding RM, this was 1.07 and 0.25. So, on the basis of these observations, it can be concluded that the [PCr] and [ATP] pool in RM is 46.9 and 47.2%, respectively (37). In normoxic common carp and rainbow trout, the [PCr] in RM was found to be 17.8 ± 2.72 and 17.11 ± 2.92 mM, respectively. The [PCr] level in the white muscle of both species was 24.9 ± 1.32 and 20.8 ± 0.91 mM, respectively (47). So, clearly the PCr content of RM is lower than that of white muscle. The same phenomenon is observed with respect to ATP levels. In normoxic common carp and rainbow trout, ATP concentrations in RM are 4.01 and 4.02 mM, respectively, whereas those in white muscle of both species are 5.87 and 6.02 mM, respectively (47). Lower levels of PCr and ATP in RM, compared with white muscle, were also observed in rainbow trout (25), sea bass (21), cod (11), and goldfish (30).

Metabolic Depression

In a former study with smaller animals of ~60–80 g, a decrease of the [ATP] to 80% was observed during

severe hypoxia (3% AS). During a 6-h recovery period, the [ATP] remained at approximately the same value (44). In this study, the ATP pool remained rather unaffected during hypoxia, even by a consecutive anoxia period. This is likely due to the size of the animals; larger animals have a relatively lower metabolic rate (23). When comparing the depletion rate of PCr and the slope of the pH_i decline during hypoxia and anoxia, it is obvious that these processes are slower during anoxia than during hypoxia. Thus the decline in pH_i in DWM during severe hypoxia is 0.13 pH units/h, which was significantly different from the decline during anoxia, which corresponded to 0.05 pH units/h. This is remarkable, because under hypoxic conditions the animals were also consuming oxygen. So, energy production under hypoxic conditions is higher than under anoxic conditions, confirming the hypothesis of flexible metabolic depression. The level of metabolic depression is dependent on the severity of the environmental stressor (40, 42). So, we can conclude that in these experiments, tilapia switch over to metabolic depression to survive the severe hypoxic conditions. Metabolic depression is observed among a range of individuals in nature, such as insects in diapause, hamsters during hibernation, diving turtles, and in some fish species (28, 29, 35, 39, 40–43, 46, 49, 50). Just like metabolism can be activated during extreme exercise, metabolism also can be directed oppositely under adverse environmental conditions. The process of metabolic depression was, for the first time, demonstrated directly for anoxic goldfish (42, 46, 50) and hypoxic tilapia (40, 41) using a specially developed 1-liter flow-through microcalorimeter. With this calorimeter, the examiner is able to measure under constant environmental stress-free conditions (1). In tilapia, the metabolic rate in this study (Table 7) was reduced to ~50% of the SMR, whereas during anoxia, goldfish could reduce their metabolic rate to even 30% of SMR. With the use of deconvolution techniques, it was demonstrated that the process of metabolic depression took place on a time scale of minutes (46). For teleosts, this is the first work that describes the response of high-energy phosphates and pH_i at different muscle tissues in the same animal with in vivo ^{31}P-NMR during a graded hypoxia load. With this information, an energy balance was calculated, estimating the aerobic and anaerobic ATP production in tilapia. Comparison with the SMR revealed that the environmental stressor hypoxia is not totally compensated for by an activation of anaerobic metabolism in tilapia, but that metabolic depression is a prerequisite for survival in this species.

Perspectives

Via two approaches, flexible metabolic depression is demonstrated in this study during anaerobioses: on one hand, by measuring the metabolic rate via respirometry based on the oxygen consumption and, on the other, by depletion of energy stores and accumulation of end products in muscle. In this study, it is demonstrated that during anaerobioses the anaerobic component remains depressed, dependent on the level of oxygen availability. In contrast the aerobic component adjusts itself dependent on the level of oxygen available. These results indicate that the control of the aerobic and anaerobic components is rather independent of each other. Similar results were observed with direct calorimetry and respirometry in two other fish species [goldfish (42, 50) and small tilapia (40, 41)] and two invertebrates, the worm *Sipunculus nudus* (13) and the bivalve *Scapharca inaequivalvis* (32). So flexible metabolic depression may generally be applied in the animal kingdom to prolong survival under adverse conditions.

Furthermore, this study proved that metabolic depression is tissue dependent. The general accepted view is that metabolic depression occurs in organs, such as muscle and alimentary tract, that are temporarily less important for the animal for survival. The question remains if vital organs, such as brain and heart, also express the mechanism of flexible metabolic depression under adverse environmental conditions. In the future we hope to elucidate this matter via ^{31}P-NMR.

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