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Note: To cite this publication please use the final published version (if applicable).

Functional Coupling of Glycolysis and Phosphocreatine Utilization in Anoxic Fish Muscle

AN *IN VIVO* ^{31}P NMR STUDY*

Aren Van Waarde[‡][§], Guido Van den Thillart[‡], Cees Erkelens[¶], Albert Addink[‡], and Johan Lugtenburg[¶]

From the Departments of ‡Biology (Animal Physiology) and ¶Organic Chemistry, University of Leiden, Gorlaeus Laboratories, Einsteinweg 5, P. O. Box 9502, 2300RA Leiden, The Netherlands

Three fish species with different strategies for anoxic survival (goldfish, tilapia, and common carp) were exposed to environmental anoxia (4, 3, and 1 h, respectively). The concentrations of high energy phosphate compounds and inorganic phosphate, besides the intracellular pH in the epaxial muscle were measured during anoxia and recovery by *in vivo* ^{31}P NMR spectroscopy. The concentration of free ADP was calculated from the equilibrium constant of creatine kinase.

During anoxia the patterns of phosphocreatine utilization and tissue acidification are remarkedly similar. Free ADP rises rapidly during the initial period of oxygen deficiency and reaches a plateau in goldfish and tilapia, while it keeps rising in the common carp. At elevated levels of free ADP, the creatine kinase reaction and anaerobic glycolysis are functionally coupled by H⁺ as a common intermediate.

The coupling between both processes disappears upon reoxygenation, when mitochondrial respiration induces a rapid drop of [free ADP]. The removal of ADP shifts the creatine kinase equilibrium toward phosphocreatine synthesis despite the low pH.

Fish which are exposed to environmental anoxia follow different metabolic strategies to ensure survival. Most species respond by Embden-Meyerhof glycolysis, leading to accumulation of lactic acid (reviews in Van den Thillart, 1982; Van den Thillart, and Van Waarde, 1985). When the metabolic rate (*i.e.* ATP turnover) is maintained at the aerobic standard level (SMR), glucose/lactate conversion results in a rapid development of acidosis. The period during which anoxia can be resisted is in such cases severely limited by anaerobic proton generation.

A better strategy consists of the combination of lactic acid production with metabolic suppression. "Metabolic arrest," as defined by Hochachka *et al.* (1982, 1985, 1987), indicates a lowering of energy demand below the SMR and requires adaptive modification of the ion channels and ion pumps in biomembranes. Metabolic arrest will lead to a retarded development of acidosis because of the lowered glycolytic flux. The period during which anoxia can be resisted is thus extended by a factor directly related to the reduced energy demand.

A third strategy is based on metabolic suppression combined with a switch to an alternative energy-producing pathway. Some Cyprinid species, like goldfish (Shoubridge and Hochachka, 1980), crucian carp (Johnston and Bernard, 1983), and bitterling (Wissing and Zebe, 1988), produce ethanol, CO_2, and NH_3 during environmental anoxia. Although Hochachka and Mommsen (1983) have suggested that proton stoichiometry does not depend on the pathway utilized, a theoretical analysis (Pörtner, 1987; Pörtner *et al.*, 1984) and measurements of intracellular pH in anoxic individuals (Van den Thillart and Van Waarde, 1989; Van den Thillart *et al.*, 1989) indicate that this suggestion is overly simplified. Glucose/ethanol conversion is not accompanied by net production of protons, whereas formation of lactate generates one acid equivalent (Pörtner, 1987). The combination of metabolic suppression with a modified metabolism further extends the period during which anoxia can be resisted. At low temperature (<5 °C), goldfish and crucian carp can survive even 4–6 months in the absence of oxygen (Blažka, 1958; Holopainen and Hyvärinen, 1985). Survival may then be limited by the amount of available substrates rather than anaerobic proton generation (Van den Thillart *et al.*, 1989).

The present paper deals with the regulation of glycolytic flux and phosphocreatine utilization in fish muscle during a period of environmental anoxia followed by aerobic recovery. Poikilothermic vertebrates are very suitable experimental animals for such a study. Since their metabolic rate is low, changes in high energy phosphate compounds can be followed easily. Three fish species were chosen, which exhibit the different strategies for anoxic survival as outlined above. Common carp (*Cyprinus carpio*) try to meet a high anoxic energy demand by conversion of glycogen to lactic acid (Van den Thillart and Van Waarde, 1989; Van den Thillart *et al.*, 1989). Tilapias (*Oreochromis mossambicus*) combine a classical glycolysis with metabolic suppression.[1] Goldfish (*Carassius auratus*) convert glucose to ethanol (Shoubridge and Hochachka, 1980), and they reduce energy demand to 30% of the aerobic SMR at 20 °C (Van Waversveld, 1988; Van Waversveld *et al.*, 1988, 1989).

Concentrations of high energy phosphate compounds and the intracellular pH of the myotomal muscles were measured in conscious animals by the noninvasive technique of *in vivo* ^{31}P NMR spectroscopy, using a special bioprobe that has been described elsewhere (Van den Thillart *et al.*, 1989). We observed a functional coupling between phosphocreatine utilization and anaerobic glycolysis by the common intermediate, H⁺, in all three species, despite their different strategies for anoxic survival.

[1] M. Van Overbeek, G. Van den Thillart, and A. Van Waarde, unpublished observations.

EXPERIMENTAL PROCEDURES

Animals—Common carp (*Cy. carpio*), goldfish (*Ca. auratus*), and tilapias (*Or. mossambicus*) were used in all experiments. Carp came from the Experimental Fish Culture Station of the Agricultural University of Wageningen, The Netherlands. Goldfish were purchased from a commercial fish dealer (Boon, Hardinxveld, The Netherlands). Tilapia's were bred in our own laboratory. The animals had a mean body weight of 50–80 g (body length 12 cm) and were acclimated to 20 °C, oxygen levels of 130–160 torr, and a 16-h light period. All fish were fed daily with trout food in pellet form (Trouvit, Trouw, Putten, Netherlands). The anesthetic ethyl *m*-aminobenzoate methanesulfonate (MS 222) was obtained from Sigma.

In Vivo ^{31}P *NMR Experiments*—The NMR studies were carried out from June to August, 1988. A Bruker MSL-400 NMR spectrometer was used, operating at 162 MHz (^{31}P) or 400 MHz (^{1}H).

For each experiment, a single fish was kept overnight in a darkened tank. Subsequently, the animal was anesthetized by addition of MS 222 to the water to a final concentration of 100 ppm. As soon as the fish lost equilibrium, it was mounted in a Perspex flow cell, which fitted inside a modified Bruker bioprobe. The design of the cell and the experimental protocol used for *in vivo* ^{31}P NMR have been described elsewhere (Van den Thillart *et al.*, 1989). The animal was immobilized by an inflatable plastic bag. The gills were irrigated with a constant flow of water, which did not contain any anesthetic. Therefore, the fish woke up rapidly and was conscious during the actual experiment. The signal of the myotome was picked up with an 18-mm surface coil placed about 2 cm behind the operculum above the lateral line. Preliminary experiments with phantoms (an array of glass tubes filled with phosphate buffer) indicated that the viscera do not contribute significantly to the total signal intensity. The homogeneity of the B_o field was optimized by shimming on the ^{1}H signal of the intracellular water. ^{31}P NMR spectra (8192 data points) were accumulated over a period of 10 min and consisted of 136 individual scans, using a pulse width of 60 ° (in the sensitive volume), and acquisition time of 0.4 s and a 4-s relaxation delay. Measurements of the T_1-values of the metabolites of interest demonstrated that the resonances of ATP, PCr,[2] and P_i are fully relaxed under these conditions, (exp($- t/T_1$) < 0.1), whereas the T_1-values are not changed significantly by anoxia. We waited for 2–3 h until the effects of handling (lowered intracellular pH and phosphocreatine/inorganic phosphate ratios) had disappeared. The fish was then in a normoxic steady state, so that control (normoxic) parameters could be determined. Oxygen lack was introduced by bubbling of the perfusion medium with nitrogen. As soon as the NMR spectra indicated virtual depletion of phosphocreatine (*i.e.* after 1 h for carp and 3 h for tilapias), the gas phase was switched back to air. In experiments on goldfish, air was reintroduced after an arbitrarily chosen period of 4 h. Measurement of the O_2 tension in the perfusion medium with oxygen electrodes indicated that 30 min of hypoxia were followed by 30, 150, or 210 min of absolute anoxia (for carp, tilapias, and goldfish, respectively). The O_2 tension dropped from 100 to ~10% air saturation during the first 10 min, followed by a slower decline to 2% during the following 10 min. After 25–30 min, O_2 became undetectable. After reintroduction of air, the pO_2 increased from 0 to 90% air saturation within the first 10 min, 100% saturation being reached after 30 min. Spectra were accumulated for another 3–4 h to monitor metabolism during the recovery period. Each animal thus served as its own control. There was no mortality; all animals survived and behaved normally after being returned to their holding tanks.

In each spectrum, the peak areas of the different high energy phosphate compounds were divided by the peak area of an external standard (a microsphere filled with a solution of methylene diphosphonate in D_2O, which was mounted at the center of the surface coil). Changes in the steady-state concentrations of metabolites were then expressed as the changes of their relative resonance intensities (RRIs). The intracellular pH (pH_i) was estimated from the difference in chemical shift between phosphocreatine and inorganic phosphate in the NMR spectra. The pH measurements were calibrated as described previously (Van den Thillart *et al.*, 1989).

Metabolite Measurements—Neutralized perchlorate extracts of freeze-clamped epaxial white muscle of fish were prepared as described in a previous paper (Van den Thillart *et al.*, 1982). ATP was determined in the extracts by an enzymatic assay (Lamprecht and Trautschold, 1970). Total creatine was determined colorimetrically

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[2] The abbreviations used are: PCr, phosphocreatine; RRI, relative resonance intensities.

(Ennor and Stocken, 1947) after complete acid hydrolysis of creatine phosphate (1 N HCl, 7 min at 100 °C).

RESULTS

In Vivo ^{31}P *NMR Spectra*—Spectra of the epaxial muscle of tilapia are presented in Fig. 1. They show well-resolved resonances of the external standard methylene diphosphonate, sugar phosphates, inorganic phosphate (P_i), PCr and ATP (*panel A*). Anoxia causes breakdown of PCr, accumulation of sugar phosphates and P_i, and the development of acidosis (*panel B*). After the return of oxygen, the control condition is restored within 3–4 h (*panel C*). Spectra of similar quality were obtained from common carp and goldfish.

Changes of PCr, P_i, and pH_i during Anoxia—The time course of the PCr concentration in fish muscle during anoxic exposure is presented in Fig. 2A. The three fish species show different patterns of phosphagen utilization in the absence of oxygen. Bubbling of the perfusion medium with nitrogen causes an immediate decline of [PCr] in carp muscle, which continues until the direct energy reserves have been depleted by more than 85%. [PCr] in goldfish does not respond immediately but only after a period of 10 min of hypoxia. The steep decline that follows comes to an end after the onset of anoxia. Then, [PCr] stabilizes at 50% of the normoxic control level. Degradation of phosphocreatine in tilapias follows an
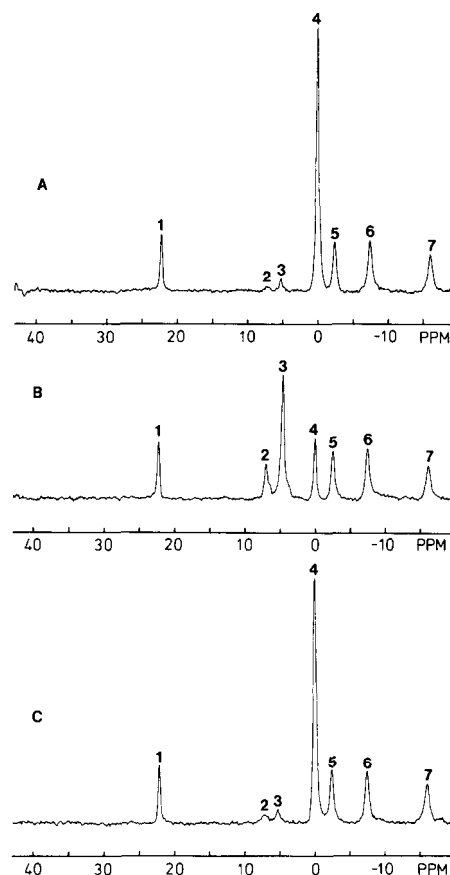

FIG. 1. *In vivo* ^{31}P **NMR spectra of the epaxial muscle of *Or. mossambicus*.** All spectra are from the same animal. *Spectrum A* was acquired after the fish had been in the probe for 2 h with well-aerated water. *Spectrum B* was taken at the end of a 3-h exposure to anoxia. *Spectrum C* was acquired 3 h after reoxygenation. The labeled resonances are *1*, methylene diphosphonate (external standard); *2*, sugar phosphates; *3*, inorganic phosphate; *4*, creatine phosphate; *5*, γ-phosphate of ATP with underlying β-phosphate of ADP; *6*, α-phosphate of ATP with underlying α-phosphate of ADP and phosphate moiety of NAD; *7*, β-phosphate of ATP. Spectra of similar quality were obtained from *Ca. auratus* and *Cy. carpio*.
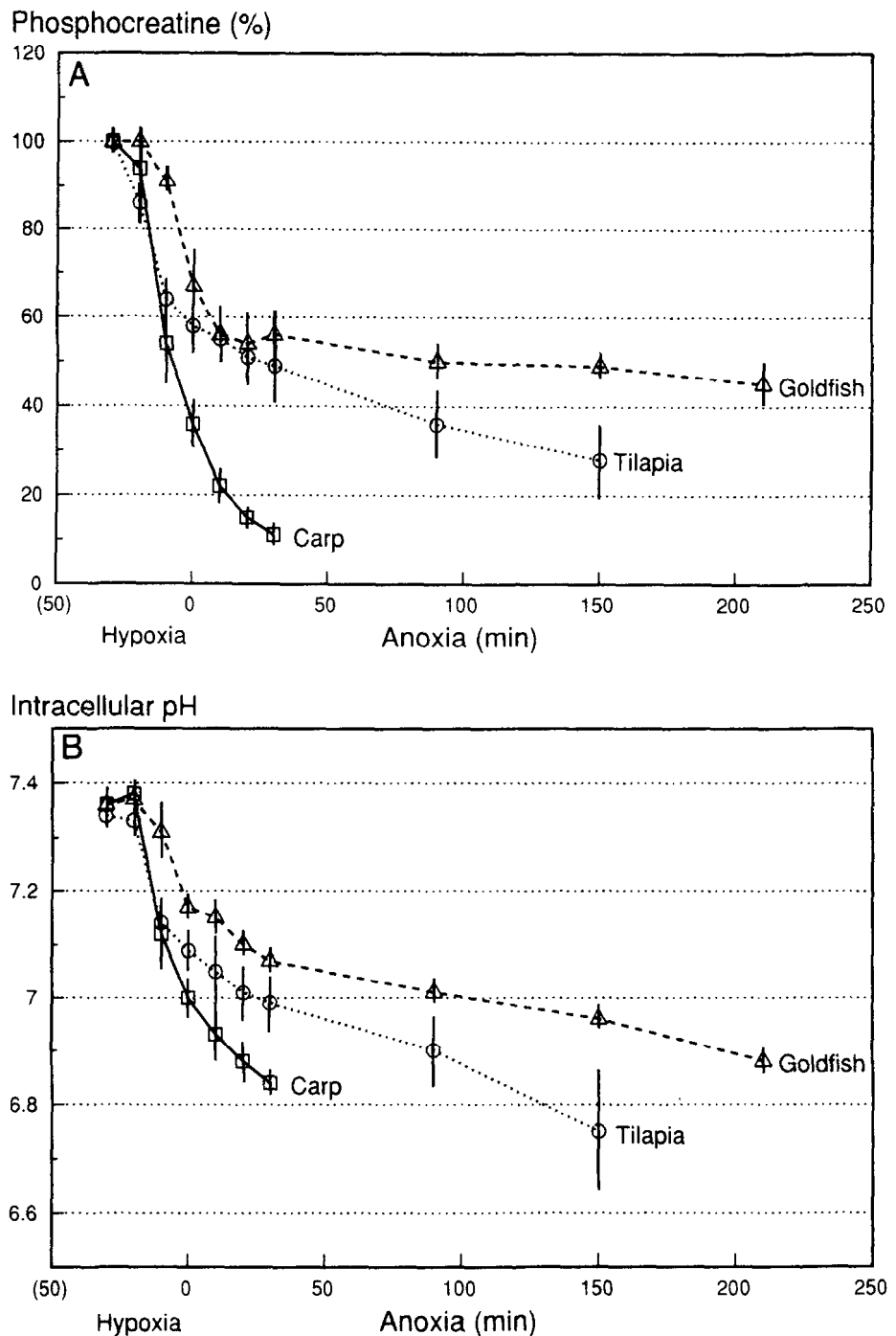
Phosphocreatine (%)

Fig. 2. Changes of phosphocreatine and intracellular pH (pH$_i$) in fish muscle during hypoxia and anoxia. Indicated are means ± standard erros of four animals of each species. Phosphocreatine levels are expressed as a percentage of the normoxic control value. The first data point at the left-hand side of each curve coincides with the onset of nitrogen bubbling of the gill perfusion fluid. Anoxia was reached after a 30-min period of hypoxia (see "Experimental Procedures").

intermediate pattern. Here, [PCr] responds immediately to a reduction of oxygen availability, but after 20 min, PCr hydrolysis slows down to a lower rate which is maintained during the whole period of anoxia.

The time course of the intracellular pH is presented in Fig. 2B. The three species show also distinct patterns of development of acidosis. During the first 10 min of hypoxia, muscle pH$_i$ does not change. Then, it starts to decline in all cases. Acidification of carp muscle continues at a high rate until pH values below 6.8 are reached. The rapid initial drop of pH$_i$ in goldfish is followed by a decreased rate of net acid production after 20 min. In tilapia muscle, the rate of acidification is also lowered after 20 min, but the dampening of acidosis is not as strong as that observed in goldfish.

When Fig. 2, A and B, are compared, it is immediately apparent that the patterns of acid production and PCr utili-

zation are remarkably similar. The two processes follow virtually identical time courses in each species. This correlation is observed in three genera (*Carassius, Cyprinus, Oreochromis*) with different strategies for anoxic survival, suggesting a universal mechanism.

Phosphocreatine utilization appears to be accompanied by a corresponding increase of inorganic phosphate. Thus, the appearance of P$_i$ is rapid in carp, but slower in tilapias, whereas the P$_i$ concentration reaches a virtual steady state in goldfish (Fig. 3A).

Changes of PCr, P$_i$, and pH$_i$ during Recovery—The time courses of [PCr], P$_i$, and pH$_i$ during postanoxic recovery are shown in Fig. 3B and Fig. 4. It is clear that the correlation between [PCr] and pH$_i$, as described above, no longer holds after the return of oxygen. [PCr] increases immediately upon reoxygenation (Fig. 4A), but the intracellular pH shows an
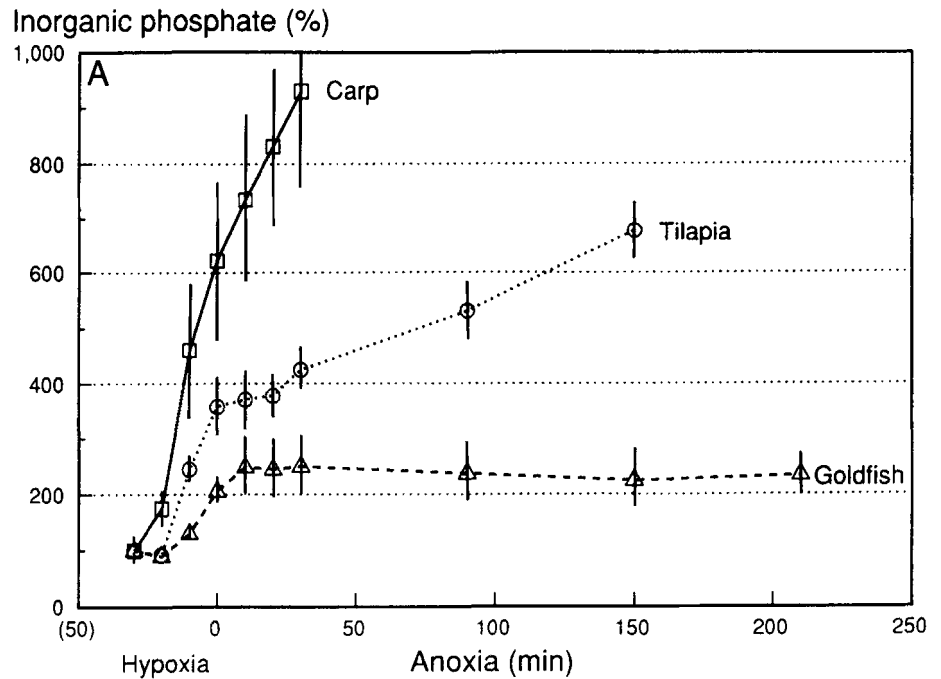
Inorganic phosphate (%)

FIG. 3. **Changes of inorganic phosphate (P_i) in fish muscle during hypoxia, anoxia, and postanoxic recovery.** Indicated are means ± standard errors of four animals of each species. Levels are expressed as a percentage of the normoxic control value. The first data point at the left-hand side of each curve coincides with the onset of nitrogen- (*upper panel*) or air- (*bottom panel*) bubbling of the gill perfusion fluid. Anoxia was reached after a 30-min period of hypoxia (see "Experimental Procedures").

Inorganic phosphate (%)
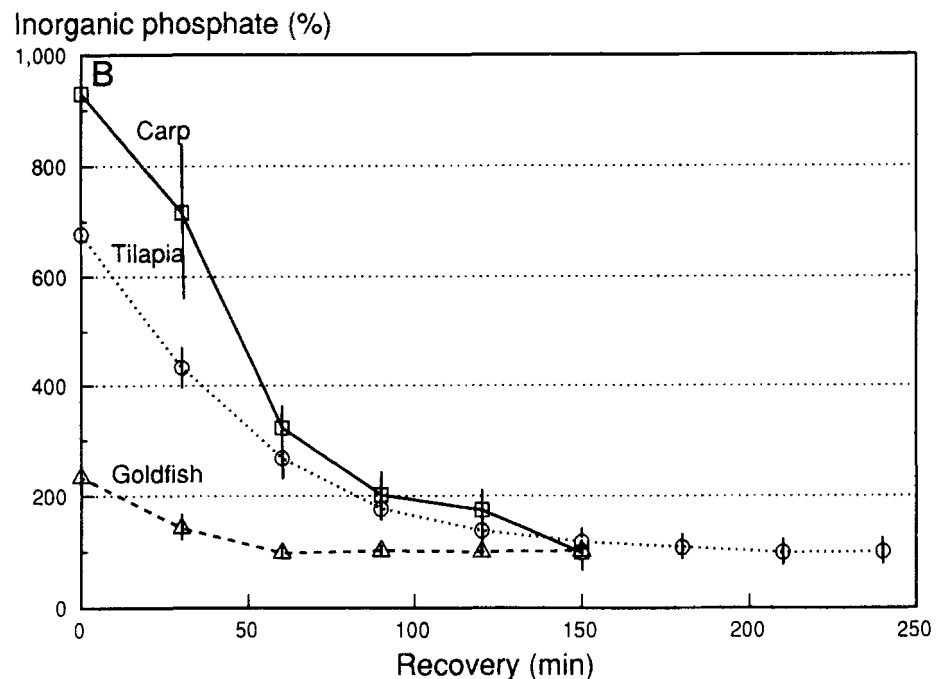
additional decline during 30 min before it finally returns to the control value (Fig. 4*B*). Carp show the highest rate of net creatine phosphorylation, followed by tilapias and, finally, goldfish. However, restoration of muscle pH_i is more rapid in goldfish and carp than in tilapias.

Resynthesis of phosphocreatine seems to be accompanied by a corresponding decrease of inorganic phosphate (Fig. 3*B*).

Changes of ATP during Anoxia and Recovery—The time course of [ATP] during anoxic exposure is presented in Fig. 5*A*. A comparison of Fig. 5*A* and Fig. 2*A* shows that creatine kinase buffers the ATP pool in anoxic muscle, since the observed changes in [PCr] are much greater than those in [ATP]. The buffering is most efficient in goldfish. There, the drop of [ATP] is <15% even after 4 h of oxygen deficiency. In tilapias, [ATP] has fallen by 15% after 2 h, and by 30% after 3 h. Carp could stabilize [ATP] only during the first 20

min of hypoxia. In this species, [ATP] fell by >50% within 1 h after the gas phase had been switched from air to nitrogen.

After the return of oxygen, carp demonstrate the highest rate of net ATP synthesis, followed by tilapias and, finally, goldfish (Fig. 5*B*). In a previous paper (Van den Thillart *et al.*, 1989), we have shown that ATP is formed from IMP by the action of the enzymes adenylosuccinate synthetase and adenylosuccinate lyase.

Changes of Cytosolic ADP during Anoxia and Recovery— Since ADP is an important allosteric modulator of energy-producing processes, we were interested in the behavior of the cytosolic ADP concentration during anoxia and recovery. It is possible to calculate free ADP from ^{31}P NMR data, if [total creatine] (*i.e.* the sum of PCr and creatine) and [ATP] are known. We measured a total creatine concentration of 29.9 ± 3.0, 25.6 ± 4.9, and 28.7 ± 3.9 μmol/g wet weight and [ATP]
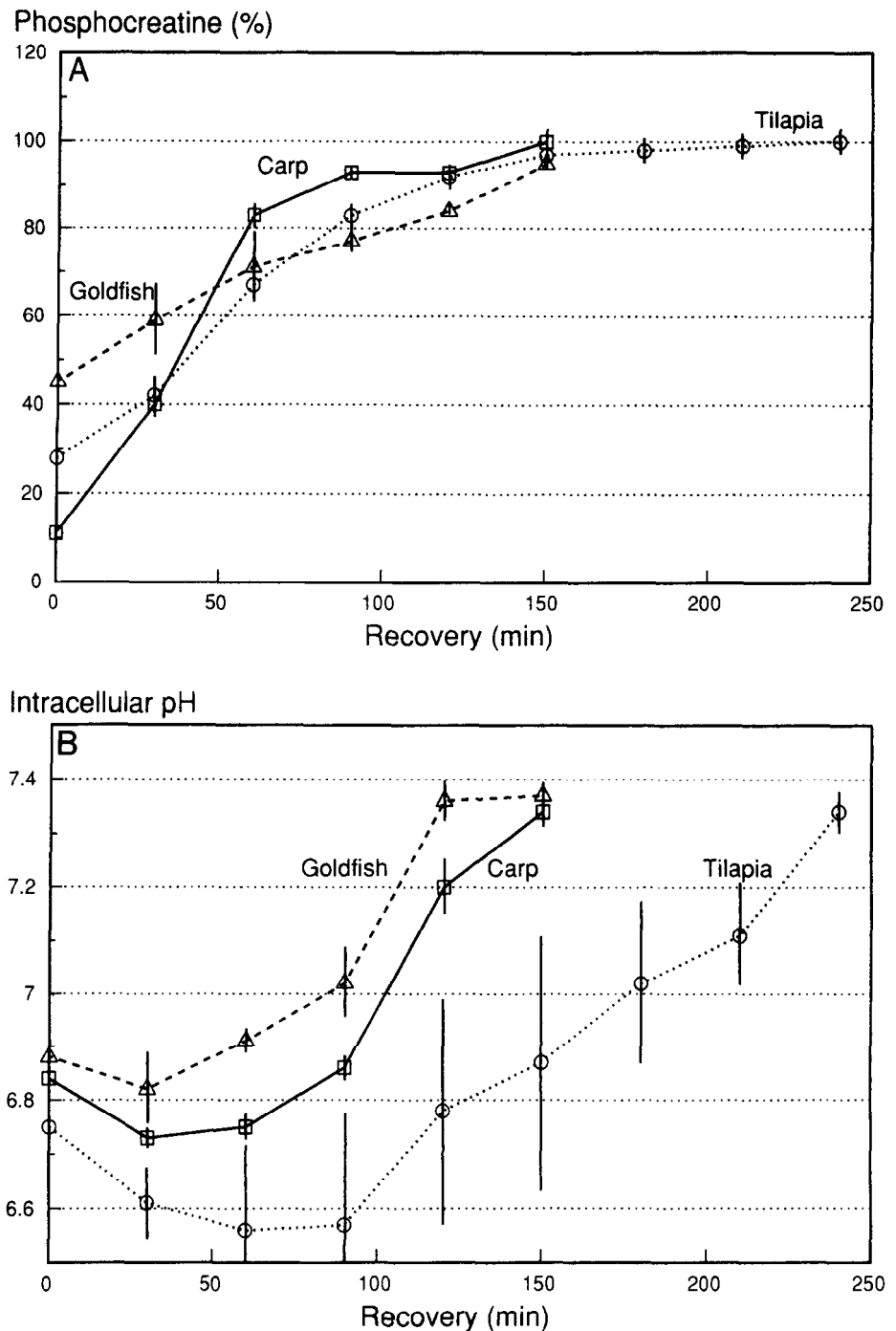
Phosphocreatine (%)

Intracellular pH

FIG. 4. **Changes of phosphocrea-tine and intracellular pH (pH$_i$) in fish muscle during postanoxic re-covery.** Indicated are means ± standard errors of four animals of each species. Levels are expressed as a percentage of the normoxic control value. The first data point at the left-hand side of each curve coincides with the onset of air bubbling of the gill perfusion fluid. 100% saturation with air was reached within 30 min (see "Experimental Proce-dures").

of 5.1 ± 0.2, 5.5 ± 0.3, and 5.5 ± 1.1 in freeze-clamped white muscle of carp, goldfish, and tilapia, respectively. In a previous paper (Van den Thillart *et al.*, 1976), white muscle of goldfish was reported to contain 30.1 ± 2.5 μmol of creatine/g wet weight. Assuming that the creatine kinase reaction is near equilibrium (see "Discussion"), we can calculate free ADP by the following formula (Lawson and Veech, 1979):

$$[\text{ADPf}] = \frac{[\text{ATP}] \cdot [\text{Creatine}]}{[\text{PCr}] \cdot [\text{H}^+] \cdot K_{eq}}$$

Peak areas of metabolites in the NMR spectra are expressed as relative resonance intensities or RRIs (area of the reso-nance of interest divided by the area of the resonance of the external standard). If the normoxic RRI of ATP corresponds to a tissue concentration of 5.5 μmol/g wet weight, [PCr] can be estimated from the ratio of the RRIs of PCr and ATP.

[Creatine] is obtained by subtraction of [PCr] from colori-metrically determined [total creatine]. The intracellular pH is measured as described under "Experimental Procedures." The K of the creatine kinase reaction is dependent on [Mg^{2+}] and temperature (Gadian *et al.*, 1981; Lawson and Veech, 1979). In our calculations, we assume K is 1.47 × 10^9 at 20 °C and a [Mg^{2+}] of 1 mM (see "Discussion").

Results are presented in Fig. 6. The initial cytosolic levels of ADP are ~20 μM. Hypoxia causes a rapid increase of the ADP concentration. Free ADP shows a continuous rise in anoxic carp, but in the other species, it reaches a plateau and is maintained at a level of 60 μM in the absence of oxygen. Reoxygenation causes a rapid decline of free ADP, probably due to mitochondrial respiration. In carp and tilapia, ADP is restored to low values within 30 min, but in goldfish, a biphasic recovery is observed. Here, ADP declines rapidly to
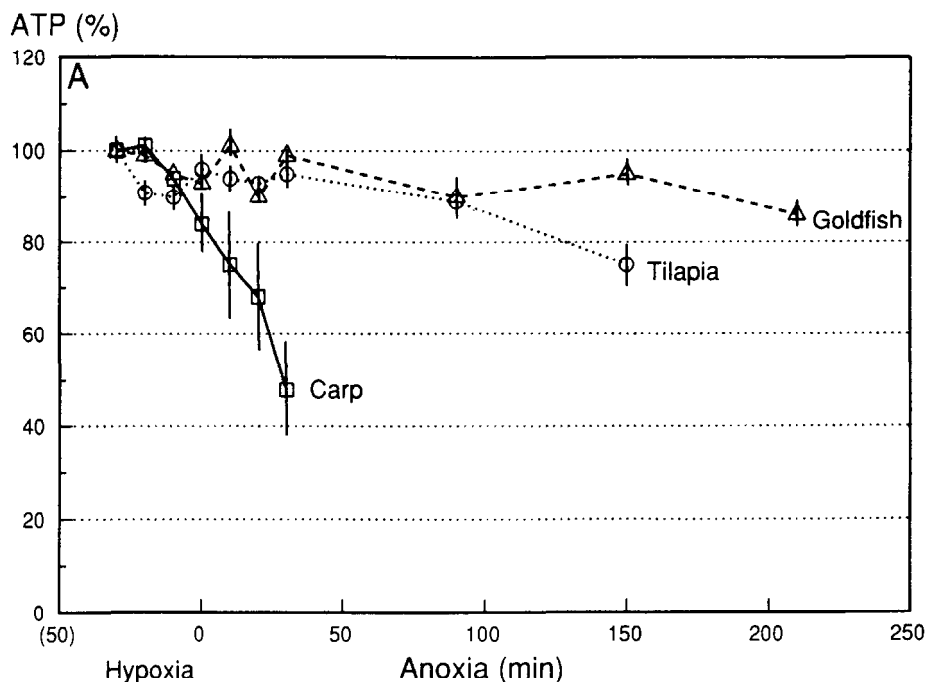
ATP (%)

FIG. 5. **Changes of ATP in fish muscle during hypoxia, anoxia, and postanoxic recovery.** Indicated are means ± standard errors of four animals of each species. Levels are expressed as a percentage of the normoxic control value. The first data point at the left-hand side of each curve coincides with the onset of nitrogen- (*upper panel*) or air- (*bottom panel*) bubbling of the gill perfusion fluid. Anoxia was reached after a 30-min period of hypoxia (see "Experimental Procedures").
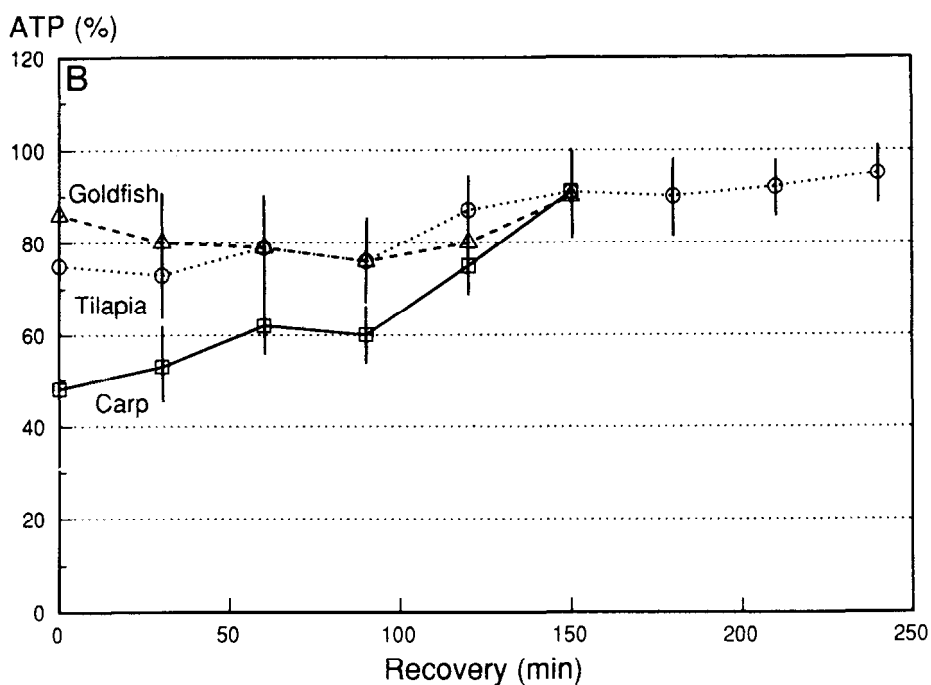
~30 μM, but control levels are not reached until 3 h after the return of oxygen.

Changes of Free AMP during Anoxia and Recovery—Free AMP in the cytoplasm can be estimated from the concentrations of free ADP and ATP, and the equilibrium constant of myokinase (Lawson and Veech, 1979):

$$[AMPf] = \frac{[ADPf] \cdot [ADPf]}{[ATP] \cdot K_{eq}}$$

The calculated time course of free AMP resembles that of free ADP, but the relative changes are much greater (values not shown). Control levels of AMP are very low, 107 ± 67, 64 ± 12, and 84 ± 27 nM in carp, goldfish, and tilapia. AMP shows a continuous rise in carp after the gas phase is switched from air to nitrogen. At the end of the anoxic exposure, AMP has increased 80-fold (concentration 8.6 μM). In tilapia and

goldfish, free AMP shows a rapid initial rise followed by a constant level during anoxia. Here, the increase is about 15-fold (final concentration 1.2 μM). Reoxygenation causes a restoration of AMP to the control values. This recovery is rapid (<30 min) in carp and tilapia, but slow (~3 h) and biphasic in goldfish.

DISCUSSION

In Vivo Levels of PCr in Fish Muscle—If the normoxic RRI of ATP in the muscle of our experimental fish corresponds to an ATP concentration of 5.5 μmol/g wet weight (see above), the PCr/ATP ratio in the NMR spectra indicates normoxic PCr levels of 25.1 ± 2.2, 24.4 ± 0.2, and 23.8 ± 1.9 μmol/g (mean ± S.D.) in carp, goldfish, and tilapia. In other words, more than 80% of the total creatine exists in the phosphory-
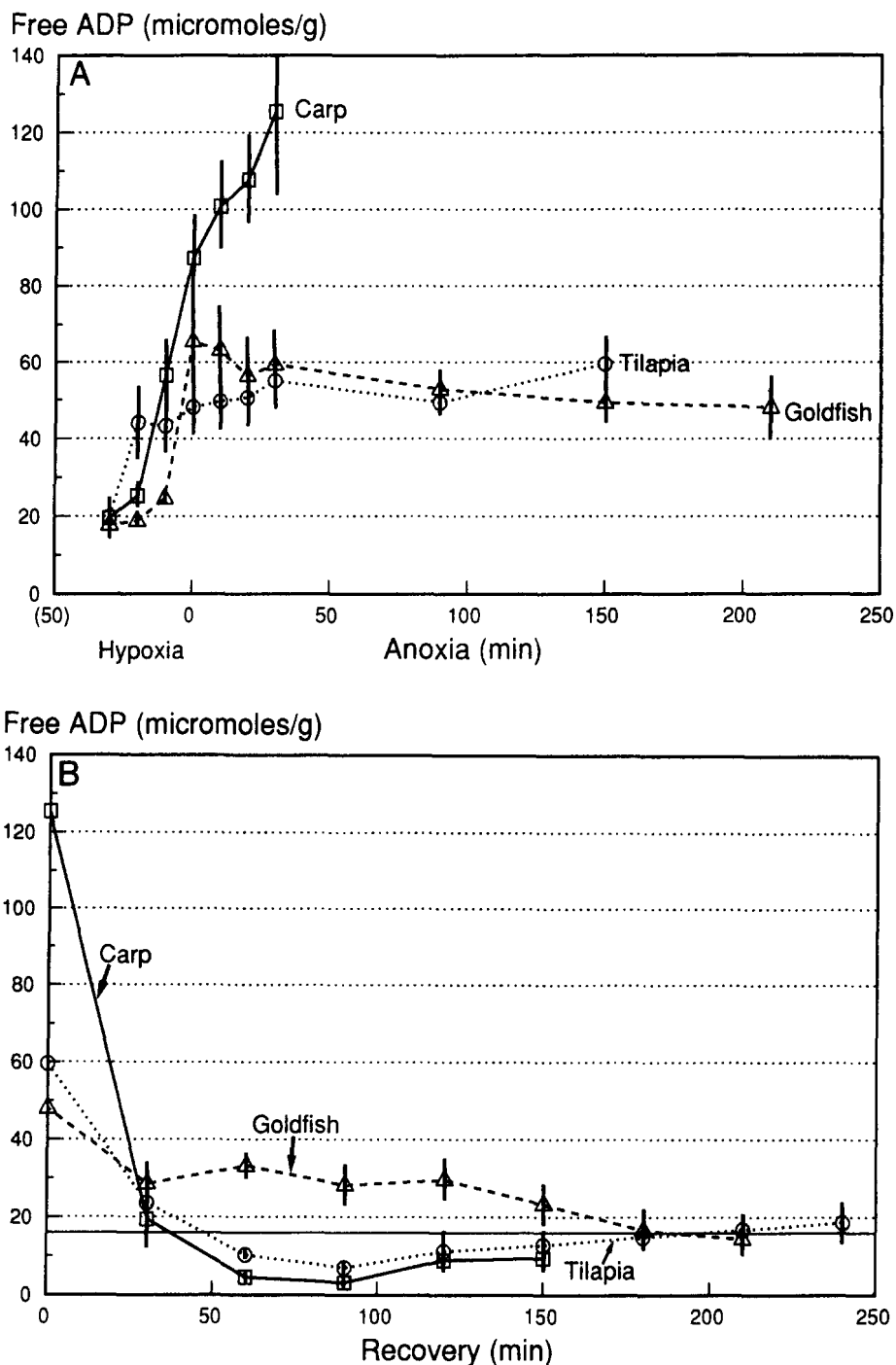
FIG. 6. **Changes of free ADP in fish muscle during hypoxia, anoxia, and postanoxic recovery.** Indicated are means ± standard errors of four animals of each species. Free ADP was calculated as described under "Results". The first data point at the left-hand side of each curve coincides with the onset of nitrogen- (*upper panel*) or air- (*bottom panel*) bubbling of the gill perfusion fluid. Anoxia was reached after a 30-min period of hypoxia (see "Experimental Procedures"). The *horizontal line* indicates the normoxic control value for [free ADP] in goldfish.

lated form (total creatine is 26–30 μmol/g wet weight, determined colorimetrically).

Enzymatic measurements of PCr in perchloric acid extracts of freeze-clamped muscle indicate levels of PCr which are much lower. For example, Beis and Newsholme (Beis and Newsholme, 1975) measured 8.8 μmol of PCr and 36.6 μmol of creatine/g wet weight in white muscle of dogfish, so less than 20% of the total creatine was found in the phosphorylated form. The low PCr/Cr ratio led to the suggestion that creatine in fish muscle plays an osmoregulatory role besides its recognized function in the creatine kinase reaction. Van den Thillart *et al.* (Van den Thillart *et al.*, 1976) likewise reported 8.3 μmol of PCr and 21.8 μmol of creatine/g wet weight of goldfish muscle. More recently, Dunn and Hochachka (Dunn and Hochachka, 1986) measured 20.8 μmol of PCr and 25.7 μmol of creatine in which muscle of rainbow

trout, *i.e.* only 45% of total creatine was phosphorylated. Dobson and Hochachka (Dobson and Hochachka, 1987) found 17.5 μmol of PCr and 27.5 μmol of creatine in the same tissue. The discrepancy between PCr/Cr ratios obtained by *in vivo* NMR and those determined in extracts is probably caused by rapid PCr breakdown during the time lag between fish killing and complete denaturation (including tissue isolation, freeze clamping, and acid extraction). If animals are killed by a blow on the head, muscle metabolism is affected by catecholamine release. Tissue excision induces stimulation of the fibers through contact potentials, which will also cause a fall of [PCr]. Enzymatic determinations of P_i in resting fish muscle indicate P_i levels in excess of 10 mM (Fraser *et al.*, 1966; Nagayama, 1961), whereas our *in vivo* NMR spectra suggest [P_i] is <1 mM. It is thus likely that enzymatic determinations result in levels of PCr which are lower, and levels of P_i which

are higher than the actual *in vivo* concentrations.

Coupling between PCr Breakdown and Glycolytic Proton Generation—The parallel decline of [PCr] and pH_i in the muscle of the three fish species (Fig. 2) suggests that the two processes (*i.e.* PCr breakdown and glycolytic proton generation) are coupled. Many authors (Brown, 1982; Dawson et al., 1978; Gadian et al., 1981; Hochachka and Mommsen, 1983; Lawson and Veech, 1979; Pörtner, 1987; Pörtner et al., 1984) have pointed out that cleavage of a phosphagen-like PCr is accompanied by net proton uptake. The creatine kinase equilibrium can be written as:

$$PCr + ADP + H^+ \rightleftharpoons ATP + Creatine$$
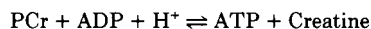
Removal of protons by the creatine kinase reaction has been actually observed *in vivo*. Ross et al. (Ross et al., 1981) reported that ischemic arm exercise in phosphorylase-deficient humans causes an *increase* of muscle pH_i due to PCr breakdown instead of the normal acidosis due to anaerobic glycolysis. The exact proton stoichiometry will depend on the intracellular pH, since the dissociation of the reactants and products is pH-dependent (Hochachka and Mommsen, 1983; Pörtner, 1987; Pörtner et al., 1984).

Comparison of Fig. 2A and Fig. 5A leads to the conclusion that the creatine kinase reaction buffers [ATP], as has been reported by others (Newsholme et al., 1978; Wilson et al., 1981). At the end of the anoxia period, PCr levels in goldfish, tilapia, and carp have fallen to 50, 20, and 10% of their respective control values, while [ATP] is reduced to 85, 70, and 50%. It is thus obvious that PCr is depleted in order to stabilize the intracellular ATP concentration. Creatine kinase is not a regulatory enzyme (Bittl et al., 1987; Veech et al., 1979), therefore PCr breakdown can only stabilize [ATP] if [ADP] and/or [H$^+$] increase. The striking similarity of the time courses of PCr depletion and anaerobic proton generation (Fig. 2) indicates that protons are involved in the shift of the PCr/ATP ratio during anoxia.

Changes in Cytosolic [ADP] Induced by Oxygen Deficiency— Most of the ADP in muscle is bound to actin (Bárány et al., 1975). This bound ADP cannot exchange freely with creatine kinase, glycolytic enzymes and mitochondrial ATPase (Balaban et al., 1983; Veech et al., 1979). The PCr/ATP ratio will thus be related to [free ADP] rather than [total ADP].

The cytosolic ADP concentration can be determined from the levels of ATP, PCr, H$^+$, and creatine, and the creatine kinase equilibrium constant. Two assumptions are involved: (a) the reaction should remain near equilibrium in the sarcoplasmatic compartment under all experimental conditions; (b) the equilibrium constant used in the calculation should be an adequate reflection of the K *in vivo*.

The first assumption seems reasonable on the following grounds. First, white muscle of Teleosts contains a large amount of creatine kinase. V_{max} ranges from 500 μmol·min^{-1}·g^{-1} in the marine coalfish to 800 μmol·min^{-1}·g^{-1} in the common carp (Johnston et al., 1987; Johnston and Moon, 1980). Second, net fluxes through the reaction are relatively low (maximally 1 μmol·min^{-1}·g^{-1}, estimated from Fig. 2A, initial [PCr] 25 mM). Third, saturation-transfer ^{31}P NMR experiments on resting muscle, using both cold- and warm-blooded vertebrates, have shown the pathway is near equilibrium (Bittl et al., 1987; Brown, 1982; Gadian et al., 1981). Since the muscle of our fish was relaxed during the experiments, a near equilibrium situation can be expected in our case as well.

The K of creatine kinase is dependent on the concentration of Mg^{2+} (Lawson and Veech, 1979). Estimations of free Mg^{2+} in frog muscle vary: >3 mM (Cohen and Burt, 1977), >2.5 mM

(Dawson et al., 1978), 0.6 mM (Gupta et al., 1983; Gupta and Moore, 1980), < 0.4 mM (Maughan, 1983), depending on the method used. Since the chemical shift difference between the α- and β-phosphate resonances of ATP in our NMR spectra indicates that more than 95% of the ATP in fish muscle is complexed with Mg^{2+}, the free magnesium concentration should be at least 10 times higher than the dissociation constant of the Mg·ATP complex. This ranges from 56 μM at pH 7.2 to 93 μM at pH 6.5 and 20 °C (Bock et al., 1985; Gupta and Moore, 1980). For our calculations of free ADP from the creatine kinase equilibrium, we have therefore assumed Mg^{2+} is about 1 mM. If the true tissue concentrations of Mg^{2+} are different (within the 0 to 5 mM range), this does not affect our conclusions regarding the time course of [free ADP] (see below). The absolute concentrations of free ADP will be slightly different, but the shape of the curves will remain the same.

The equilibrium constant of creatine kinase is temperature-dependent. At 38 °C, it ranges from 3.78×10^8 M^{-1} (free Mg = 0) to 1.66×10^9 M^{-1} (free Mg = 1 mM; enzyme from rat muscle, (Lawson and Veech, 1979)). At 4 °C and [Mg^{2+}] = 1 mM, it is reported as 1.30×10^9 M^{-1} (enzyme from frog muscle, (Gadian et al., 1981)). Our experiments were performed at 20 °C rather than 4 or 38 °C; we have therefore assumed an intermediate value for K of 1.47×10^9 M^{-1}.

Our calculated values of [free ADP] in normoxic fish muscle (18 ± 2 μM in goldfish, 20 ± 3 μM in tilapia, and 20 ± 7 μM in carp) are in good agreement with those reported elsewhere (37 μM in rat muscle (Veech et al., 1979), 20 μM in frog muscle (Gadian et al., 1981)). Hypoxia causes an increase of free ADP in all our experimental animals (Fig. 6). The increase takes place within 10 min in tilapia, 20 min in carp, and 30 min in goldfish. Initially, the level of free ADP will be determined mainly by oxidative phosphorylation. The lag phase between the onset of nitrogen bubbling and the rise of [free ADP] may therefore reflect the ability of a species to extract oxygen from the external medium. It is well known that goldfish can take up O$_2$ even at very low O$_2$ tensions, whereas the ability of the other species for O$_2$ extraction is more limited (Van den Thillart, 1982).

During anoxia, free ADP in carp continues to rise until levels >120 μM are reached. In contrast, the ADP-concentration in the other species stabilizes at a value of 50–60 μM, which is maintained during the whole anoxic period (Fig. 6). Apparently, anaerobic glycolysis in goldfish and tilapia is well regulated and results in steady-state values of free ADP, but in carp the anaerobic metabolism cannot quite meet the energy demand.

In the absence of mitochondrial activity and at relatively stable levels of free ADP, the creatine kinase reaction in anoxic fish muscle is driven forward by the increase of the H$^+$ concentration caused by anaerobic glycolysis. This explains the striking similarity of the time courses of [PCr] and [H$^+$] during anoxia (Fig. 2).

Postanoxic Recovery of [PCr], pH_i, and [free ADP]—The coupling between [PCr] and pH_i is lost upon reoxygenation (Fig. 4). The phosphocreatine concentration increases immediately, but the intracellular pH continues to decline for another 30 min. The return of [PCr] toward control values is much more rapid than that of [ATP] (Figs. 4A, 5B). It is thus obvious that postanoxic PCr/ATP ratios are not mainly determined by [H$^+$]. There is a steep decline of [free ADP] in the muscle of all species after the return of oxygen, probably due to mitochondrial respiration (Fig. 6). The removal of ADP drives the creatine kinase reaction toward net PCr synthesis despite the low pH_i.

In a previous paper (Van den Thillart *et al.*, 1989), we have presented evidence which suggests that the initial drop of pH*i* upon reoxygenation is due to the production of acid equivalents by phosphocreatine synthesis. On the basis of known rates of H^+ and lactic acid efflux, lactate oxidation and gluconeogenesis, we concluded that the rapid recovery of muscle pH toward control values (<2 h in carp and goldfish, <4 h in tilapias, see Fig. 4*B*) is mainly caused by H^+ efflux.

Species Differences during Anoxia and Recovery—The time courses of phosphocreatine and pH*i* during anoxia (Fig. 2) indicate adaptive differences between the three fish species. In goldfish and tilapias, there is a slowdown of the rate of acidification and phosphocreatine utilization after 30–40 min of nitrogen bubbling of the gill perfusion medium. However, such a transition is not observed in the common carp. The observed metabolic pattern in goldfish is known to be due to the occurrence of two different mechanisms: (*a*) a lowering of energy demand to 30% of the aerobic standard metabolic rate (Van Waversveld, 1988; Van Waversveld *et al.*, 1988, 1989), and (*b*) a switch to a modified metabolism, resulting in the conversion of glycogen to ethanol (Shoubridge and Hochochka, 1980; Van den Thillart, 1982; Van den Thillart and Van Waarde, 1989).

The first mechanism causes a slow down of the rate of acidification which is directly proportional to the reduction of energy demand. The switch to a modified metabolism dampens acidosis even further since alcoholic fermentation of carbohydrates is not accompanied by net production of acid equivalents. The presence of both mechanisms in anoxic goldfish causes virtual stabilization of [phosphocreatine] and pH*i* at 20 °C (Fig. 2) and complete stabilization at environmental temperatures ≤15 °C (Van den Thillart and Van Waarde, 1989; Van den Thillart *et al.*, 1989). In contrast to goldfish, anoxic tilapias do not produce ethanol. Mechanism b thus does not operate and the transition to lower rates of muscle acidification (Fig. 2) and stable [free ADP] (Fig. 6) must be solely due to a lowering of energy demand (mechanism a). The absence of any transition in the common carp seems to indicate that this species is unable to lower its anoxic energy demand, at least at the experimental temperature of 20 °C.

When the postanoxic time courses of [free ADP] in the animals are compared, another species difference is apparent (Fig. 6). Cytoplasmic ADP in carp and tilapia returns within 1 h to values below the control level. In contrast, goldfish maintain a rather high ADP concentration ($\sim 30~\mu M$) for 2 h before ADP finally returns to normal. So initially, goldfish muscle mitochondria seem to have a lower affinity for ADP than the mitochondria of carp and tilapia.

Whereas carp and tilapia rely on a classical anaerobic glycolysis to meet their anoxic energy demand, goldfish have a modified metabolism which produces ethanol, CO_2, NH_3, and lipids (Shoubridge and Hochachka, 1980; Van Waarde, 1988; Van Waversveld *et al.*, 1988, 1989). Here, mitochondria play a vital role during anaerobiosis and a modification of the mitochondrial pyruvate dehydrogenase complex is required (Mourik *et al.*, 1982; Van den Thillart and Van Waarde, 1989).

We assume therefore that goldfish mitochondria are modified during anoxia. Upon reoxygenation, there is a lag phase during which vital enzymes like pyruvate dehydrogenase are slowly restored to their aerobic form and the affinity of the mitochondria for ADP is still reduced. In contrast, the mitochondria of carp and tilapia are not altered by anoxic exposure. This explains the observed rapid decline of free ADP in carp and tilapia, as opposed to a slow recovery in goldfish.

Conclusion—The NMR results described in this paper in-dicate a coupling between PCr breakdown and anaerobic glycolysis through the common intermediate, H^+, in the muscle of anoxic fish. This coupling disappears upon reoxygenation because the oxidative phosphorylation reduces the level of free ADP. The removal of ADP drives the creatine kinase equilibrium toward net PCr synthesis despite the low pH*i*. The behavior of free ADP during the initial hours of recovery suggests goldfish mitochondria are modified during an anoxic interval, whereas the mitochondria of carp and tilapia are unaffected.

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