Intracellular pH transients in rainbow trout tissues measured by dimethadione distribution

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MILLIGAN, C. LOUISE, AND CHRIS M. WOOD. *Intracellular pH transients in rainbow trout tissues measured by dimethadione distribution.* Am. J. Physiol. 248 (Regulatory Integrative Comp. Physiol. 17): R668–R673, 1985.—Intracellular pH transients in response to P_{CO_2} changes were measured in vitro with the weak acid dimethadione (DMO) in gas-equilibrated red blood cells and a perfused trunk preparation (white muscle) of the rainbow trout. Red cell intracellular pH (pH_i) was also measured directly on cell lysates. At an extracellular pH (pH_e) of 7.81 ± 0.04, P_{CO_2} = 2 Torr, red cell $pH_{i_{lysate}}$ averaged 7.40 ± 0.02, and $[DMO]_i/[DMO]_e$ averaged 0.37 ± 0.02, corresponding to a mean $pH_{i_{DMO}}$ of 7.39 ± 0.02. Decreasing pH_e to 7.53 ± 0.04 by increasing P_{CO_2} to 8 Torr caused $[DMO]_i/[DMO]_e$ to increase to 0.50 ± 0.03 and resulted in a decline in pH_i to a mean of 7.19 ± 0.03 as measured by both techniques. With both methods red cell pH_i responded rapidly (<5 min) to the P_{CO_2} change, paralleling the response of pH_e. In the isolated perfused trunk preparation at a perfusate pH_e of 7.79 ± 0.04 and P_{CO_2} of 2 Torr, $[DMO]_i/[DMO]_e$ averaged 0.38 ± 0.03, yielding an average white muscle pH_i of 7.35 ± 0.04. Decreasing pH_e to 7.34 ± 0.02 by elevating P_{CO_2} to 15 Torr caused pH_i to drop to a mean of 7.11 ± 0.03, as indicated by the significant increase in $[DMO]_i/[DMO]_e$ to 0.58 ± 0.03. The response of $[DMO]_i/[DMO]_e$ was complete within 15 min. In both preparations the pH_i changes were fully reversible. The DMO distribution method for measuring intracellular pH transients proved to be rapid and reliable in fish tissues.

Salmo gairdneri; erythrocytes; white muscle

USE OF THE WEAK ORGANIC ACID 5,5-dimethyloxazolidine-2,4-dione [dimethadione (DMO)] as an in vitro marker for intracellular (subscript i) pH has been widespread since its introduction by Waddell and Butler (26). The DMO distribution method has been shown, repeatedly, to produce reliable estimates of pH_i under steady-state conditions in various tissues from a wide range of animals (10, 23). However, many interesting aspects of the pH_i regulatory mechanism have been revealed by studying pH_i transients (see Ref. 23 for review). The transients have been measured with pH-sensitive microelectrodes and as a consequence have been studied only in isolated tissue preparations. Such pH_i transients have not been studied in vivo, because the DMO distribution method has not been proven reliable under conditions where pH_i may be changing rapidly.

The DMO technique has been employed to describe pronounced steady-state differences in intracellular acid-base status in fish associated with acclimation temperature (4, 9, 27) and exogenously or endogenously induced hypercapnia (2, 11). However, the pH_i transients associated with these treatments remain unknown, as do those suspected to occur after exercise (12).

The present study was undertaken to assess the reliability of the DMO method for measuring pH_i, and particularly pH_i transients, in fish tissues with specific reference to applicability in vivo. In particular, we wished to evaluate the DMO method for detecting the pH_i transients thought to occur in fish white muscle after exercise, when pH_e drops from ~7.85 to ~7.35 with a concomitant increase in extracellular (subscript e) P_{CO_2} and decrease in extracellular HCO_3^- (e.g., Ref. 12). Two in vitro preparations were employed: rainbow trout whole blood in tonometers and an isolated-perfused trout trunk. The former was used to provide optimal conditions for rapid redistribution of DMO across the intracellular-extracellular interface and to permit independent validation of the DMO distribution estimate of pH_i by direct measurement on red cell lysates. This technique has been extensively used and is considered to yield reliable estimates of erythrocytic pH_i, at least in mammalian blood (1, 23). The isolated perfused-trunk preparation, in contrast, represented a situation more closely resembling the conditions in skeletal muscle in vivo that may be suboptimal for DMO redistribution across the cellular boundary, i.e., where tissue perfusion may be limiting. In both preparations pH_i transients were induced by altering extracellular P_{CO_2}, because CO_2 is known to readily penetrate cell membranes, in contrast to H^+ or HCO_3^- (23).

MATERIALS AND METHODS

Experimental Animals

Adult rainbow trout (*Salmo gairdneri*; 200–600 g) from Spring Valley Trout Farm, Petersburg, Ontario, were held in large (600 liters) fiberglass tanks supplied with a continuous flow of dechlorinated Hamilton tap water (5–18°C; seasonal fluctuations) and fed twice weekly with commercial trout pellets. At least 1 wk before experimentation, fish were acclimated to experimental temperature (15°C), during which period they were starved.

To facilitate collection of blood for in vitro tonometry, 12 fish were cannulated in the dorsal aorta while under MS-222 (1:10,000, Sigma) anaesthesia using a modification of the technique of Smith and Bell (24). Fish were allowed to recover in darkened Plexiglas boxes supplied with a continuous flow of freshwater for 24–48 h.

In Vitro Experiments

Whole blood. Whole blood (25 ml) was drawn from the dorsal aorta cannulas of several fish, pooled, heparinized (5,000 IU/ml Na-heparin, Sigma) and 0.03 μCi/ml ^{14}C-DMO (sp act, 50 mCi/mmol; New England Nuclear) was added. Blood was equilibrated in shaking tonometer flasks (15°C) to a typical resting arterial P_{CO_2} of 2 Torr (11) (balance air) and sampled (600 μl) at 5-min intervals for measurement of pH_e and red cell pH_i. Once these parameters had stabilized (40–75 min), four control measurements were taken over a 1-h period (−60, −45, −30, and 0 min). P_{CO_2} was then increased (*time 0*) to 8 Torr, a typical postexercise arterial value (12), and samples drawn at 5-min intervals for the 1st h, with further samples at 75, 90, and 120 min. In most experiments P_{CO_2} was then returned to 2 Torr to further test reliability of the DMO method, with samples drawn on the same schedule over the next 90 min. Gas mixtures were supplied with Wosthoff gas-mixing pumps.

To determine intra- and extracellular [DMO], several replicates of 80 μl whole blood were centrifuged in Radiometer hematocrit tubes for 5 min at 5,000 *g*. Plasma and red cell pellets were counted separately and corrected for trapped extracellular fluid in the red cell pellet, as described below. Red cell pH_i was also measured directly on red cell lysates.

Trunk preparation. An isolated-perfused trunk preparation very similar to that described by Turner and Wood (25) was employed. Trout were quickly killed by a cephalic blow, the head severed posterior to the cleithrum, and the trunk eviscerated, leaving the kidney intact. The dorsal aorta was cannulated at the cut surface with PE-50 polyethylene tubing (Clay-Adams), through which the trunk was perfused. Inflow pressure was maintained at physiological levels (20–35 cmH_2O) and was monitored using a Narco pressure transducer attached to a Gilson chart recorder. Dorsal aortic flow was maintained at 6.0–7.0 $ml \cdot kg^{-1} \cdot min^{-1}$, using Buchler or Gilson peristaltic pumps and a windkessel to reduce pulse pressure to the normal range (4–8 cmH_2O). Cardiac output in resting fish in vivo has been estimated, with the Fick principle, at ~18 $ml \cdot kg^{-1} \cdot min^{-1}$ (3, 5, 14, 15). The Fick principle, however, neglects gill O_2 consumption and as a result tends to overestimate cardiac output (6). Systemic flow is also somewhat reduced by venous return from the gills (13). In addition, blood flow to the skeletal muscle is estimated at 5–10 $ml \cdot kg^{-1} \cdot min^{-1}$ in trout in vivo (21). Thus the flow rates in the present study appear to be within the physiological range. Temperature was maintained at 15°C.

The perfusion medium consisted of a basic Cortland saline to which 40 g/l polyvinylpyrrilidone (PVP; mol wt 40,000; Sigma), 55 g/l bovine hemoglobin (Sigma), 30 μCi/l ^{14}C-DMO, and 100 μCi/l [^{3}H]mannitol (sp act, 27.4 mCi/mmol; New England Nuclear) were added, the last as an extracellular fluid volume (ECFV) marker. When equilibrated at P_{CO_2} of 2 Torr (balance O_2) this perfusate closely resembled trout whole blood with respect to O_2 content (~2.3 mM/l), acid-base status (pH = ~7.8, $[HCO_3^-]$ = ~5.5 mM/l), and buffer capacity (8–9 $mmol \cdot l^{-1} \cdot pH^{-1}$) (25).

Perfusate (100 μl) was sampled from the arterial sample port ~10 cm from point of entry of the cannula into the trunk. White muscle samples (~100 mg) were taken from the epaxial muscle mass anterior to the dorsal fin by punching biopsy needles through the trunk. Biopsy needles were stainless steel trocars of 4.78 mm ID (Arnold-Nasco, Guelph, Ontario). Samples were immediately frozen in liquid N_2 until they were counted, as described below. In pilot experiments, initial mixing and equilibration time for the markers was determined to be ~90 min, so in all following experiments trunks were perfused for 90 min at a P_{CO_2} of 2 Torr (balance O_2) before experimentation. Control samples for muscle pH_i, total water, ECFV, and perfusate pH (pH_e) were taken at 15-min intervals for the next 30–45 min. The perfusate was then quickly switched by means of a stopcock to one equilibrated to a P_{CO_2} of 15 Torr (balance O_2). A P_{CO_2} of 15 rather than 8 Torr (used in blood studies) was used to ensure that a large pH_i change would occur, because muscle has a greater buffer capacity than blood. At the new perfusate P_{CO_2}, muscle and perfusate samples were taken every 15 min for 90 min. In four additional experiments the protocol was reversed, with initial perfusion at P_{CO_2} of 15 Torr, followed by a decrease to 2 Torr for 60 min.

Analytic Techniques

Samples of perfusate from the isolated perfused trunk were treated as whole blood. Blood pH (pH_e) was measured on 40-μl samples with a Radiometer pH microelectrode maintained at experimental temperature (15°C) and linked to a Radiometer PHM 71 or 72 acid-base analyzer. For measurement of pH_i of red cell lysates, red cell pellets, obtained by centrifuging 400 μl of whole blood at 9,000 *g* for 2 min, were repeatedly frozen and thawed under anaerobic conditions in dry ice-ethanol and water, respectively (30). pH was then measured directly on 40 μl of lysate as described.

For determination of pH_i of epaxial muscle and red blood cells by the DMO distribution method, duplicate samples of perfusate (50 μl) or plasma (50 μl) and muscle (~100 mg) or packed red blood cells (50–100 μl) were digested in 2 ml tissue solubilizer (NCS; Amersham) until a clear solution was obtained (7–10 days). The solution was neutralized with 60 μl glacial acetic acid; then 10 ml fluor (OCS; Amersham) were added. Samples were stored overnight in the dark to reduce chemiluminescence and counted on a Beckman LS-250 scintillation counter. Single- (blood samples) or dual- (perfusate and muscle samples) label quench correlation was performed using the external standard ratio method in conjunction with a series of quench standards prepared from the tissue of interest. Water contents of plasma, red cell pellets, perfusate, and muscle samples were determined by drying duplicate samples to a constant weight at 85°C.

Corrections for trapped ECFV in red cell pellets were determined in a separate series of experiments in which whole blood was equilibrated to P_{CO_2} values of 2 and 8 Torr in the presence of the extracellular marker alone, [^{14}C]mannitol (30 μCi/l; sp act, 50 mCi/mmol). The percentage trapped ECFV, determined by counting sam-

ples of packed red blood cells as described, ranged from 0.8 to 1.5%, depending on hematocrit.

Muscle ECFV, intracellular fluid volume (ICFV), and pH_i and red cell pH_i were calculated from pH_e, pK_{DMO}, and the distribution of [^{3}H]mannitol and ^{14}C-DMO according to standard equations (16).

Statistical Analyses

Means ± SE are reported throughout, unless otherwise stated. Significant differences within each group were tested ($P < 0.05$) with Student's two-tailed t test (paired design). Lines were fitted using the method of least-squares linear regression, and the significance of simple Pearson's correlation coefficients was assessed.

RESULTS

Whole Blood

The four whole blood experiments in which the complete sequence P_{CO_2} = 2, 8, and 2 Torr was imposed represent all six that were performed and are shown in Fig. 1. The hematocrits ranged from 14 to 26%, averaging 18.3 ± 2%. Water contents of plasma and red blood cells at P_{CO_2} of 2 Torr were 962.5 ± 0.4 ml/kg (n = 6) and 664.3 ± 1.1 (n = 5), respectively, and at 8 Torr were 953.5 ± 1.0 ml/kg (n = 6) and 696.3 ± 2.0 (n = 4); this significant change in water distribution reflected red cell swelling at the higher P_{CO_2}.

At P_{CO_2} of 2 Torr, pH_e was 7.81 ± 0.04, and the $[DMO]_i$-to-$[DMO]_e$ ratio averaged 0.37 ± 0.02, corresponding to an average pH_i of 7.39 ± 0.02 (n = 6), which was not different than that measured directly on cell lysates (7.40 ± 0.02) (Fig. 1, *A* and *B*). On increasing P_{CO_2} to 8 Torr, pH_e fell to 7.53 ± 0.04, with the $[DMO]_i$-to-$[DMO]_e$ ratio increasing significantly to 0.50 ± 0.03, resulting in a significant drop in pH_i to 7.19 ± 0.03. Again, red cell pH_i measured directly on cell lysates (7.19 ± 0.02) did not differ significantly. Red cell pH_i measured on cell lysates and by DMO distribution reached a plateau within 15 min of the P_{CO_2} change that remained stable throughout the 120-min equilibration period. On returning P_{CO_2} to 2 Torr, both pH_e and pH_i, as measured by both methods, returned to initial levels within 15 min.

The time courses of pH_e and pH_i changes were identical for changes in both the acid and alkaline direction. At no point during the experimental period, either at a P_{CO_2} of 2 or 8 Torr, did red cell pH_i measured by DMO distribution differ significantly from that measured directly on red cell lysates. Red cell pH_i, as measured by both techniques, responded as quickly to the P_{CO_2} change as pH_e. The limiting factor for all three measurements therefore appeared to be the time for complete gas changeover and equilibration (~15 min); the actual DMO redistribution occurred within ~5 min as shown by the rapid change in $[DMO]_i$-to-$[DMO]_e$ ratio and the correspondence between $pH_{i_{DMO}}$ and $pH_{i_{lysate}}$ values.

The transmembrane distribution ratio for H^+ ($r_{H^+} = [H^+]_e/[H^+]_i$) across the erthrocyte was calculated separately from $pH_{i_{DMO}}$ and $pH_{i_{lysate}}$ values and plotted against pH_e in Fig. 2. The regression lines describing the relationship between pH_e and $r_{H^+_{DMO}}$ ($r_{H^+_{DMO}} = -0.37\ pH_e + 3.26$) and $r_{H^+_{lysate}}$ ($r_{H^+_{lysate}} = -0.36\ pH_e + 3.24$) are not significantly different, with r_{H^+} significantly correlated with pH_e ($r = -0.73$; $P < 0.001$).

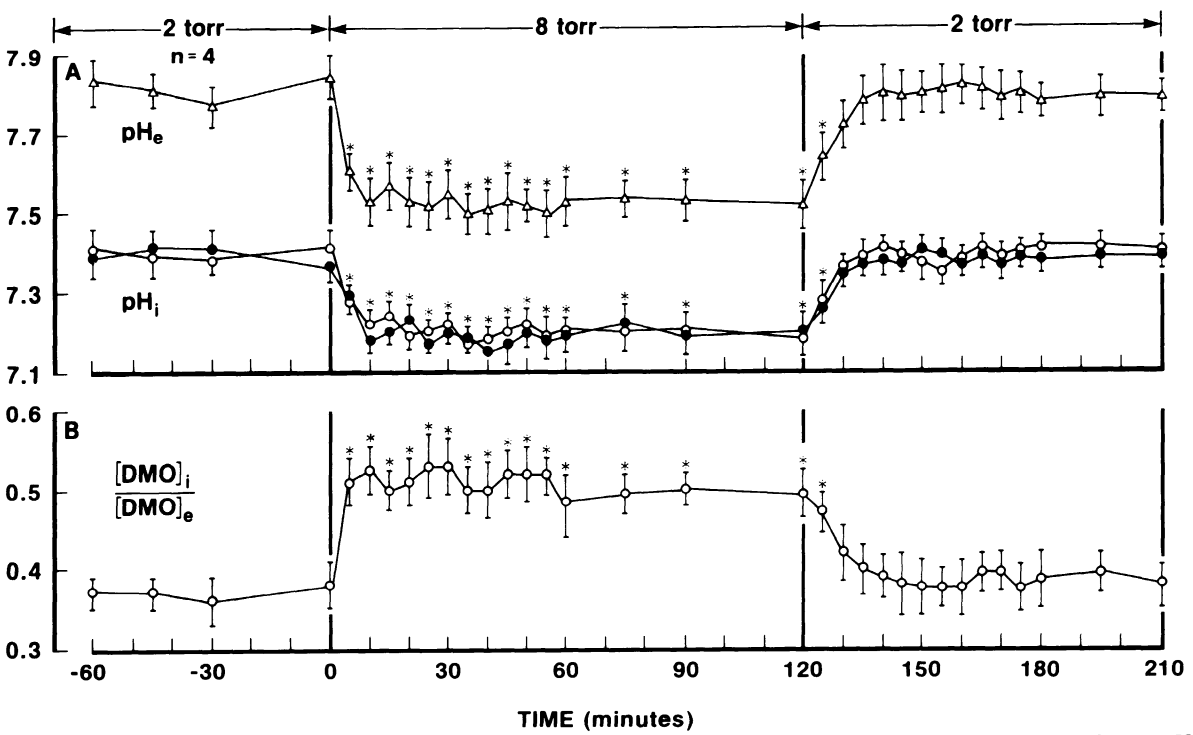

FIG. 1. Response of (*A*) plasma pH_e and red cell pH_i and (*B*) dimethadione (DMO) distribution ratio in whole blood to changes of P_{CO_2} in vitro. *Open triangles*, pH_e; *closed circles*, pH_i measured on cell lysates; *open circles*, pH_i measured by DMO distribution. Values are means ± SE. *Asterisks*, significant difference from initial values at P_{CO_2} of 2 Torr; $P < 0.05$.

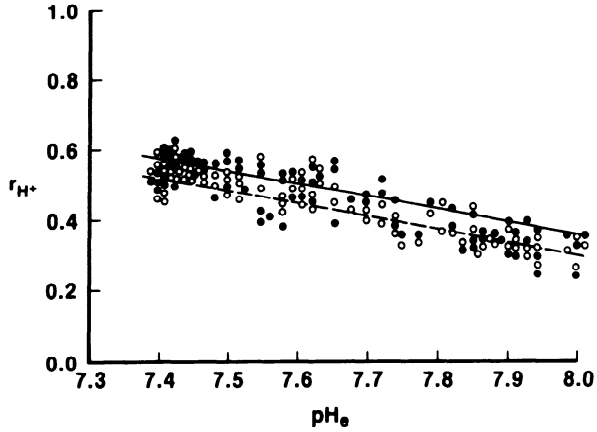

FIG. 2. Relationship between pH_e and transmembrane distribution ratio for H^+ (r_{H^+}) across red cell membrane in blood of rainbow trout in vitro. *Open circles*, r_{H^+} determined from $pH_{i_{DMO}}$; *closed circles*, r_{H^+} determined from $pH_{i_{lysate}}$. Regression lines are $r_{H^+_{DMO}} = -0.37\ pH_e + 3.26$, $r = -0.74$, $n = 88$, $P < 0.001$ (*broken line*); $r_{H^+_{lysate}} = -0.36\ pH_e + 3.24$, $r = -0.73$, $n = 88$, $P < 0.001$ (*solid line*). See text for abbreviations.

Trunk Preparation

In initial experiments duplicate muscle samples were taken throughout for determination of muscle water. However, total muscle water did not change during the perfusion period, so in subsequent experiments muscle samples for total water were taken only at the beginning (P_{CO_2} = 2 Torr; 768.6 ± 5.1 ml/kg; n = 6) and end (P_{CO_2} = 15 Torr; 770.4 ± 3.2 ml/kg; n = 6) of the perfusion period. The average of these two values was used for calculation of muscle ICFV. Muscle ECFV was also stable for the duration of the perfusion period, yielding mean values of 94.1 ± 5.0 ml/kg (n = 8) at 2 Torr and 97.6 ± 3.1 ml/kg (n = 8) at 15 Torr.

After 90 min perfusion at P_{CO_2} of 2 Torr, pH_e 7.79 ± 0.04 (n = 6), the mean DMO distribution ratio for white muscle was 0.38 ± 0.03, corresponding to a mean pH_i of 7.35 ± 0.04 which was not significantly different at 120 min (Fig. 3*A*). Switching to a perfusate equilibrated to a P_{CO_2} of 15 Torr and pH_e of 7.34 ± 0.02 resulted in a rapid significant decrease in muscle pH_i to 7.11 ± 0.03 (Fig. 3*A*). The change in $[DMO]_i/[DMO]_e$ to 0.58 ± 0.03 was complete within 15 min, and this ratio remained unchanged for 60 min (Fig. 3*B*). To further test the reliability of the DMO distribution method, the reciprocal experiment was performed, where trunks were initially perfused with the acidic perfusate (P_{CO_2} = 15 Torr, pH_e = 7.37 ± 0.01; n = 4) then switched to a more alkaline perfusate (P_{CO_2} = 2 Torr, pH_e = 7.81 ± 0.02). Muscle pH_i increased significantly from a mean of 7.00 ± 0.02 to a mean of 7.29 ± 0.03 (Fig. 3*C*). The DMO distribution ratio decreased significantly (Fig. 3*D*), again emphasizing that DMO was indeed redistributing in response to the pH change.

As expected, both white muscle pH_i and red cell pH_i were significantly correlated ($P < 0.01$) with pH_e (Fig. 4, *A* and *B*). The slope of the white muscle pH_i vs. pH_e relationship (0.53) was significantly less than the slope of the red cell pH_i vs. pH_e relationship (0.75), presumably reflecting a difference in buffer capacity of the two cell types.

DISCUSSION

Values of pH_i

In trout blood in vitro, there was good agreement between DMO and direct lysate measurements of erythrocytic pH_i over a wide range of pH_e and pH_i (Figs. 1 and 4*A*), which agrees with numerous studies on mammalian erythrocytes (see Ref. 23 for review) and indicates that in this tissue, DMO provides reliable estimates of pH_i. It must be pointed out, however, that fish erythrocytes are nucleated; thus their intracellular compartment is heterogeneous. Whatever the effect of this heterogeneity on intracellular H^+ distribution, both the cell homogenate and DMO method measure the same pH_i.

The values (7.39 ± 0.02) for red cell pH_i in the present study at pH_e 7.81 agreed well with that (7.37) reported for trout erythrocytes in saline at the same pH_e (20). The observed regression relationship between r_{H^+} and pH_e (Fig. 2) is very similar to that described for $r_{HCO_3^-}$ in trout erythrocytes (29) and r_{Cl^-} and $r_{HCO_3^-}$ in human

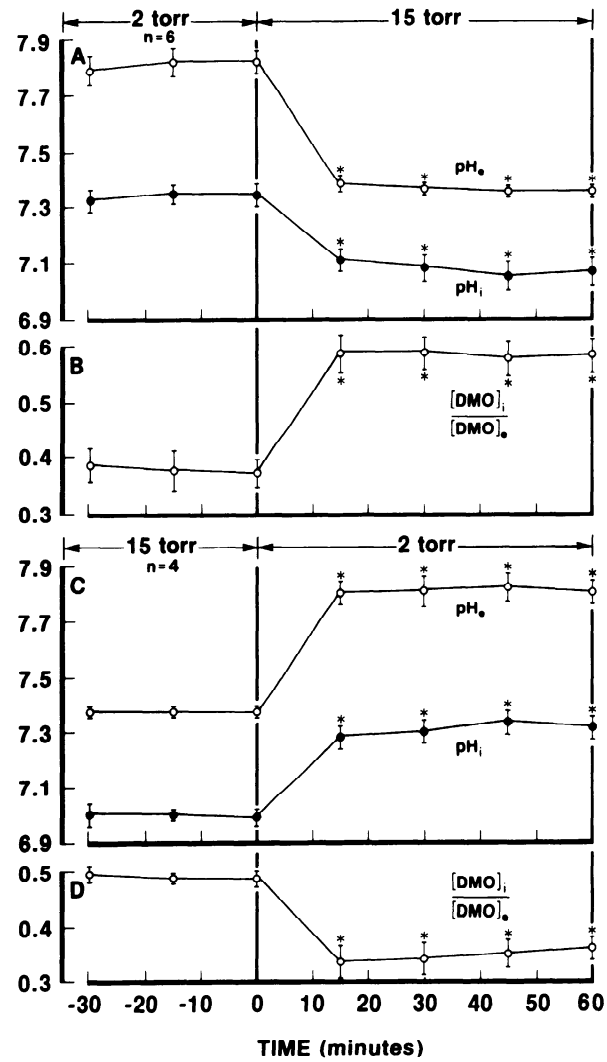

FIG. 3. Response of pH_e, $[DMO]_i/[DMO]_e$, and white muscle pH_i in perfused trunk preparation to step changes in P_{CO_2} from 2 to 15 Torr (n = 6; *A* and *B*) and from 15 to 2 Torr (n = 4; *C* and *D*). *Asterisk*, significant difference from values at *time 0* ($P < 0.05$). DMO, dimethadione.

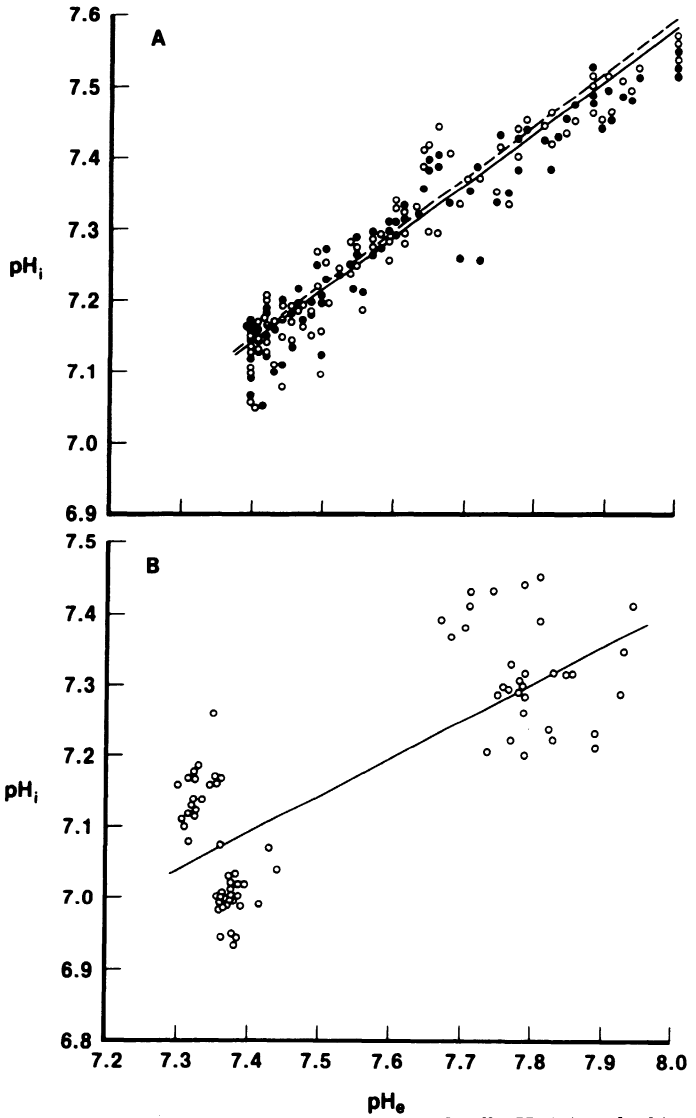

FIG. 4. Relationship between pH_e and red cell pH_i (*A*) and white muscle pH_i (*B*). *Open circles*, $pH_{i_{DMO}}$; *closed circles*, $pH_{i_{lysate}}$. Regression lines are red cell $pH_{i_{DMO}} = 0.75\ pH_e + 1.59$, $r = 0.86$, $n = 88$, $P < 0.001$ (*broken lines*); red cell $pH_{i_{lysate}} = 0.73\ pH_e + 1.74$, $r = 0.90$, $n = 88$, $P < 0.001$ (*solid line*); muscle $pH_i = 0.53\ pH_e + 3.00$, $n = 68$, $r = 0.75$, $P < 0.001$.

erythrocytes (1, 7). In the latter, this relationship is thought to reflect a passive Gibbs-Donnan distribution of these species. The linear relationship between r_{Cl^-} and pH_e observed by Haswell et al. (8) in tilapia blood also suggests that this is true of fish erythrocytes. However, definitive proof of Gibbs-Donnan distribution awaits simultaneous determination of the membrane potential.

Once P_{CO_2} was changed, red cell pH_i changed in concert with pH_e and then was stable over time in the present study, as indicated by the constancy in the $[DMO]_i$-to-$[DMO]_e$ ratio. This suggests that apart from the pH_i regulation attributable to passive physicochemical buffering, trout erythrocytes in vitro, under the particular conditions of these experiments, were unable to regulate pH_i back toward control values. However, recent evidence suggests that in vivo catecholamine mobilization during postexercise acidosis permits pH_i regulation by net H^+ extrusion (20).

At a perfusate P_{CO_2} of 2 Torr and pH_e 7.79, typical resting arterial levels in fish, values for white muscle pH_i from the perfused trunk (7.34 ± 0.02) are in good agreement with those measured in vivo at 15°C with the DMO distribution method in trout [7.30–7.32 (11)], eels [7.34 (27)], and channel catfish [7.36 (4)].

The only pH_i regulation observed during high P_{CO_2} perfusion of the trunk was that attributable to passive physicochemical buffering reflected in the decreased $pH_e - pH_i$ gradient (Fig. 3*A*). This apparent lack of recovery of muscle pH_i over the 90-min perfusion period may have been due to an inhibition of the pH_i regulatory mechanism(s) caused by the persistent depression of pH_e (see Ref. 23). It is also possible that some rapid recovery occurred within the first 15 min of high P_{CO_2} so that the observed values are "partially recovered." However, in intact fish in vivo, recovery of both pH_e and pH_i in the face of high blood P_{CO_2} are lengthy processes with time courses well beyond the present experimental period (2, 11).

pH_i Transients

The results from both the whole blood and perfused trunk experiments demonstrated that the DMO distribution method could detect pH_i transients within 15 min of the extracellular P_{CO_2} change as indicated by the rapid and significant changes in$[DMO]_i/[DMO]_e$. It therefore should be capable, for example, of detecting the pH_i changes suspected to occur over such a time course after exhaustive exercise in fish (12). Traditionally the DMO distribution method has not been used to measure pH_i transients, because it was thought that DMO equilibration time was limiting (22, 23). There is, however, some ambiguity over the interpretation of equilibration time. The initial equilibration period, i.e., the time requirement for convective mixing, can indeed be quite lengthy; 4–8 h in fish (4, 11, 27) and 1–2 h in humans(17). Indeed the mixing time for mannitol alone throughout the ECFV in intact trout is ~3 h (18). The in vitro trunk preparation of the present study required 90 min for initial equilibration of markers. However, once DMO had equilibrated throughout the system, redistribution of DMO across the intracellular-extracellular interface occurred quickly, as indicated by the rapid response of the DMO distribution ratio to changes in P_{CO_2} observed in both whole blood and muscle (Figs. 1 and 3). Similarly, in isolated eel hepatocytes (28) and isolated barnacle muscle fibers (10), DMO diffusion across the cell membrane was quite rapid (<15 min).

In a study of isolated rat diaphragms, Roos and Boron (23) successfully demonstrated pH_i transients with the DMO technique and furthermore showed that their results agreed reasonably well with a similar study using pH microelectrodes. The temporal resolution of the transients was 30 min, which may reflect the slower nature of the pH_i change compared with the present study. In addition, DMO equilibration may have been limited by lack of perfusion as the extracellular fluid was bathing, rather than perfusing, the diaphragm.

The DMO technique was also successfully employed

in isolated perfused rat hearts to detect pH_i transients in response to ischemia and anoxia (19). These authors could detect pH_i changes within 5 min of ischemia, the faster resolution probably reflecting the faster pH_i change.

In conclusion, the DMO technique appears suitable for measurement of pH_i transients over ~15 min in fish tissues in vivo, provided the markers have already equilibrated throughout the system.

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