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Determination of intracellular pH and P_{CO_2} after metabolic inhibition by fluoride and nitrilotriacetic acid

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Abstract. Mean intracellular pH (pHi) and P_{CO_2} (Pi_{CO_2}) have been analysed based on pH and total CO_2 measurements in tissue homogenates. Tissues were sampled from undisturbed worms (*Sipunculus nudus*), squid (*Illex illecebrosus*), trout (*Salmo gairdneri*), toads (*Bufo marinus*), and rats. Homogenate metabolism was inhibited by the addition of potassium fluoride and nitrilotriacetic acid (NTA). Model calculations revealed that the influence of dilution, medium buffers, and contamination by extracellular fluids was negligible. In white muscle tissue the resulting pHi values were virtually the same as found in studies using DMO (dimethyloxazolidinedione). If large fractions of mitochondria were present (e.g. in heart muscle), DMO derived pHi values were considerably higher, probably representing overestimates. Homogenate derived pHi values are concluded to represent the effective mean pHi by taking into account pH gradients, and the volumes and buffering of cellular compartments. High time resolution and small variability make this method especially useful to assess rapid changes in pHi, e.g. in exercising animals.

Animal, rat, sipunculid, squid, toad, trout; Buffer value, heart, muscle; Drug, fluoride, nitrilotriacetic acid; Heart, buffer value; Muscle, buffer values; pH, intracellular

For a quantitative analysis of acid–base regulation in intact tissues or animals changes in mean intracellular pH must be determined. Among the available techniques, ^{31}P-NMR seems suitable for monitoring changes in pHi and organic and inorganic phosphates in isolated tissues or exposed organs but is only rarely applicable to the living unrestrained organism since the animal must remain motionless. Therefore, pHi is most frequently analysed by the use of a weak acid, DMO (5,5-dimethyl-2,4-oxazolidinedione). More rarely, weak bases like methylamine are used. These substances are distributed between intra- and extracellular compartments according to the pH difference (see Roos and Boron, 1981, for review). Accurate measurement of

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extracellular pH, which, in the ideal case, is performed in arterial and venous blood close to the respective tissue, is required for the calculation of intracellular pH from weak acid or base distribution. The basis of these techniques and the meaning of the calculations in terms of their accuracy have been discussed, especially for DMO (e.g. Roos and Boron, 1981). DMO requires time to reach equilibrium and, as a corollary, this method may generally be inadequate to follow rapid pHi changes, e.g. during anaerobic exercise.

For this reason pH has frequently been measured in tissue homogenates, predominantly of human muscle, in order to yield approximate data for pH changes during activity. Uncertainties about the influence of metabolism on the measured pH have, so far, only led to an empirical procedure of pH analysis which is certainly not suitable for all tissues (cf. Costill *et al.*, 1982; Spriet *et al.*, 1986). The present study tries to improve on these drawbacks. ATP dependent metabolism, which is assumed to include most, if not all, pH relevant reactions in the homogenate, is inhibited by the removal of magnesium and calcium ions. Since disturbances of the measurements by homogenate metabolism are expected to be largest in tissues from control animals, the present study focuses on the analysis of pHi values in tissues excised from resting animals of different species. The validity of the resulting values is tested by model calculations, by an analysis of homogenate metabolism, and by comparison to pHi values obtained in studies using DMO.

Based on total CO_2 measurements in tissue homogenates mean intracellular P_{CO_2} can also be evaluated. If mean intracellular pH is analysed and appropriate values for pK and CO_2 solubility are selected (Heisler, 1986), intracellular P_{CO_2} values can then be calculated.

Materials and methods

Animals. Worms (*Sipunculus nudus*, 5–15 g) were dug out on intertidal flats of Brittany, France. They were kept in Düsseldorf for several weeks in an aquarium containing a bottom layer of sand (10 to 20 cm) from the worms' original habitat. The tanks were circulated with well aerated artificial sea water at 12 to 15 °C. Animals were allowed to recover from the transport for at least 2 weeks before being used in the experiments.

Squid (*Illex illecebrosus*, 300–500 g) were caught by commercial fishermen in St. Margaret's Bay or close to Herring Cove, Nova Scotia during October through December of 1986. When obtained from the fishermen the animals were placed in plastic bags filled with oxygenated sea water at 2–6 °C for transportation to Halifax. There they were kept in running (ambient) sea water 8–15 °C. At ambient water temperatures close to 15 °C they were used, as soon as they were recovered (after 2–4 h). Otherwise, the animals were brought close to the experimental temperature for 12 to 24 h before being used.

Rainbow trout (*Salmo gairdneri*, 220–340 g, delivered by Merlin Fish Farms, Wentworth, N.S.) were maintained at 15 °C in 4 m^3 fibreglass tanks under running fresh water for at least 4 weeks before being used in the experiments.

Toads (*Bufo marinus*, 200-500 g) were obtained from a commercial dealer (Sullivan Inc., Nashville, TN, USA). After shipment they were kept at room temperature in boxes with a bottom layer of sand and were provided with ample water and food for at least one week. The animals were used after a period of starvation of at least 24 h.

Anaesthesia and sampling procedure. Sipunculid worms were brought into darkened tanks containing normoxic artificial sea water (salinity 34‰, 15 ± 0.5 °C) but no sand. Exclusion of light was found to minimize activity. After 24 h control animals were removed and dissected as quickly as possible. The musculature (body wall and introvert retractor muscles, introvert excluded, *cf.* Pörtner, 1987b) was freeze-clamped immediately (Wollenberger *et al.*, 1960), wrapped in aluminium foil and stored under liquid nitrogen until analysed. Sipunculids were not anaesthetised since their phosphagen and other metabolite concentrations were close to those of resting muscle without anaesthesia (Pörtner, 1987b).

Squid, some of them being permanently cannulated in the *vena cava*, were brought into a respirometer which was filled with approximately 100 L of sea water at 15 ± 0.5 °C. With continuous overflow, the water was circulated through the system at approximately 0.1 m sec^{-1}. After about 1.5 h of recovery from handling a blood sample was taken, the respirometer closed and the animal anaesthetized by adding 2 L of pure ethanol to the water circulation downstream of the animal. Full anaesthesia (indicated by the cessation of ventilation) was reached after 3 to 5 min. The animal was thereafter removed from the respirometer and quickly decapitated. A piece of muscle (6 to 10 cm long) was immediately excised from the left or right ventral mantle using two scalpel blades arranged in parallel at a distance of 11 mm. An aluminium ruler, 2.5 cm wide, was inserted into the mantle cavity and used as a counterpart during the excision. The muscle sample was freeze-clamped and treated as described above.

Twenty-four hours before tissue sampling, individual rainbow trout were brought into darkened 2.8 L Lucite flux boxes of the design described by McDonald (1983). A continuous fresh water flow (15 ± 0.2 °C) was maintained through the animal chambers by aeration of the surrounding system. Then the flux box was closed and a concentrated solution of MS 222 (Sigma, St. Louis, MO, U.S.A.) in fresh water (neutralized by the addition of $NaHCO_3$) was slowly introduced to a final concentration of 0.2 g · L^{-1}. In some cases, addition of the anaesthetic provoked a slight increase in swimming activity but no severe struggling. Fish lost control of balance after 1 to 2 min and were usually fully anaesthetized after 3 to 4 min. A sample of white epaxial muscle was then immediately excised from beside the spine starting at the middle of the dorsal fin and cutting 3 to 4 cm backward. The excision was done before the animal was decapitated and killed, in order to avoid any muscle contraction. Muscle samples were freeze clamped as described above.

Toads, cannulae placed in the right ischiadic artery (Boutilier *et al.*, 1979) and the left femoral vein, were put into darkened 4 L flasks containing 400 ml of dechlorinated tap water. Air, saturated with water at 20 ± 0.1 °C, was fed into the animal chambers. At the end of a 24 h control period blood samples were taken via the indwelling catheters.

The water was replaced with a pH-neutralized solution of MS 222 ($3\,g \cdot L^{-1}$) in dechlorinated tap water. After about 10 min the flask was opened and the immobilized animal was removed. If the animal proved to be fully anaesthetized, the gastrocnemius muscle of the left leg, both sartorius muscles, ventricle and kidneys were excised and freeze-clamped, and the animal was pithed. Blood frozen on the surface of the ventricle could easily be removed after freeze-clamping.

The hearts of 250–300 g male Wistar rats, anaesthetized by urethane ($1.5\,g \cdot kg^{-1}$ body weight), were isolated and perfused according to the technique of Langendorff as described by Rösen and Reinauer (1984).Perfusion medium was Krebs-Henseleit bicarbonate buffer equilibrated with 95% O_2/5% CO_2, pH 7.4. Venous effluent pH was 0.02 pH units below arterial pH (P. Rösen, pers. comm.). At the end of 15 min of perfusion the hearts were freeze-clamped and kept under liquid nitrogen until analysed.

Blood samples from toads and squid were withdrawn anaerobically and analysed for pH and P_{CO_2} using Radiometer (Copenhagen) equipment (BMS 3, thermostatted to the ambient temperature of the animals, $\pm 0.1\,°C$). Plasma bicarbonate levels were calculated using appropriate values for pK''' and CO_2 solubility (Heisler, 1986).

Preparation of homogenates and pH analysis. All tissue samples were ground under liquid nitrogen using mortar and pestle. During grinding, skin fragments were removed from trout muscle. Repeated sampling was possible from tissue powder in bags folded from aluminium foil which were kept on liquid nitrogen in a closed Dewar flask. Exposure of tissue samples and powder to air was minimized in order to exclude water and CO_2 condensation as well as CO_2 release. In order to test whether pH values were affected by these processes grinding and subsequent handling of the tissue powder of *Sipunculus nudus* was performed with and without use of a glove bag (Instruments for Research and Industry, Cheltenham, USA) purged with pure nitrogen gas.

For the measurement of pH and total CO_2 aliquots of the powder were resuspended in ice-cold media containing KF and NTA (nitrilotriacetic acid). KF concentrations were adjusted to the intracellular free potassium levels. Sodium and chloride turn out to be present close to intracellular activities, as a result of ions from the extracellular compartments and the application of the disodium salt of nitrilotriacetic acid. The latter was added to the medium up to a level sufficiently high to minimize metabolic changes in the homogenate, but far below that of KF in order to prevent acidification of the homogenate by proton release during Mg^{2+} or Ca^{2+} binding (table 1). The pH of the respective medium was selected below the expected range of pHi values in order to minimize the CO_2 content and, thus, the disturbing influence of medium buffers. The maximum pH difference between tissue and medium was 0.5 pH units (see table 2 for an estimate of the introduced error). Stock reagents were kept in polyethylene flasks to prevent contamination with ions originating from fluoride action on glassware.

Frozen tissue powder (100–200 mg) was added to 0.2 ml of ice-cold medium in 0.5 ml Eppendorf tubes (preweighed for CO_2 analysis). After determination of the wet weight (necessary only for CO_2 analysis) the Eppendorf tube was filled with medium, the mixture stirred briefly with a needle to release bubbles and the tube capped. After brief

TABLE 1

Composition of media ($mmol \cdot L^{-1}$) utilized for the analysis of intracellular pH in homogenates of tissues originating from vertebrate and invertebrate species. Note the lower levels of nitrilotriacetic acid (NTA) in media used for the marine invertebrates *Illex illecebrosus* and *Sipunculus nudus*.

Species	KF	Na_2NTA
Sipunculus nudus	160	1
Illex illecebrosus	160	2.9
Salmo gairdneri	150	6
Bufo marinus	130	5
Rattus	150	5

mixing on a Vortex mixer (Scientific Industries Inc., Bohemia, NY, U.S.A) (and weighing of the added volume of medium, if necessary), the insoluble fraction of the homogenate (which contains a large fraction of the ATPases) was spun down (for 15 sec maximum in an Eppendorf centrifuge). Aliquots of the supernatant were taken for repeated measurements of pH in a capillary pH electrode (Radiometer, Copenhagen G299A or E5021) thermostatted to the ambient temperature of the animal (± 0.1 °C) and calibrated with precision phosphate buffers (Radiometer, Copenhagen). For reproducible pH measurements, pH electrodes with a fast response in weakly buffered solutions were selected and preconditioned with medium or a KCl solution of similar ionic strength. After an initial sample, replicates were rapidly taken from above the pellet, the Eppendorf tube always being recapped and kept on ice, in order to minimize the pH changes produced by CO_2 loss. Variations in pH between replicates usually ranged below ± 0.005 pH units in samples from the same homogenate and below ± 0.010 pH units in different homogenates from the same muscle powder.

The measurement of total CO_2 in 50 μl aliquots from at least two homogenates prepared from the same muscle powder was performed using the gas chromatographic method outlined by Lenfant and Aucutt (1966) as modified by Boutilier *et al.* (1985). Total CO_2 of the homogenate aliquot was transformed into CO_2 by injection into a 5 ml Hamilton syringe containing 2 ml 0.01 N HCl (preequilibrated with pure N_2), the residue being N_2. Gas and liquid phases were equilibrated by repeated shaking of the closed syringe for at least 2 min. The gas phase was injected through a drying filter [$Mg(CLO_4)_2$] into a 1 ml sample loop of the gas chromatograph (Hach Carle Series 100 equipped with a thermal conductivity detector, sample and reference column packed with Porapack Q, carrier gas: helium, 28 $ml \cdot min^{-1}$, column temperature 30 °C). Calibration was performed by using 5 $mmol \cdot L^{-1}$ bicarbonate standard solutions subjected to the same procedure. CO_2 readings were corrected for the CO_2 contamination of reagents and syringes and for the CO_2 content of the fractional volume of medium in the homogenate.

Arginine phosphate, arginine, and octopine in muscle tissue and homogenate supernatants were measured as described by Pörtner (1990).

TABLE 2

Influence (ΔpHi) of buffering and pH in media (equilibrated with air) used for pHi analysis, on the pH found in tissue homogenates (at 15 °C, dilution factor = 8). The system is closed for CO_2. The influence of both HF/F^- and CO_2/bicarbonate is calculated according to eqs. 8,9 and 6. It can be minimized by selecting a medium pH below tissue pH. Readjusting the pH of the medium close to intracellular pH is not usually required (for apparent pK values see Pörtner, 1990; assumed tissue parameters: pH = 7.3, β_{NB} = 17.7, β_{CO_2} = 0.8, β_{tot} = 18.5 mmol · pH^{-1} · kg^{-1} wet weight).

	Medium (KF, 0.16 mol · L^{-1}; NTA, 2.9 mmol · L^{-1})	
pH	6.8	7.8
C_{CO_2}	0.06	0.46
ΔpHi_{HF}	− 0.0055	+ 0.0017
ΔpHi_{CO_2}	− 0.0032	+ 0.0090
ΔpHi	− 0.0087	+ 0.0107

Calculations. The influence exerted by the medium on homogenate pH depends on the relative magnitude of the buffering in the medium and the tissue (table 2). The effect of dilution on pH only becomes significant if, in a buffer solution, the concentration of buffer components falls below a value of 100 · K_a'. If, in a simplified model, β_{tot} is assumed to be 0.020 mol · pH^{-1} · kg^{-1} equivalent to 0.0269 mol · pH^{-1} · L^{-1} tissue water (cf. Pörtner, 1990), a minimal concentration C_{min} of buffering groups and substances can be evaluated as

$$C_{min} = \beta/0.575 \ (\text{mol} \cdot L^{-1}, \text{van Slyke, 1922}) \tag{1}$$

valid with a pK_a' value of 7.3 at pH 7.3 (C_{min} = 0.0468 mol · L^{-1} tissue water). Considering a dilution factor of 10 in the dissociation equilibrium

$$K_a' = \frac{a_{H^+} \cdot [A^-]}{[HA]} = \frac{y \cdot (0.00234 + y)}{(0.00234 - y)} = 10^{-7.3} \tag{2}$$

$$y = a_{H^+} = 10^{-7.3} = 5.0 \cdot 10^{-8} = K_a'$$

leads to the conclusion that the level of buffer substances is large as compared to the potential effect of dilution on a_{H^+} ($[A^-]/y = [HA]/y = 46\,689 \gg 100$). The change in the dissociation equilibrium and, thus, the effect on pH is negligible ($\Delta pH_{i-j} < 0.0001$).

A major problem for estimating intracellular pH from pH measurements in tissue homogenates arises from contamination by the extracellular fluid of the tissue. If the intracellular space is treated as one compartment, the resulting change in pH (ΔpH−j) can be quantified for each extracellular subcompartment j by calculating a theoretical amount of base equivalents (ΔH^+j−t <0) to be transferred from the respective extracellular compartment j into the mixture of tissue water. The extracellular space may

comprise two compartments in animals without blood cells or even three or more compartments in animals possessing blood cells. The calculation (eqs. 3–9) is based on differences in pHj and measured tissue pHtw and on extra- and intracellular buffering.

During homogenisation the CO_2/bicarbonate system acts like any other buffer component, since the whole system remains closed for CO_2 between the intact subcompartments of the frozen tissue and the homogenate mixture. Therefore, the buffer equation by van Slyke (1922) applies leading to a CO_2 buffer value (β_{CO_2}) for each subcompartment j (at the measured pHtw) and a total buffer value βj_{tot}. In the intracellular spaces of the tissue and the erythrocytes β_{CO_2} is small as compared to β_{NB} (< 5%). Therefore, the following treatment, valid with a linear relationship between ΔpH and ΔH^+ owing to pH-independent buffer values, is possible with negligible error:

$$\Delta pHj\text{–}tw = pHj - pHtw \quad (3)$$
$$\beta j_{tot} = \beta j_{NB} + \beta j_{CO_2}\ (mmol \cdot pH^{-1} \cdot L^{-1}\ jw) \quad (4)$$
$$\Delta H^+ j\text{–}tw = -|\beta j_{tot}| \cdot \Delta pHj \cdot Fjw\ (mmol \cdot L^{-1}\ tw) \quad (5)$$
$$\Delta pHi\text{–}j = \Delta H^+ j\text{–}tw / -|\beta cw_{tot}| \cdot Fcw \quad (6)$$
$$pHi = pHtw + \Delta pHi\text{–}j \quad (7)$$

cw: cell water, tw: tissue water, jw: water of compartment j
Fj: fraction of jw in the tissue water

In the interstitial fluid and plasma, however, β_{CO_2} becomes large as compared to β_{NB}. Since β_{CO_2} varies with pH, buffering has to be analysed as a non-linear process. The transfer of protons (or base) from each of these subcompartments into the mixture of tissue water is calculated by analysing the change in dissociation equilibrium of CO_2 between compartment j and the tissue water according to

$$pHj = pK''' + \log \frac{Fj_{Bic}}{1 - Fj_{Bic}}; \quad pHtw = pK''' + \log \frac{Ftw_{Bic}}{1 - Ftw_{Bic}} \quad (8)$$

$$\Delta H^+ j\text{–}tw_{CO_2} = -(Fj_{Bic} - Ftw_{Bic}) \cdot Cj_{CO_2} \cdot Fj / \sum Fj\ (mmol\ L^{-1}\ tw) \quad (9)$$

where F_{Bic} is the fractional value of bicarbonate in the dissociation equilibrium. The contribution of Cj_{CO_2} to the final tissue pH ($\Delta H^+ j\text{–}tw_{CO_2}$) depends on the difference of F_{Bic} in j and the homogenate mixture of tissue water and on the quantity of Cj_{CO_2} released into this mixture. The deviation from pHi caused by extracellular CO_2/bicarbonate buffering can be calculated according to eq. (6), since intracellular buffering is assumed to be linear (see above). The effect of non-bicarbonate buffering by plasma and interstitial fluid is analysed according to eqs. (3,5–7) using β_{NB} instead of β_{tot} in eq. (5).

pH values may be lower in interstitial fluid than in venous blood owing to respiratory acidification (*cf.* fig. 1). A mean difference of 0.1 pH units between values in plasma and interstitium is accepted to be valid for control conditions (for results of all of these calculations see table 5).

Total CO_2 was calculated for the tissue water by using eq. 10:

$$Ctw_{CO_2} = Ch_{CO_2} \cdot \frac{VM + (FW \cdot Ftw)}{FW \cdot Ftw} \; (mmol \cdot L^{-1}\, tw) \tag{10}$$

FW : fresh weight (mg)
Ch_{CO_2} : total CO_2 in the homogenate
Ftw : fraction of water in tissue (tw: tissue water)
M : medium
V : volume (μl)

For the calculation of cell water total CO_2 in toad tissues [eq. (11)] the high (*i.e.* contaminating) CO_2 levels present in the venous blood and interstitial fluid of the muscle had to be taken into account. Owing to free exchange between these two extracellular fluids, levels of total CO_2 higher in the interstitial fluid than in the plasma are considered to be determined only by the Donnan factor (assumed to be 1.05 as in mammalian muscle, fig. 1). This correction procedure was not necessary for squid mantle muscle, due to the small difference between intracellular and extracellular pH values and the minor error involved:

$$Ccw_{CO_2} = \frac{Ctw_{CO_2} - Q \cdot Cew_{CO_2}}{1 - Q} \tag{11}$$

cw: cell water
ew: extracellular water (interstitial fluid + plasma)
Q : fraction of extracellular water in tissue water (for toad tissues adopted from Boutilier *et al.*, 1987).

Measured and calculated values of pHi and intracellular C_{CO_2} were used to evaluate mean intracellular P_{CO_2} based on eq. (12):

$$P_{CO_2} = C_{CO_2} / (10^{pH - pK'''} \cdot \alpha + \alpha) \tag{12}$$

pK''' and α values were calculated according to Heisler (1986). The validity of these values was tested in an experimental analysis for squid mantle muscle. For this purpose pH and total CO_2 were analysed in homogenates prepared in 0.16 $mol \cdot L^{-1}$ KF and 2.9 $mmol \cdot L^{-1}$ NTA and equilibrated with varying CO_2 tensions by tonometry (cf. Pörtner, 1990). Applying calculated values of α, apparent pK''' values were evaluated from the Henderson-Hasselbalch equation. Calculations for the cell water were performed assuming I = 0.13 $mol \cdot L^{-1}$ (toad) or 0.16 $mol \cdot L^{-1}$ (squid), $[Na^+]$ = 0.01 to 0.02 $mol \cdot L^{-1}$, [Protein] = 180 to 220 $g \cdot L^{-1}$ for the calculation of pK''', and assuming $[M]$ = 0.560 $mol \cdot L^{-1}$ for squid and $[M]$ = 0.210 $mol \cdot L^{-1}$ for toad as molarity of dissolved species for the calculation of CO_2 solubility (based on the analysis

of intracellular ion levels by Robertson, 1965 for cephalopod muscle, Boutilier *et al.*, 1986 for amphibian muscle).

Results and Discussion

Methodology. In any tissue the interrelationship between the function of ATPases (ATP consumption) and kinases (ATP production) depends not only on the presence of ATP but also of Mg^{2+} as a cofactor. Moreover, an acceleration of ATP turnover by myosin ATPase in muscle tissue is triggered by the release of Ca^{2+} from the sarcoplasmic reticulum. During homogenisation, destruction of tissue and cell compartments occurs and metabolic equilibria are displaced by dilution. Oxidative phosphorylation is likely to be uncoupled and aerobic ATP regeneration ceases. ATP consumption increases in muscle tissue homogenates. This is linked to the utilization of anaerobic metabolism, which predominantly includes a rapid depletion of the phosphagen, utilization of the glycolytic pathway and finally a depletion of ATP and an accumulation of ADP, AMP and further degradation products (table 3).

Since H^+ ions are usually both released and consumed in homogenate metabolism (table 3), recording of pH in tissue homogenates is not sufficient to correct for metabolic changes. When pH measurements were first tried in muscle homogenates prepared with water or physiological saline, the rapid and variable drift in pH readings did not allow to extrapolate back to tissue pH. Generally, if no control of metabolic pathways and ATPase activities is achieved, pH and buffer values (see Pörtner, 1990) measured in tissue homogenates cannot be regarded as representing true values. Moreover, the time course of metabolic changes is likely to be different in tissue homogenates from control and experimental animals.

Arrest of metabolism was attempted by adding Mg^{2+} and Ca^{2+} binding substances to the homogenisation medium. Removal of these ions is expected to inhibit both ATPases and kinases and to exclude the stimulation of myosin ATPase. Since both ATP synthesis and consumption are inhibited, those processes in aerobic and anaerobic

TABLE 3

Metabolic processes influencing the acid–base parameters (pH, C_{CO_2} = CO_2 content, β_{NB} = non-bicarbonate buffer value) in tissue homogenates under a closed system (based on Pörtner, 1987a; ↑ positive change, ↓ negative change). Processes are listed as they chronologically occur in the homogenate.

Process	Effect
Phosphagen breakdown	pH ↑, β_{NB} ↑
Anaerobic glycolysis	pH ↓
Depletion of ATP, ADP, or AMP	pH ↓, β_{NB} ↑
Deamination of AMP, adenosine	pH ↑
Oxidative decarboxylation	pH ↓ or ↑, C_{CO_2} ↑

metabolism are cut down, which show a high proton turnover. This includes any pathway, which depends on the use or formation of ATP, especially the glycolytic pathway, phosphagen depletion or resynthesis, and the citric acid cycle (for review see Pörtner, 1987a).

Methodological procedure: pH analysis. KF and nitrilotriacetic acid were selected, since both substances show minimal buffering in the range of cell pH (table 2). With maintenance of ionic strength, approximate adjustment of medium pH, and adequate dilution factors, the influence of dilution or of medium buffers on the analysed pHi values can be kept negligible.

The accuracy of pH measurements in homogenates proved to depend strongly upon the following factors: (1) sufficient inhibition of metabolism; (2) minimization of NTA levels; (3) minimization of CO_2 contamination or loss.

(1) As evidenced by the minimization of metabolic changes (table 4) and the agreement of DMO and homogenate derived pHi values in white muscle tissue (table 6) inhibition of metabolism is sufficient in media containing potassium fluoride and nitrilotriacetic acid. Very slow changes in pH, which may occur in the supernatant at the measurement temperature, do not affect the accurate analysis, which is reproducible in aliquots repeatedly taken from the supernatant kept on ice. NTA levels can be adjusted to allow for a period which is sufficiently long for the pH measurement to take place. In the species analysed, it was found not to be necessary to add another substance for maximizing Mg^{2+} and Ca^{2+} binding (*e.g.* sulphoxine).

(2) At neutral pH, one acid group of NTA releases protons during the chelation reaction. This proton release is minimized by the addition of excess fluoride, which precipitates* Mg^{2+} and Ca^{2+}. Nevertheless, the concentration of nitrilotriacetic acid must be kept low, in order to avoid any possible disturbance of pH by this process. This is a problem especially acute in marine invertebrates (*Illex illecebrosus, Sipunculus nudus*) where homogenate levels of calcium and magnesium are high owing to contamination with high extracellular levels of these ions. For measuring pHi in these animals, NTA levels had to be reduced below those applicable to vertebrate tissues (table 1). The very slow drift in pH or the minor changes in metabolite levels (table 4), still, do not influence the accuracy of the analysis.

(3) The contamination with CO_2 during grinding had to be considered as pointed out in an early paper by Ponten and Siesjö (1964). These authors found a 4.9% increase in the total CO_2 content of 40–140 mg brain tissue powder under liquid nitrogen during

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* Some transient proton release was found when a concentrated solution of Ca^{2+}/Mg^{2+} was added to the reagent containing 160 mmol · L^{-1} KF, 2.9 mmol · L^{-1} NTA, to similar final concentrations as found in squid mantle homogenates, 5 mmol · L^{-1} Mg^{2+}, 0.8 mmol · L^{-1} Ca^{2+}. The maximum pH change seen was <0.5 pH-units in the unbuffered solution. The final pH difference between solutions before and after the addition of ions was <0.03 pH units. A similar effect was not seen in homogenates prepared using the same solution of reagents, very likely due to proton buffering, lower concentration gradients for the ions and, possibly, a more rapid precipitation of the fluorides.

TABLE 4

Comparison of phosphagen and glycolytic end product (octopine) levels (given in $\mu mol \cdot g^{-1}$ fresh weight) in homogenate supernatants and muscle tissue of *Sipunculus nudus* after perchloric acid extraction (for the procedure see Pörtner, 1990). Despite the reduction in NTA levels for this species (see text and table 1), only minor changes occur during the extraction and storage on ice. A change in phospho-L-arginine (PLA) levels according to the slight drop in the ratio of phospho-L-arginine over the sum of PLA and L-arginine (L-Arg) contents would be equivalent to a slight upward shift by 0.020 pH-units ($\bar{x} \pm SD$, $n = 7$, *denotes a significant change, paired sample t-test, $P < 0.05$).

Sample	Octopine	PLA	L-Arg	$\frac{[PLA]}{[PLA] + [L\text{-}Arg]}$
Muscle tissue	0.04	31.9	6.3	0.83
	± 0.05	± 8.7	± 2.6	± 0.06
Homogenate	0.06	31.4	9.3*	0.78*
supernatant	± 0.07	± 8.6	± 4.1	± 0.07

5 min of exposure to room air. In the present study, 10 to 100 times this tissue weight was used. The time required for grinding usually did not exceed 3–5 min and pHi values were found to be equal to the values found with DMO. Moreover, no difference was found between pHi values obtained with and without use of the glove bag. These observations suggest that contamination with CO_2 was negligible. For tissue samples of small size the use of liquid nitrogen in CO_2 free air or nitrogen, *e.g.* in a glove box or glove bag, should be considered. The risk of CO_2 loss from homogenates is considered to be minimal under the experimental conditions applied. The CO_2 gradient is reduced by dilution and by keeping the homogenate on ice. By repeated sampling from the (cold) Eppendorf tube no early increase in pH attributable to CO_2 loss was seen.

Methodological procedure: Pi_{CO_2} calculations. The validity of Pi_{CO_2} calculations largely depends on the accurate analysis of pH and C_{CO_2} and on the accuracy of applied pK''' and α values. Since solubility values measured in tissue homogenates are too high due to CO_2 dissolved in the lipid fraction of the tissue, Siesjö (1962) utilized the solubility coefficient for CO_2 in 0.16 M NaCl in his pK calculations. Alternatively, values for CO_2 solubility as determined according to Heisler (1986) may be applicable. If these values are used for the determination of pK''' from total CO_2 and pH measurements in tissue homogenates, the resulting constant (6.252 ± 0.025, $\bar{x} \pm SD$, $n = 10$, valid for a mean pH of 7.02, T = 15 °C) is close to the value (6.233) calculated according to Heisler (1986) in this and in Siesjö's study. These considerations suggest that lipid-solubilized CO_2 should be excluded from the analysis. Therefore, the measurement is restricted to the homogenate supernatant which contains the water-solubilized CO_2 fraction, *i.e.* the one to be related to the acid–base parameters of the cell.

Contamination by extracellular fluids. The correction for extracellular CO_2, which is required for Pi_{CO_2} calculations, seems to be complicated by the observation that extracellular pH may be different in interstitial fluid and plasma (*e.g.* de Hemptinne and Huguenin, 1984). A P_{CO_2} gradient between tissue and blood could cause this pronounced pH difference, since the level of protein and, correspondingly, non-bicarbonate buffering is much lower in interstitial fluid than in plasma. The increase in C_{CO_2} with high P_{CO_2}, however, would be small (fig. 1). Moreover, the excess base equivalents (bicarbonate and carbonate ions) may be released into the plasma. The calculation procedure for cellular total CO_2, therefore, does not need to consider a heterogeneity for extracellular CO_2 other than that taken into account by the application of the Donnan factor.

Table 6 shows the mean intracellular P_{CO_2} values of toad and squid muscle obtained by calculation. Adopting the values of intracellular P_{CO_2} presented for toad gastrocnemius muscle, fig. 1 demonstrates that differences in intracellular and venous P_{CO_2} values may cause pH to be 0.1 to 0.15 units lower in the interstitial fluid than in the plasma. A pH difference or gradient of 0.07 to 0.2 pH units between interstitial fluid and plasma has been described for mammalian skeletal muscle (*e.g.* de Hemptinne and Huguenin, 1984), supporting the validity of the calculated P_{CO_2} values and the analysis presented in fig. 1.

The presence of extracellular fluids may also affect the analysis of pHi (table 5). The influence of extracellular compartments on the measured pH is minor in the squid (*Illex*

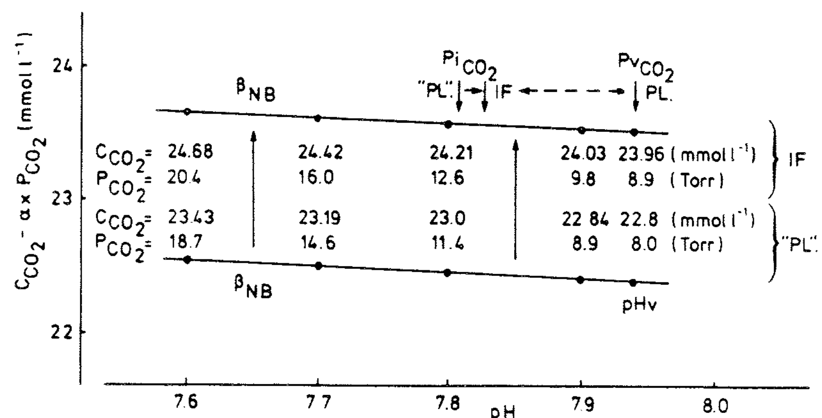

Fig. 1. An analysis of the effect of intracellular P_{CO_2} on interstitial pH in toad gastrocnemius muscle. The calculation is based on a low non-bicarbonate buffer value in the interstitial fluid (IF) and represents the maximum effect close to the cellular surface. If a Donnan factor of 1.05 is considered, the shift in the buffer line caused by the increase in apparent bicarbonate levels (↑) reduces (→) the pH gradient (←---→) which results from the difference between intracellular and venous P_{CO_2}. Downward arrows (↓) point at the pH value valid for the plasma, PL., at the pH which would be valid for the interstitial fluid with plasma total CO_2 levels, 'PL.', and at interstitial pH, IF, which results after consideration of the mammalian Donnan factor. Consequently, the range between $pH_{'PL'}$ and pH_{IF} gives the range of interstitial pH values expected with lower Donnan factors in the amphibian.

TABLE 5

Quantitative analysis of the influence of extracellular compartments and erythrocytes (based on Tufts *et al.*, 1987) on pH measurements in tissue homogenates. For the toad, buffer values, haematocrit, values for Q are based on Boutilier *et al.* (1979, 1987), Pörtner (1990), Pörtner and Toews (in preparation). Fractional values of blood were adopted from data for mammalian muscle (Hoppeler *et al.*, 1981, Weibel, 1984). For squid similar fractional blood values were assumed as for *Sepia officinalis* (Robertson, 1965). (Q: fraction of extracellular water in tissue water, h: haematocrit, bl: blood, pl: plasma, IF: interstitial fluid, tw: tissue water, cw: cell water, ew: extracellular water = water fractions of plasma (or blood) + interstitial fluid, ery: erythrocytic, t: tissue, F: water fraction in tissue (valid for Ftw) or in tissue water (valid for Fbl; Fcw; Fpl = Fbl − Fery; Fery = Fh · Fbl · 0.7; FIF = Q − Fbl).

Species:	Squid	Toad			
Muscle:	mantle				
Cond. (T):	Control (15 °C)	Gastrocnemius	Sartorius		
		Control (20 °C)			
Q	0.09	0.145	0.202		
Fh	-	0.20	0.20		
Fbl	0.01	0.01	0.01		
Fpl	-	0.008	0.008		
FIF	0.08	0.137	0.194		
Ftw	0.75	0.78	0.79		
Fcw	0.91	0.855	0.798		
βbl_{NB}	5.0	-	-	$mmol \cdot pH^{-1} \cdot L^{-1}$	bl
βpl_{NB}	-	4.1	4.1	$mmol \cdot pH^{-1} \cdot L^{-1}$	pl
βIF_{NB}	0.5	0.4	0.4	$mmol \cdot pH^{-1} \cdot L^{-1}$	IF
Cbl_{CO_2}	4.4	-	-	$mmol \cdot L^{-1}$	bl
Cpl_{CO_2}	-	22.8	22.8	$mmol \cdot L^{-1}$	pl
CIF_{CO_2}	4.4	24.0	24.0	$mmol \cdot L^{-1}$	IF
$Cery_{CO_2}$	-	7.1	7.1	$mmol \cdot L^{-1}$	ery
Ccw_{CO_2}	4.8	7.0	5.5	$mmol \cdot L^{-1}$	cw
βery_{CO_2}	-	1.4	1.3	$mmol \cdot pH^{-1} \cdot L^{-1}$	ery
βery_{NB}	-	67.1	67.1	$mmol \cdot pH^{-1} \cdot L^{-1}$	ery
βery_{tot}	-	68.5	68.4	$mmol \cdot pH^{-1} \cdot L^{-1}$	ery
βcw_{NB}	25.8	29.8	31.5	$mmol \cdot pH^{-1} \cdot L^{-1}$	cw**
βcw_{CO_2}	0.7	1.2	0.9	$mmol \cdot pH^{-1} \cdot L^{-1}$	cw
βcw_{tot}	26.5	31.0	32.4	$mmol \cdot pH^{-1} \cdot L^{-1}$	cw
Δ*pHbl−t	−0.12	-	-		
ΔpHpl−t	-	0.69	0.66		
Δ*pHIF−t	−0.12	0.59	0.56		
ΔpHery−t	-	0.18	0.15		
$\Delta pHi, bl_{NB}$	−0.00025	-	-		
$\Delta pHi, bl_{CO_2}$	−0.00002	-	-		
$\Delta pHi, pl_{NB}$	-	+0.0009	+0.0008		
$\Delta pHi, pl_{CO_2}$	-	+0.0004	+0.0004		
$\Delta pHi, IF_{NB}$	−0.0002	+0.0012	+0.0017		
$\Delta pHi, IF_{CO_2}$	−0.0002	+0.0070	+0.0092		
ΔpHi	−0.001	+0.010	+0.013		

* pH differences for squid represent maximum estimates based on Pi_{CO_2} calculations.

** βcw_{NB} assumed to be identical in gastrocnemius and sartorius muscles of the toad.

illecebrosus), since blood pH is close to the value of pHi. In the amphibian (*Bufo marinus*) the influence of the extracellular compartments is more pronounced. Based on a ratio of mitochondrial volume/capillary blood volume of 3 (Weibel, 1984), blood fractions higher than 3 to 4%, however, hardly occur even in aerobic skeletal musculature (*cf.* Hoppeler *et al.*, 1981). The fact that the major buffer components of vertebrate blood are located inside of the blood cells reduces the effect of these buffers on the pH measurement owing to the low intracellular pH of the erythrocytes (for *Bufo marinus* see Tufts *et al.*, 1987). Thus, the fraction of plasma and interstitial fluid and, among buffer systems, the CO_2/bicarbonate buffer system in these compartments is much more important. Generally, mean tissue pH in skeletal muscle is likely to be less than 0.02 pH units higher than mean intracellular pH. This deviation can usually be considered negligible.

The ratio of mitochondrial volume/blood volume may be lower in heart muscle than in skeletal muscle. This is valid the more so for the amphibian ventricle since coronary circulation is negligible and the oxygen is extracted from the blood passing the ventricular lumen. Because blood is largely removed during freeze clamping the error in estimating the mean intracellular pH is still considered to be far below + 0.05 pH units. This conclusion is supported by the observation that the large difference found between DMO and homogenate derived pHi is close to the upper limit of the difference expected from a consideration of the cytosolic-mitochondrial pH gradient (see below) and, thus, excludes that the homogenate derived pH represents a high estimate owing the contamination with blood. Similar considerations are valid for the mean pHi evaluated for the isolated perfused rat heart, the more so, since the perfusion medium contained no non-bicarbonate buffers.

Cellular compartmentation. Table 6 compares pHi values in muscle tissues from different vertebrate and invertebrate species analysed by means of DMO and/or the controlled homogenate technique presented herein. As shown by the differences in standard deviations the variability of pHi estimates is higher in DMO studies than in measurements performed in homogenates under metabolic control. The additive effect of non-directional experimental errors in the measurements of extracellular pH, extracellular DMO, extracellular inulin, tissue DMO, tissue water content, tissue inulin possibly explains the high variability experienced with DMO. The high correlation between octopine levels and intracellular pH in the mantle of exercising squid may be considered as additional, indirect evidence for the validity of the homogenate technique (Pörtner, 1989).

It is evident that in muscle tissue exhibiting high capacities for anaerobic function (*e.g.* vertebrate white muscle, body wall musculature of *Sipunculus nudus*) pHi values agree well between the two methods. Considering the small fraction of mitochondria in white muscle (*e.g.* 2–4% in gastrocnemius and sartorius muscle of mammals, Hoppeler *et al.*, 1981) the contribution of mitochondrial matrix fluid (the one with the high pH) to the total volume of the cell would range below 2%. This fraction only negligibly influences homogenate and DMO based pHi values, which both are close to cytosolic pH. The

TABLE 6

Comparison of intracellular pH values ($\bar{x} \pm SD$) in tissues of different animals under resting conditions. At the temperature indicated, pH was determined in tissue homogenates under metabolic control (see text). DMO values were adopted from literature studies. For Pi_{CO_2} calculations in toad tissues the following extracellular (venous) parameters were used: pHv = 7.94 ± 0.05, Pv_{CO_2} = 8.0 ± 0.5 Torr, $[HCO_3^-]v$ = 22.4 ± 2.5 mmol · L^{-1} plasma ($\bar{x} \pm SD$, n = 5).

Species	Muscle tissue	T (°C)	pHi	Pi_{CO_2} (Torr)	Technique	(n)	Source
Sipunculus nudus	Body wall musc.	15	7.29 ± 0.01	-	Homogenate	(4)	This study
			7.27 ± 0.05	-	DMO	(10)	Pörtner, 1987b
Illex illecebrosus	Mantle muscle		7.38 ± 0.02	5.8 ± 1.3	Homogenate	(5)	This study
Salmo gairdneri	Epaxial muscle	15	7.30 ± 0.01	-	Homogenate	(5)	This study
		12–14	7.30 ± 0.08	-	DMO	(9)	Höbe *et al.*, 1984
		15	7.21 ± 0.12	-	DMO	(9)	Milligan and Wood, 1986
Bufo marinus	Sartorius	20	7.28 ± 0.03	-	Homogenate	(5)	This study
			7.27 ± 0.10	-	DMO	(10)	Boutilier *et al.*, 1987
	Gastrocnemius	20	7.25 ± 0.02	11.2 ± 1.6	Homogenate	(5)	This study
			7.28 ± 0.09	-	DMO	(10)	Boutilier *et al.*, 1987
	Ventricle	20	7.13 ± 0.01		Homogenate	(5)	This study
				11.1* ± 1.3			
			7.35 ± 0.16		DMO	(10)	Boutilier *et al.*, 1987
	Kidney	20	6.99 ± 0.04	-	Homogenate	(5)	This study
Rattus	Heart	37	6.90 ± 0.02	-	Homogenate	(3)	This study
			7.05 ± 0.06	-	DMO	(10)	Steenbergen *et al.*, 1977

* Pi_{CO_2} calculations for toad ventricle are baded on total CO_2 measurements in the homogenate and on the mean of DMO-derived pHi.

accuracy of the absolute values is further substantiated by the recent finding that DMO and ^{31}P-NMR pHi values agree well in white muscle tissue of *Mytilus edulis* (Zange *et al.*, 1990).

However, in the ventricle of the toad *Bufo marinus* and the isolated perfused rat heart

homogenate pH is lower than the pHi found with DMO (table 6). The fact that this difference only arises in highly aerobic organs suggests that the influence of a large volume of mitochondria on DMO-derived pH may be substantial. DMO overestimates the mean pHi in these cases based on the distribution characteristics of weak acids (Roos and Boron, 1981). In the isolated perfused rat heart 19% of the cell fluid are attributable to the mitochondrial matrix (Kauppinen, 1983). Assuming a ΔpH of 0.63 between cytosol and mitochondria (Kauppinen, 1983) the DMO derived value is expected to lie 0.08 pH units above a volume weighted mean pHi (with equal, pH-independent buffer values in the two compartments). The observed difference, however, is 0.15 pH units for the Langendorff perfused rat heart and 0.22 pH units for toad ventricle muscle (table 6). Acknowledging that this difference has not been evaluated based on a direct comparison of DMO and homogenate techniques in the same samples, this large discrepancy suggests that the degree to which mean pHi is overestimated by means of DMO may be even higher than derived from the consideration of weak acid distribution alone. If other reasons for high DMO-derived pHi values (like lipid solubility of protonated DMO) are neglected, mitochondria can be concluded not to determine homogenate-derived mean pHi according to their volume fraction. The discrepancy could be explained assuming lower buffering in mitochondria than in the cytosol. It can be stated, however, that homogenate-derived pHi values reflect these differences in buffering and, therefore, represent effective mean pHi values, whereas those derived from DMO distribution do not. The contribution of each cellular subcompartment to the mean pHi depends on subcompartmental values of pH, β_{NB}, C_{CO_2} and volume fraction according to eqs. (3–7) for the (sum of) non-bicarbonate buffers and according to eqs. (8, 9 and 6) for the CO_2/bicarbonate buffer.

In most cases DMO will still be useful to evaluate relative changes in intracellular pH. This may, however, no longer be the case in aerobic tissues, when the pH gradient across the mitochondrial membrane changes due to a shift of H^+ between cytosol and mitochondria. If protons do not leave the cell, no change in effective mean pHi should occur. Such an apparent change will, however, be detected by DMO and, at constant P_{CO_2}, will be erroneously attributed to metabolic processes or proton equivalent ion exchange between cells and extracellular fluid. Such an error is not expected to occur with the homogenate technique since all important factors determining the effective mean pHi are operative.

Different pHi values resulting from homogenate or DMO techniques will lead to different values of mean intracellular P_{CO_2} in the calculation procedure for organs containing high amounts of mitochondria. Like DMO, CO_2 is distributed as a weak acid between cell compartments. The calculation of P_{CO_2} [eq. (12)] uses the Henderson Hasselbalch equation for the calculation of the concentration of undissociated acid (HDMO or $\alpha \cdot P_{CO_2}$). Owing to the identical distribution characteristics of CO_2 and DMO, obviously, a weak acid (DMO) derived pHi is applicable to calculate mean intracellular P_{CO_2}. (The difference in pK′ of DMO and CO_2 does not influence the results of this calculation.) The effective mean pHi is only suitable for this purpose when weak acid and homogenate derived pHi values are identical as in anaerobic muscle. If

the mean of DMO derived pHi values is adopted from the study by Boutilier *et al.* (1987) similar Pi_{CO_2} values as in the gastrocnemius result for the ventricle of the toad *Bufo marinus*. Obviously, DMO, despite of its failure to give close estimates of the effective mean intracellular pH in aerobic organs, appears still useful for the evaluation of mean intracellular P_{CO_2}.

However, the resulting Pi_{CO_2} values show a higher scatter than the related venous P_{CO_2} values (table 6). Since, analogous to the procedure with DMO, Ci_{CO_2} values have been evaluated from tissue and plasma total CO_2, the same reasons apply which cause the variability in DMO-derived pHi (see above). The variability of Pi_{CO_2} values would increase even further if highly scattered individual DMO pHi values would be used for the calculation. If applicable (see above), however, the validity of each individual homogenate pHi value permits to calculate Pi_{CO_2} based on pHi and Ci_{CO_2} of each individual tissue sample.

Conclusions. The homogenate technique yields mean pHi values which are determined by the relative volume and buffering of cellular compartments. This is most important for studies of acid–base regulation, which focus on the movement of proton quantities between intra- and extracellular compartments. The method is suitable for analysing the effective mean intracellular pH with less variability than the determination by use of DMO and is not subject to errors inherent with weak acid distribution techniques. It is, therefore, an adequate method for recording small differences in mean intracellular pH even between small sized muscle populations. The considerable time delay which is associated with DMO distribution, and to some extent, also with ^{31}P-NMR, is not inherent to the homogenate technique. The low cost of this technique (*e.g.* no radio-labelled compounds and associated apparatus required) and the simple methodological procedure should, generally, make it a useful alternative to other methods of pHi analysis.

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