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Determination of intracellular buffer values after metabolic inhibition by fluoride and nitrilotriacetic acid

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Abstract. Non-bicarbonate buffer values have been analysed in homogenates of squid mantle muscle and toad gastrocnemius muscle using the P_{CO_2} equilibration technique. Homogenate metabolism was inhibited by the addition of potassium fluoride and nitrilotriacetic acid. After an initial change in the concentration of organic phosphates a new metabolic equilibrium was achieved which was insensitive to pH changes during P_{CO_2} variations. Inaccurate measurements of homogenate buffer values could, thereby, be prevented. The accumulation of inorganic phosphate during the initial metabolic shift caused an increase in homogenate buffer values. Levels of unbound inorganic phosphate, which are effective in buffering, were estimated by enzymatic analysis in homogenate ultrafiltrates. Based on the evaluation of apparent dissociation constants pK' , and buffer values for fluoride and inorganic phosphate, the non-bicarbonate, non-phosphate, (i: inorganic) buffer value of the tissue was calculated. The level of free inorganic phosphate in the resting tissue was considered for an estimate of the intracellular non-bicarbonate buffer value under control conditions.

Animal, squid, toad; Buffer value; Dissociation constant, apparent; Drug, fluoride, nitrilotriacetic acid; Metabolic inhibitor; Muscle, gastrocnemius – of toad, mantle – of squid

In the past, buffer values of tissue homogenates from a variety of animals have been measured using two different methods (see Roos and Boron, 1981 for review): (1) tissue homogenates have been titrated with strong acid or base (HCl, NaOH, *e.g.* Castellini and Somero, 1981) and (2) tissue homogenates have been equilibrated (or “titrated”) with different partial pressures of CO_2 (*e.g.* Heisler and Piiper, 1971). During titration with fixed acid or base, the influence of the CO_2 /bicarbonate buffer system is not correctly assessed. During titration with CO_2 , it is possible to clearly distinguish non-bicarbonate buffers from the CO_2 /bicarbonate buffer, since the buffering of non-

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bicarbonate buffers is calculated from the changes in pH and apparent bicarbonate levels (Heisler and Piiper, 1971). Other discrepancies between buffer values obtained by titration with fixed acid or base or with volatile acid (CO_2) are discussed to be related to the denaturation or hydrolysis of proteins or organic phosphates during exposure to strong acid or base (Heisler, 1986). However, metabolic changes in homogenates both before and during the titration procedure have never been investigated.

Metabolic processes may cause the buffer value of the homogenate to deviate from the buffer value in the intact tissue under control conditions. Analogous to the situation in exhausted or anaerobic muscle (*e.g.* Pörtner, 1986), anaerobic homogenate metabolism causes inorganic phosphate to accumulate especially in homogenates from white muscle with high phosphagen contents, thus leading to an increase in buffer value (Pörtner, 1989). In addition, since the phosphagen kinase (*e.g.* creatine or arginine kinase) equilibrium is influenced by pH, a decrease (increase) in homogenate pH during the titration procedure would trigger phosphagen depletion (repletion), leading to fluctuations in the buffer value. Since, generally, pH sensitivity causes metabolic processes to respond to the titration process by proton production (*e.g.* glycolysis, ATP degradation, phosphagen repletion) or proton consumption (phosphagen hydrolysis, Pörtner *et al.*, 1984), pH values result which are different from those produced by physicochemical buffering alone.

In the present study buffer values were analysed by CO_2 equilibration. This method is limited by the fact that each equilibration with a different P_{CO_2} value lasts about 20 min, so that the homogenate is used during a prolonged experimental period. Therefore, even with the use of chemical control by KF and nitrilotriacetic acid (Pörtner *et al.*, 1990), it was necessary to examine whether the endogenous metabolism of the homogenate still influences the overall analysis.

Materials and methods

Squid and toad tissues analysed for the present paper were collected as described by Pörtner *et al.* (1990).

Experimental procedure. For the analysis of buffer values all tissue samples were ground under liquid nitrogen using mortar and pestle. Powder, which was not used immediately, was wrapped in aluminium foil and stored in liquid nitrogen.

Approximately 1 g of muscle powder was placed into preweighed tonometer vessels precooled on ice. 4 ml of ice-cold medium were added. The mixture was stirred and allowed to thaw. The different media (which were kept in polyethylene bottles) included deionized water, $0.16 \text{ mol} \cdot \text{L}^{-1}$ KF + $2.9 \text{ mmol} \cdot \text{L}^{-1}$ or $10 \text{ mmol} \cdot \text{L}^{-1}$ NTA (nitrilotriacetic acid, Sigma Corp. St. Louis, U.S.A.), $0.54 \text{ mol} \cdot \text{L}^{-1}$ NaF + $0.01 \text{ mol} \cdot \text{L}^{-1}$ NTA or $0.54 \text{ mol} \cdot \text{L}^{-1}$ KF + $0.01 \text{ mol} \cdot \text{L}^{-1}$ NTA for squid muscle and $0.54 \text{ mol} \cdot \text{L}^{-1}$ KF + $0.01 \text{ mol} \cdot \text{L}^{-1}$ NTA + $5 \text{ mmol} \cdot \text{L}^{-1}$ KHCO_3 for toad muscle. Tonometry (Instrumentation laboratory, Padorno Dugano, Italy) started with a medium P_{CO_2} and changed to different P_{CO_2} values in the following order (*cf.* Heisler and Piiper, 1971):

medium–low (high)–medium–high (low)–medium P_{CO_2} . Normoxia was maintained during equilibration (each step lasting 15 to 30 min). Gas mixtures were prepared from pure nitrogen, oxygen and carbon dioxide by using gas mixing pumps (Type 303/a-F, Wösthoff, Bochum, F.R.G.). In some cases (for the presentation in figs. 1 to 5) pH was continuously recorded by placing a Radiometer pH electrode (Type GK2401) into the tonometer vessel. Aliquots of the homogenate were withdrawn after each step of equilibration.

Samples were taken into a syringe equilibrated with the respective gas mixture and were centrifuged for 3 to 5 sec in an 0.5 ml Eppendorf tube. The supernatant was analysed for total CO_2 and pH as described by Pörtner *et al.* (1990). For the analysis of free inorganic phosphate aliquots of the supernatant were subjected to ultrafiltration (at 0–4 °C) using Millipore (Bedford, U.S.A.) Ultrafree PF units (100 000 d during 15 to 45 min). The filtrate was collected in vials containing 10 or 20 μl of $3 \text{ mol} \cdot \text{L}^{-1}$ perchloric acid. After filtration the volume of perchloric acid was adjusted to 20% (v/v) and the precipitate (predominantly KClO_4) was centrifuged. The supernatant was neutralized to pH 7.5–8 by two steps of titration, firstly by using KOH ($5 \text{ mol} \cdot \text{L}^{-1}$, 10% of the extract volume) and, secondly (in order to minimize the risk of overtitration in small extract volumes) by using a mixture of solid $\text{K}_2\text{CO}_3/\text{KHCO}_3$ (1/6, w/w).

In addition, the levels of high energy phosphates and related metabolites were analysed in aliquots of the homogenates of squid mantle muscle (neither centrifuged nor subjected to ultrafiltration). Perchloric acid ($3 \text{ mol} \cdot \text{L}^{-1}$) was added to a final concentration of $0.6 \text{ mol} \cdot \text{L}^{-1}$. After mixing on a Vortex mixer (Scientific Industries Inc., Bohemia, NY, U.S.A.) the precipitate was removed by centrifugation (about 1 min at maximum speed) in an Eppendorf centrifuge. Neutralization of the supernatant was performed as described above. For comparison with the metabolic status of the homogenates, frozen muscle powder was directly subjected to extraction in perchloric acid ($0.6 \text{ mol} \cdot \text{L}^{-1}$) according to Beis and Newsholme (1975).

The neutralized extracts were analysed for ATP, ADP, AMP and α -glycerophosphate by using standard enzymatic procedures (Bergmeyer, 1974; Lowry and Passonneau, 1972). Arginine phosphate, octopine, and arginine were measured according to Grieshaber *et al.* (1978). Inorganic phosphate was measured by using an enzymatic test modified after Cornell *et al.* (1979). The assay mixture contained (in $\text{mmol} \cdot \text{L}^{-1}$) 100 Triethanolamine/HCl pH 8.0, 0.25 EDTA, 1 glucose, 2.5 MgCl_2 , 2.5 NAD^+ , 2.5 ADP, 0.6 fructose 1,6-bisphosphate, 0.5 U ml^{-1} hexokinase, 0.3 U ml^{-1} aldolase and 0.7 U ml^{-1} of both phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase. All chemicals were purchased from Sigma, St. Louis, MO, U.S.A. Aqueous solutions of lyophilized enzymes were preferred to suspensions in ammonium sulfate ($3.2 \text{ mol} \cdot \text{L}^{-1}$), in order to prevent inhibition of glyceraldehyde 3-phosphate dehydrogenase by ammonium sulfate itself and precipitation or binding of inorganic phosphate as MgNH_4PO_4 . The assay was started by the addition of standards or samples with known and unknown phosphate concentrations. The final readings were corrected for the blank absorbance of standards and samples in the reagent mixture without enzymes.

For the analysis of the apparent dissociation constant of inorganic phosphate under

homogenate (and cellular) conditions, pK_2' was measured as the pH of an equimolar solution of potassiumdihydrogenphosphate and dipotassiumhydrogenphosphate in excess potassium chloride of different ionic strengths ($I_{P_i} : I_{KCl} = 1 : 10$). Measurements were performed at $25 \pm 0.1^\circ C$ using a thermostatted pH-microelectrode (BMS 3, Radiometer, Copenhagen) calibrated with precision phosphate buffers. The pH of the phosphate stock solution was compared with the pH expected from the dependence of pK_{P_i}' on ionic strength in pure phosphate solutions (Strang, 1981). The measured pK' values were used to fit empirical equations derived from an extended Debye-Hückel approach (see below).

Calculations. The calculation of non-bicarbonate buffer values b in the homogenate H basically followed the procedure outlined by Heisler and Piiper (1971):

$$b_H = - \frac{0.5 (\text{Bic}_1 + \text{Bic}_3) - \text{Bic}_2}{0.5(\text{pH}_1 + \text{pH}_3) - \text{pH}_2} (\text{mmol} \cdot \text{pH}^{-1} \cdot \text{L}^{-1} \text{ homogenate water}) \quad (1)$$

(1,3: at medium P_{CO_2} , 2: at high or low P_{CO_2})

The buffer value of fluoride in the reagent mixture had to be considered in order to correct for the respective change in the buffer value b_H .

$$b_{H'} = b_H - b_F \quad (2)$$

Moreover, since metabolism may cause the buffer value of inorganic phosphate (P_i) to fluctuate it was useful to evaluate the non-bicarbonate, non-phosphate, buffer value.

$$b_{H''} = b_{H'} - b_{P_i} \quad (3)$$

The buffer values of fluoride and the system $\text{HPO}_4^{2-}/\text{H}_2\text{PO}_4^-$ can be calculated by comparing the fractional values F of HPO_4^{2-} and F^- in their respective dissociation equilibrium between pH_1 , pH_3 and pH_2 according to

$$F = 1 - [1/(10^{\text{pH}-\text{pK}_a'} + 1)] \quad (4)$$

$$b = [F_2 - 0.5 \cdot (F_1 + F_3)] \cdot C / [\text{pH}_2 - 0.5 \cdot (\text{pH}_1 + \text{pH}_3)] (\text{mmol} \cdot \text{pH}^{-1} \cdot \text{L}^{-1} \text{ homogenate water}) \quad (5)$$

where C is the total concentration of the buffer ($\text{mmol} \cdot \text{L}^{-1}$) and K_a' its apparent dissociation constant.

Finally, the non-bicarbonate, non-phosphate, buffer value β_{NB, NP_i} of the tissue ($\text{mmol} \cdot \text{pH}^{-1} \cdot \text{kg}^{-1}$ wet weight) results from $b_{H''}$ (eq. 3) by considering the dilution factor of the tissue (wet weight) in the homogenate water (volume of medium + tissue water). Consideration of the levels of free inorganic phosphate in a tissue under control conditions leads to the non-bicarbonate buffer value of this tissue in the resting control animal. The buffer value of the system $\text{HPO}_4^{2-}/\text{H}_2\text{PO}_4^-$ can then be calculated

according to

$$\beta = 2.303 \cdot a_{H^+} \cdot \frac{K'_a \cdot C}{(a_{H^+} + K'_a)^2} \text{ (mmol} \cdot \text{pH}^{-1} \cdot \text{kg}^{-1}\text{)} \quad (6)$$

where C is the concentration of the buffer ($\mu\text{mol} \cdot \text{g}^{-1}$ wet weight) and K'_a its apparent dissociation constant in the cell water (van Slyke, 1922).

For correct calculation of the buffer values of fluoride and inorganic phosphate, apparent dissociation constants have to be used which are valid for the ionic strength of the homogenate or of the cell. An apparent change in dissociation constants is caused by the fact that activity coefficients γ usually change differently for conjugated acids or bases.

$$\begin{aligned} \text{pH} &= \text{pK}_a + \log \frac{\gamma_{\text{base}} \cdot [\text{base}]}{\gamma_{\text{acid}} \cdot [\text{acid}]} \\ &= \text{pK}_a + \Delta\text{pK}_a + \log \frac{[\text{base}]}{[\text{acid}]} \end{aligned} \quad (7)$$

$$\text{pK}'_a = \text{pK}_a + \Delta\text{pK}_a \quad (8)$$

An extended Debye-Hückel approach (Robinson and Stokes, 1959) provides the mathematical basis for the calculation of activity coefficients for ionic strengths above $0.01 \text{ mol} \cdot \text{L}^{-1}$ [$I = 0.5 \sum (c_i \cdot z_i^2)$, c : molar concentration, z : number of charges of the respective ion i]. In some of these solutions the ionic atmosphere or effective ion radii of the participating ions must be considered (eq. 9).

$$\log \gamma_i = \frac{-A \cdot z_i^2 \cdot \sqrt{I}}{1 + Bd \cdot \sqrt{I}} + X \cdot z_i^2 \cdot I \quad (9)$$

$$A = 1.82481 \cdot 10^6 \cdot (\varepsilon \cdot T)^{-3/2} \text{ dm}^{3/2} \cdot \text{mol}^{-1/2}$$

$$B = 502.90 \cdot (\varepsilon \cdot T)^{-1/2} \text{ dm}^{3/2} \text{ mol}^{-1/2} \cdot \text{nm}^{-1}$$

For F^- , HPO_4^{2-} and H_2PO_4^- the radius d (in nm) amounts to 0.35 (F^-), 0.4 (HPO_4^{2-}) and 0.45 (H_2PO_4^-) (Koryta and Dvorak, 1987, ε : dielectric constant of water, $\varepsilon = 81.95$, 15°C ; 80.10 , 20°C ; 78.30 , 25°C ; CRC Handbook of Chemistry and Physics, 1975–1976, T : absolute temperature). The linear component $X \cdot z_i^2 \cdot I$ takes into account that some electrolytes, when an increase of ionic strength occurs in their own solution, show an increase in activity coefficients after an initial, but transient decrease. Effects specific for the respective ion mixture predominate in a range of ionic strength above 0.1 to $0.2 \text{ mol} \cdot \text{L}^{-1}$. Therefore, X has to be determined by empirical analysis in order to predict the relationship between activity coefficients and ionic strength for a single electrolyte in the respective electrolyte solution. In addition, the term Bd may vary for the same ion species between different mixtures of electrolytes.

TABLE 1

Apparent dissociation constants at different temperatures for inorganic phosphate (pK_2) under different ionic strengths. Thermodynamic constants ($I = 0$) are based on Perrin (1969). It becomes evident that phosphate solutions do not reflect the apparent pK' values valid for homogenate conditions, but that the presence of excess KF/KCl has to be considered. For further details see text.

I	Phosphate solution		Phosphate in KCl			
	Eq. (11)	Measured	Eq. (12)		Measured	
	25 °C		15 °C	20 °C	25 °C	25 °C
0	7.198	–	7.231	7.213	7.198	–
0.1	6.862	6.854	6.861	6.849	6.831	6.819
0.16	6.807	6.816	6.801	6.780	6.762	6.765
0.19	6.787	6.802	6.776	6.753	6.736	6.731
0.25	6.754	6.773	6.730	6.708	6.689	6.690
0.47	6.682	6.713	6.619	6.597	6.577	6.594
0.75	6.638	6.652	6.533	6.510	6.489	6.515

Equations (10–12) result for the calculation of pK' values of fluoride [eq. (10)] and phosphate, for the latter in pure phosphate solutions [eq. (11)] and in excess KCl [eq. (12)]. The validity of these equations is substantiated based on table 1, where calculated values of pK' agree well with pK' values measured in solutions of pure phosphate and phosphate under excess KCl.

$$F^- : \log \gamma = \frac{-A \cdot z^2 \cdot \sqrt{I}}{1 + B \cdot 0.35 \cdot \sqrt{I}} \quad (10)$$

$$P_i^- / P_i^{2-} : \log \gamma = \frac{-A \cdot z^2 \cdot \sqrt{I}}{1 + Bd \cdot \sqrt{I}} + 0.032 \cdot z^2 \cdot I \quad (11)$$

$$P_i^- / P_i^{2-} : \log \gamma = \frac{-A \cdot z^2 \cdot \sqrt{I}}{1 + \sqrt{I}} \quad (12)$$

Potassium fluoride is the predominant electrolyte in the homogenate and determines the ionic strength of the medium. Below $0.5\text{--}1 \text{ mol} \cdot \text{L}^{-1}$, the dependence of γ_i on I is similar for the chlorides and fluorides of potassium and sodium, which show an exponential decrease of γ_i with increasing ionic strength up to this range of concentration (based on reported activity coefficients, CRC Handbook of Chemistry and Physics). Therefore, the term X in eq. (9) is assumed to be close to zero (eq. 10). The calculation substantiates that the pK' of hydrofluoric acid varies between 2.8 and 3.2 (depending on T and I)

and is far enough from the pH of the tissue homogenate in order to only minimally influence its buffer value.

The apparent dissociation constant of inorganic phosphate in tissue homogenates is predominantly influenced by the ionic strength of the excess electrolyte (mainly KF). For analysing pK' , however, the use of KCl is more adequate owing to its lower buffer value. Under excess KCl, γ_{P_i} more closely follows eq. (12), which assumes B_d to be close to 1, X being zero under these conditions. Equation (12) is used to calculate pK'_2 for homogenate and intracellular conditions.

Results and Discussion

Methodological procedure. The main goal of the present paper is to evaluate non-bicarbonate buffer values of the intracellular space of tissues under control conditions. Since buffer values are calculated from values measured in tissue homogenates, differences in metabolic status between the homogenate and the intact tissue must be defined in terms of metabolic processes that can potentially influence non-bicarbonate buffer values. The following factors turned out to be essential for an accurate estimate: (1) control of endogenous pH fluctuations in the homogenate; (2) minimal sensitivity of metabolic processes to pH changes = maintenance of a metabolic equilibrium; (3) correction for changes in free inorganic phosphate levels.

Factors 1 and 2. In homogenates prepared in water the first step of equilibration seemed to be complete at low pH (fig. 1). A reduction in P_{CO_2} from 3.5 to 0.7 Torr, however, did not cause pH to increase by the expected order of magnitude. The observed alkalosis was only transient. As a corollary, it is very likely that the metabolic equilibrium is highly pH sensitive and is determined by the velocity of proton-producing and -consuming processes and their feedback control by pH. If pH increases, proton-

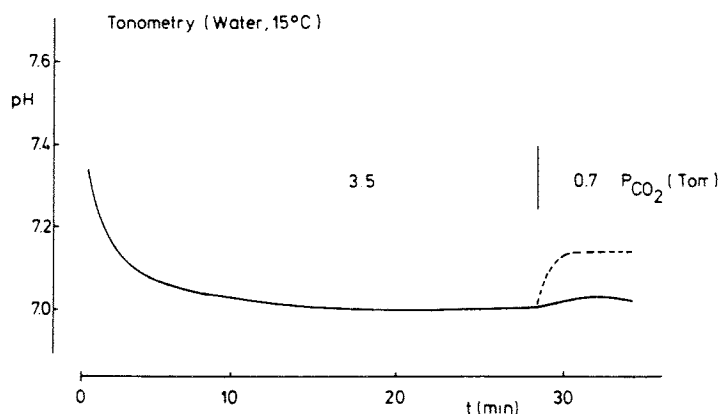


Fig. 1. pH changes in a homogenate prepared from squid mantle muscle (*Illex illecebrosus*) using deionized water as a medium. During equilibration at $P_{CO_2} = 3.5$ Torr a rapid, initial pH drop is observed, exceeding that expected from respiratory acidification (cf. figs. 3 and 4). After a steady state is achieved, the reduction in P_{CO_2} does not lead to the expected alkalosis (broken line).

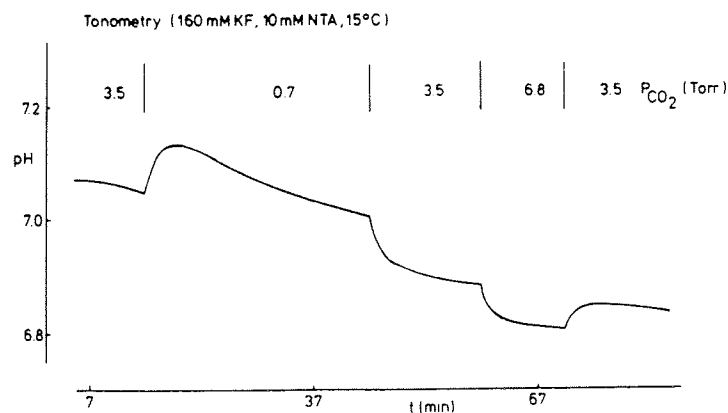


Fig. 2. The medium selected for this homogenate prepared from squid muscle slows down the metabolic response to respiratory alkalosis (transition from 3.5 to 0.7 Torr). A strong trend to continue acidification remains which is reduced by transition to higher P_{CO_2} values. For further explanations see text.

producing processes are stimulated, proton-consuming processes are inhibited and *vice versa*.

If medium was utilized which contained levels of KF and nitrilotriacetic acid, close to intracellular ionic strength, the velocity of compensation for the alkalosis was reduced, but compensation still occurred (fig. 2). If, in a similar medium, P_{CO_2} was first increased (fig. 3) a reduction in the initial pH drift resulted which is consistent with a slow-down in glycolytic rate or with phosphagen depletion caused by the acidosis. After subsequent return to medium P_{CO_2} , the homogenate was equilibrated with low P_{CO_2} . Finally, a discrepancy was observed between pH values before (pH_1) and after (pH_3) this step of equilibration. The increase in the difference between pH_2 and pH_3 as

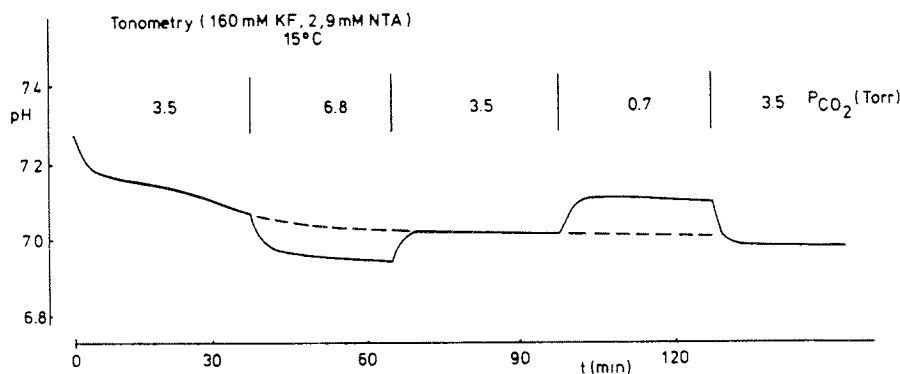


Fig. 3. Incomplete metabolic inhibition causes high P_{CO_2} to reduce the acidotic trend in squid muscle homogenates (transition from 3.5 to 6.8 Torr). If, later on, P_{CO_2} changes from 3.5 to 0.7 Torr the buffer value of the homogenate is reduced during the period of exposure to 0.7 Torr as indicated by the pH change being larger during return to 3.5 Torr (see text).

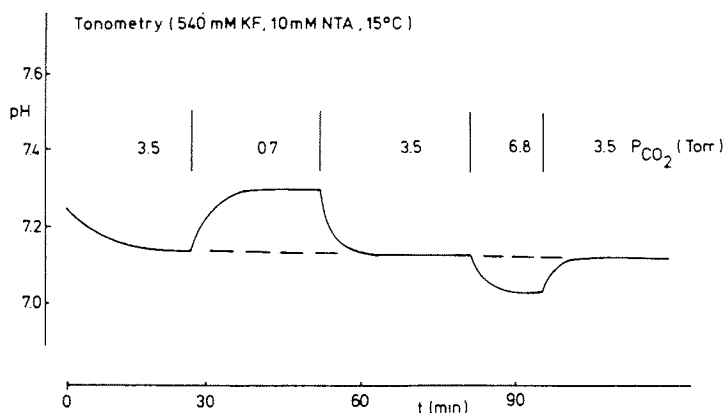


Fig. 4. After an initial metabolic shift the final concentrations of fluoride and NTA in squid mantle muscle homogenates render the new metabolic equilibrium insensitive to P_{CO_2} changes.

compared to the difference between pH_1 and pH_2 suggests that phosphagen was rephosphorylated, leading to a reduction in the buffer value of the homogenate. Obviously media such as this do not fulfill the requirement that metabolism of the homogenate be unaffected by the experimental change in P_{CO_2} .

Generally, the results presented in figs. 1, 2 and 3 lead to the conclusion that, when pH at medium P_{CO_2} returns to previous values after reversal of an experimental increase or decrease in P_{CO_2} , metabolic equilibria may be insensitive to pH changes. Accordingly, fig. 4 suggests that an increase in the levels of fluoride and nitrilotriacetic acid is adequate to minimize the sensitivity of homogenate metabolism to pH changes caused by P_{CO_2} variations. After the initial equilibration period only a small unexplained trend to acidify remained ($\Delta pH/h = -0.02 \pm 0.01$ SD in squid tissue, dilution factor of tissue = 5.17 ± 0.64 SD, $n = 6$) but this pH drift remained constant during subsequent changes in P_{CO_2} and was considered in the calculation procedure. The respective medium could not only be demonstrated to be effective in squid mantle muscle but, possibly owing to lower Mg^{2+} levels in vertebrate tissues, even more so in toad gastrocnemius muscle (fig. 5, $\Delta pH/h = -0.004 \pm 0.006$ SD, dilution factor of tissue = 5.16 ± 0.41 SD, $n = 6$).

Table 2 demonstrates that the concentration difference between essential metabolites was insignificant between the first and last samples of the experiments. Fluctuations exclusively occurred during the first step of equilibration which lasted 20 to 30 min. During this time the phosphagen was 75% depleted, but there was no glycolytic activity, as indicated by constant octopine levels. α -Glycerophosphate was formed to a small extent and ATP levels were depleted by 90%, whereas ADP and AMP accumulated. Consequently, steady state was reached after the initial equilibration period. Measurements of buffer values were now possible without a significant continuation in the accumulation of inorganic phosphate and with metabolism being insensitive to pH. These observations demonstrate the efficiency of the reagents to allow for a correct estimate of homogenate buffer values (see Introduction).

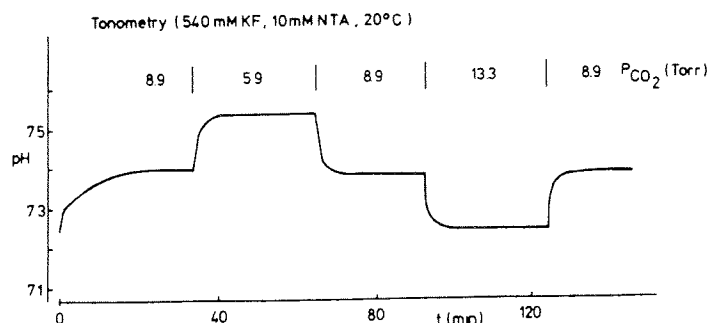


Fig. 5. In toad (*Bufo marinus*) gastrocnemius muscle the initial pH drop is not observed, partly due to the fact that the initial respiratory acidification is suppressed by the addition of bicarbonate to the medium. An accumulation of inorganic phosphate occurs (table 3) indicating a metabolic shift during the first equilibration period.

Factor 3. Traditionally, non-bicarbonate buffering of a tissue is thought to be constant over a wide range of pH since a large number of buffer systems with different pK' values are contributing (Heisler, 1986). This allows to assess buffer values in media of different ionic strengths, assuming that the shift of pK' values does not change the sum of the individual buffer values. For a single one out of these buffer systems, however, calculations have to be performed for the specific pH values reached during the process of buffering. This is valid for the consideration of buffering by fluoride and by inorganic phosphate. Of the two, fluoride contributed less than 2% to the buffer value of

TABLE 2

Levels of high energy phosphates and of octopine ($\mu\text{mol} \cdot \text{g}^{-1}$ wet weight, $\bar{x} \pm \text{SD}$, $n = 5$) in the mantle muscle of squid (*Illex illecebrosus*) compared to the levels found in homogenates of the same tissues after different steps of tonometry (first and last sample in $0.54 \text{ mol} \cdot \text{L}^{-1}$ NaF, $0.01 \text{ mol} \cdot \text{L}^{-1}$ NTA). Data given for "last" were analysed in homogenates prepared under insufficient metabolic inhibition. In these homogenates the depletion of the phosphagen and of ATP is almost complete at the end of the experimental procedure. This observation demonstrates that there is still some protective effect by the reagents on the levels of high energy phosphates. For further explanations see text. (-: not determined, * denotes a significant change compared to intact tissue, * compared to the first sample, $P < 0.05$, Student's t -test).

Sample	Phospho-L-arginine	L-arginine	Octopine	α -glycero-phosphate	ATP	ADP	AMP
Intact tissue	33.8 ± 2.8	14.8 ± 4.2	0.6 ± 0.3	0.1 ± 0.1	6.1 ± 0.4	1.4 ± 0.2	0.1 ± 0.02
first	8.6* ± 3.0	39.8* ± 3.7	-	0.3 ± 0.2	0.5* ± 0.2	2.0 ± 0.6	1.9* ± 0.7
last	6.8* ± 2.8	43.0* ± 5.1	0.5 ± 0.8	0.5 ± 0.6	0.5* ± 0.2	1.9 ± 0.6	1.9* ± 1.1
last' (n = 3)	0.6** ± 0.1	44.1* ± 1.6	3.9* ± 1.6	-	0.09** ± 0.06	0.8** ± 0.3	5.2** ± 1.2

homogenates from toad gastrocnemius and less than 5% to the buffer value of homogenates from squid mantle muscle.

The measured non-bicarbonate buffer values are about 89% higher in squid and about 29% higher in toad muscle than the buffer values calculated for control conditions (table 3). These considerations demonstrate that, after elimination of the risk of measurement errors (see above), the change in buffer values during degradation of organic phosphates must still be taken into account. The drop in organic phosphate levels by itself (*cf.* table 2) is considered unimportant: the buffer value of the adenylate mixture in the homogenate is not very much different from that in the intact cell ($\Delta\beta = +0.4 \text{ mmol} \cdot \text{pH}^{-1} \cdot \text{kg}^{-1}$ at $\text{pH} = 7.3$, calculated based on pK' values compiled by Pörtner *et al.*, 1984). The decrease in phosphagen levels in the homogenate represents a drop in buffer value by $0.05 \text{ mmol} \cdot \text{pH}^{-1} \cdot \text{kg}^{-1}$ (at $\text{pH} = 7.3$). These differences are small in relation to the magnitude of the total buffer value and can, therefore, be neglected. Thus, no other process except the accumulation of inorganic phosphate is essential for the quantification of the metabolic influences on homogenate buffering. Although these processes are most important in white muscle tissues, changes in the levels of free inorganic phosphate should also be monitored in aerobic tissues with minor amounts of phosphagen. In the rat ventricle, for example, an increase in free phosphate levels by about $30 \text{ mmol} \cdot \text{L}^{-1}$ cell water may result from the complete degradation of creatine phosphate and adenylates, leading to a potential overestimate of the non-bicarbonate buffer value by about 60 to 70% (based on the buffer value measured by Gonzalez *et al.*, 1979).

The subtraction of the buffer value of inorganic phosphate found in the ultrafiltrates yields the non-bicarbonate, non-phosphate, buffer value of the tissue (table 3). This value is of some physiological importance since it represents the constant fraction of the non-bicarbonate buffer value. Depending on the degree of inorganic phosphate

TABLE 3

The amount of inorganic phosphate (measured $\text{P}_{i, \text{free}}$, $\mu\text{mol} \cdot \text{g}^{-1}$ wet weight) found in homogenate ultrafiltrates ($0.54 \text{ mol} \cdot \text{L}^{-1}$ KF, $0.01 \text{ mol} \cdot \text{L}^{-1}$ NTA) permits to calculate the non-bicarbonate (NB), non-phosphate, (NP_i) buffer value (β , $\text{mmol} \cdot \text{pH}^{-1} \cdot \text{kg}^{-1}$, $\bar{x} \pm \text{SD}$, $n = 5$ for squid, $n = 6$ for toad) of intact tissue from the measured values of β_{NB} by subtraction of the phosphate buffer value. Adding the buffer value of free inorganic phosphate in the tissue ($\text{P}_{i, \text{free}}$ control, $\mu\text{mol} \cdot \text{g}^{-1}$ wet weight) leads to β_{NB} of intact tissue under control conditions (see text), and finally to $\beta_{i\text{NB}}$ (control) of the intracellular space ($\text{mmol} \cdot \text{L}^{-1}$ cell water). pHi values for the calculation of phosphate buffer values from $\text{P}_{i, \text{free}}$ (control) and values for the fractional volume of tissue and cell water were adopted from Pörtner *et al.* (1990).

Species	$\text{P}_{i, \text{free}}$ measured	$\text{P}_{i, \text{free}}$ control	β_{NB} measured	pH range	$\beta_{\text{NB, NP}_i}$	β_{NB} control	$\beta_{i\text{NB}}$ control
<i>Illex</i> (15 °C)	39.7 ± 3.4	≤ 6	33.5 ± 5.4	6.9–7.3	16.0 ± 5.1	≤ 17.7 ± 5.1	25.8
<i>Bufo</i> (20 °C)	20.9 ± 2.5	1.6	25.7 ± 4.9	7.1–7.6	19.1 ± 4.8	19.8 ± 4.8	29.8

accumulation the buffer value of a tissue may increase during functional or environmental hypoxia (Pörtner, 1986).

The contribution of inorganic phosphate to intracellular buffering in the intact tissue under normoxic resting conditions can be calculated from a consideration of free intracellular inorganic phosphate levels and of pK'_2 values valid for the intracellular space ($pK' = 6.80$ for squid and 6.81 for toad muscle, *cf.* table 1, based on values for intracellular ionic strength selected by Pörtner *et al.*, 1990). For squid muscle a maximum value of around $6 \mu\text{mol} \cdot \text{g}^{-1}$ wet weight of inorganic phosphate may be valid as was found by ^{31}P -NMR in other (isolated) molluscan muscle preparations (*e.g.*, Graham *et al.*, 1986). Even lower levels may be possible since Schank *et al.* (1986) detected less than $0.5 \mu\text{mol} \cdot \text{g}^{-1}$ wet weight in *Mytilus edulis* muscle tissue. In resting toad gastrocnemius muscle Pörtner and Toews (in preparation) found $1.6 \mu\text{mol} \cdot \text{g}^{-1}$ wet weight by enzymatic analysis after tissue extraction, a value which is close to the free level of $2 \mu\text{mol} \cdot \text{g}^{-1}$ wet weight found in frog muscle by use of NMR (Tanokura and Yamada, 1984). Consequently, the contribution of inorganic phosphate to cellular buffering is low under control conditions and does not exceed $1\text{--}2 \text{mmol} \cdot \text{pH}^{-1} \cdot \text{kg}^{-1}$ (for a comparison of non-bicarbonate, non-phosphate, buffer values and non-bicarbonate buffer values see table 3).

The question arises as to how the buffer values presented in table 3 compare to those obtained by using other techniques? The value for squid mantle muscle is far below the one of $50 \text{mmol} \cdot \text{pH}^{-1} \cdot \text{kg}^{-1}$ found by Morris and Baldwin (1984). This difference can be attributed to the lack of metabolic control in their homogenates. It is in the same order of magnitude as the difference (by a factor of 2.8) only recently described between high buffer values obtained by fixed acid titration of uncontrolled homogenates and much lower values obtained by use of ^{31}P -NMR (Wiseman and Ellington, 1989). Actually, the buffer value for squid ($17.7 \text{mmol} \cdot \text{pH}^{-1} \cdot \text{kg}^{-1}$) is close to the one ($16.4 \text{mmol} \cdot \text{pH}^{-1} \cdot \text{kg}^{-1}$) found in the study of Wiseman and Ellington for another molluscan (*Busycon canaliculum*) muscle. For isolated amphibian muscle, the techniques used include ^{31}P -NMR (Tanokura and Yamada, 1984) and microelectrodes (Curtin, 1986), both being applied to frog sartorius. In both studies was a buffer value close to the one found in toad gastrocnemius obtained. In the NMR study ($18 \text{mmol} \cdot \text{pH}^{-1} \cdot \text{kg}^{-1}$) the authors took the proton absorption by inorganic phosphate accumulating during 5 sec of tetanic contraction into account. In the study of Curtin (1986) a mean value of $36.2 \text{mmol} \cdot \text{pH}^{-1} \cdot \text{L}^{-1}$ cell water equivalent to $21 \text{mmol} \cdot \text{pH}^{-1} \cdot \text{kg}^{-1}$ wet weight was obtained during alkalization with NH_3 . These comparisons also demonstrate that the non-bicarbonate buffer values given in table 3 for the whole tissue are very likely to be determined by the intracellular β_{NB} (control). In support of this conclusion, the extracellular contribution to tissue buffering is minor. The error in neglecting this contribution is 0.4% in squid and 0.7% in toad muscle (calculated based on Pörtner *et al.*, 1990, their table 5).

Conclusions. The estimate of intracellular buffer values β_{NB} in tissue homogenates under metabolic control strongly suggests that buffer values have largely been overesti-

mated in many studies described in the literature, both due to measurement errors and the influence of accumulating inorganic phosphate (Pörtner, 1989). Measurement errors can be eliminated during metabolic control of the homogenate by the application of fluoride and nitrilotriacetic acid. After metabolism has shifted towards a new equilibrium, the metabolic compensation of pH changes provoked by titration ceases. Correction for the shift in metabolism is possible by quantifying the influence of free inorganic phosphate levels on the analysed buffer values. Finally, this procedure leads to the intracellular non-bicarbonate buffer value of the intact tissue under resting conditions.

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