J. Physiol. (1972), **221**, *pp.* 15–27
With 6 text-figures
Printed in Great Britain

INTRACELLULAR pH, H ION FLUX AND H ION PERMEABILITY COEFFICIENT IN BULLFROG TOE MUSCLE

By K. T. IZUTSU*

From the Department of Physiology and Biophysics, University of Washington School of Medicine, Seattle, Washington 98105, U.S.A.

(*Received 27 April 1971*)

SUMMARY

1. The dimethyloxazolidinedione (DMO) technique was used to estimate intracellular pH (pH_i) in bullfrog toe muscles incubated *in vitro*. The control value of pH_i was 7·16+ ± 0·01 (S.D.).

2. pH_i was affected by changes in P_{CO_2} and external bicarbonate ion concentration ($[HCO_3^-]_0$). At a given P_{CO_2}, decreasing the external $[HCO_3^-]$ was more effective in lowering pH_i than increasing the external $[HCO_3^-]$ was in increasing pH_i.

3. On the assumption that the changes in pH_i were due to hydrogen ion (H^+) movements across the membrane, a H^+ flux of 10^{-13} mole/cm^2. sec was calculated. The corresponding H^+ permeability coefficient was 10^{-3} cm/sec.

4. The variability of the tissue CO_2 buffer value was examined.

INTRODUCTION

There are currently three methods used for estimating intracellular pH (pH_i): the DMO (dimethyloxazolidinedione), the carbonic acid (CO_2), and the glass micro-electrode techniques. Although the theory behind each of these techniques is straightforward, there is a great deal of controversy in interpreting the resulting data (Waddell & Bates, 1969; Carter, Rector, Campion & Seldin, 1967). In general, there is at least one major criticism that may be applied to each of the three techniques. If these criticisms are valid, then pH_i is approximately 6·0 in agreement with the value predicted from a passive distribution of hydrogen ions (H^+s). If these criticisms are invalid then pH_i is approximately 7·0. Since this value is higher than the value expected for a passive distribution of H^+s, and if the membrane is permeable to H^+ and/or bicarbonate ions (HCO_3^-s), then an

* Present address: K. T. Izutsu, Center for Research in Oral Biology, University of Washington School of Dentistry, Seattle, Washington 98105, U.S.A.

additional active outward H^+ (or inward HCO_3^-) flux must be postulated. Thus, if the validity of the techniques for measuring pH_i can be established, then the fundamental question concerning the active or passive distribution of H^+s across the cell membrane can be answered.

There are a number of ways to test this validity of the current techniques. One method would be to test each of the criticisms individually. This is currently being pursued in some laboratories (Butler, Poole & Waddell, 1967; Campion, Carter, Rector & Seldin, 1967; Paymaster & Englesson, 1966). Another tack would be to compare the results of the three techniques in a given tissue. Since the criticisms for each of the techniques are fundamentally different, it would be highly improbable that all three techniques would yield the same value of pH_i if the criticisms are valid. Thus, by comparing the three techniques in a given tissue, one should be able to reach some conclusion on the validity of the techniques. If this question could be settled, the study of the factors regulating the distribution of H^+s could then proceed.

Rationale of the present experiments. The CO_2 (Fenn & Maurer, 1935) and the glass micro-electrode techniques (Kostyuk & Sorokina, 1961) have previously been used to estimate the pH_i of frog skeletal muscle. Hence it is efficient to apply the DMO technique to this tissue also. In the present experiments, the pH_i of bullfrog (*Rana catesbeiana*) skeletal muscle (flexor IV) incubated *in vitro* was estimated using the DMO technique. The effects of external DMO concentration, muscle mass, duration of incubation, bicarbonate ion concentration, and CO_2 concentration (P_{CO_2}) on the calculated pH_i were investigated.

METHODS

Bullfrog toe muscles were incubated *in vitro* under various acid/base conditions. The pH_is of these muscles were calculated from their uptake of [$^{14}C_2$] DMO and [$^3H_1$-methoxy] inulin.

Incubation procedure. The frogs were stunned with a quick blow to the back of the head and pithed. Their feet were then cut off and the skin removed. During dissection of the toe muscle, care was taken to leave enough tendon attached for easy handling. After two pairs of muscles were dissected, crossed pairs were transferred to Warburg flasks to incubate. The average dissection time was 15 min.

Warburg flasks with side arms capped with rubber serum tube stoppers were used. This arrangement permitted sampling of the flask contents without exposing them to the atmosphere. Fluid samples were withdrawn directly into a Radiometer capillary pH electrode (type E5021a) and their pHs measured anaerobically. The pH electrode was calibrated using Radiometer precision buffers (S 1500 and S 1510).

The flasks were continuously gassed and shaken at about 110 cycles per minute during incubation in a Warburg apparatus. The gas was saturated with water vapour prior to being admitted to the flasks. All incubations were done at room temperature which varied between 23·5 and 26° C during the course of the year.

Changes in the pH of the medium during incubation were negligible.

Sample preparation. After the sample was incubated for the designated period of time, the pH of the incubation medium was measured. The muscles were then removed, blotted and placed in counting vials. These vials were then weighed and placed in a glass desiccator and dried under a 10 mm Hg vacuum at 40° C for 36 hr (steady weight was reached after 12 hr). The samples were reweighed and total muscle water was calculated from the difference between wet and dry weights.

100 μl. of distilled water and 1 ml. toluene were added to each vial and the tissue was solubilized by heating the vials for 2 hr at 60° C. After cooling, 50 μl. glacial acetic acid were added to each vial in order to reduce thermal and photoactivation during counting (Herberg, 1958; Vaughan, Steinberg & Logan, 1957). Finally, 15 ml. of a scintillation cocktail containing 6 g 2,5-diphenyloxazole (PPO) and 0·5 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl-benzene) (M_2 POPOP) per litre toluene were added to each vial. The samples were refrigerated until they were counted.

The two isotopes were counted simultaneously on a Packard Tri-Carb liquid scintillation counter. Each sample was counted several times and probable counting errors were less than 1 %. Counting efficiencies were determined in each vial by the internal standard method. A control experiment was performed to verify that no radioactivity was lost in the tissue preparation procedure.

In the bullfrog, a second muscle bundle which may be easily overlooked is often found attached to the flexor IV. One experiment (six muscle pairs) was performed to verify that the presence or absence of this muscle bundle did not affect the calculated pH_i.

The calculation of pH_i. pH_i was calculated from the following equation:

$$pH_i = pK + \log([C_r(1+V_r) - V_r][10^{pH_o - pK} + 1] - 1, \quad (1)$$

where pH_i = intracellular pH.

pK = $-\log K$ where K is the equilibrium constant for the dissociation of DMO.

pH_o = extracellular pH.

$C_r = C_T/C_e$ where C_T is the total DMO in the muscle divided by the total water of the muscle, and C_e is the concentration of DMO in the extracellular space.

$V_r = V_e/V_i$ where V_e is the extracellular water volume of the muscle and V_i is the intracellular water volume.

This equation was first described by Waddell & Butler (1959) and its derivation is given in Irvine, Saunders, Milne & Crawford (1960).

The parameters of the above equation were determined as follows. The [$^{14}C_2$] DMO concentration and the pH of the medium were measured directly for each flask. The extracellular water of the muscle was obtained by dividing the tritiated inulin content of the muscle by the concentration of tritiated inulin in the external medium. The total muscle water was determined from the difference in the weight of the sample before and after desiccation. The intracellular water was taken as the difference between total muscle water and extracellular water. The total DMO content was measured by counting the ^{14}C radioactivity. pK was taken as 6·27 (Steinmetz, 1969).

Composition of the solutions. Incubation medium (2 ml.) was put in each Warburg flask. The control medium contained 24 mM sodium bicarbonate, 96·4 mM sodium chloride, 2·5 mM potassium chloride, 1·8 mM calcium chloride and 5 mM glucose. Solutions with different bicarbonate ion concentrations were adjusted so that the sum of the sodium chloride and the sodium bicarbonate concentrations was equal to 120·4 mM (these solutions will be referred to by their respective bicarbonate ion concentrations). There were three types of variations of the incubation parameters

during incubation: (i) P_{CO_2} was held constant and the $[HCO_3^-]$ was varied ('metabolic' variations), (ii) the $[HCO_3^-]$ was held constant and P_{CO_2} was varied ('respiratory' variations), and (iii) both P_{CO_2} and the $[HCO_3^-]$ were varied.

The effect of varying the external DMO concentration on pH_i. [$^{14}C_2$] DMO (0·35 mc/ml. and [$^3H_1$-methoxy] inulin (2·95 μc/ml.) were added to each flask resulting in tissue samples with activities of at least 2000 counts/min. (The background activity was about 50 counts/min in each channel.) The radioisotopes were obtained from New England Nuclear (Boston, Mass.) and International Chemical and Nuclear Corporation (Irvine, Cal.).

To test for tissue binding of DMO, experiments were performed in which a large amount of unlabelled DMO was added to the incubation medium. This DMO was first titrated to neutral pH with 2 N-NaOH. The final concentration of DMO in these 'loaded' solutions was about 50 × the DMO concentration of the control solutions. There were no significant differences between the pH_i of muscles incubated in either solution under control conditions and under conditions of metabolic acidosis and alkalosis. The DMO concentration of the control solutions was approximately 50 μM.

RESULTS

Two types of experiments were performed. The initial experiments were designed to test the experimental procedure, i.e. the effects of the DMO concentration and of muscle mass on the calculated pH_i. The second group of experiments was designed to measure the transient and steady-state effects of the $[HCO_3^-]_o$ and P_{CO_2} on pH_i.

The relationship between muscle mass and pH_i. Calculations were made to determine the relationship between muscle mass and pH_i to test for the possibility of diffusion, limitation of oxygen or of other metabolites affecting the results. There was no correlation between pH_i and muscle weight over a fourfold range in any instance.

Temporal variations of pH_i. The effect of the length of incubation on the average pH_i of groups of muscles exposed to different external conditions was also investigated. If the $[HCO_3^-]_o$ was 24 mM or greater at 5 % CO_2 ($pH_o \geqslant 7{\cdot}35$) then the pH_i rapidly attained a steady value. Under these conditions, the pH_i as calculated after 1 hr of incubation was not significantly different from that after 8 and 10 hr of incubation (Figs. 1 and 2). In fact, experiments performed using solutions with $[HCO_3^-]_o$s of 24 and 50 mM (pH_os of 7·30 and 7·61) equilibrated with 5 % CO_2 indicated that the pH_i reached a steady state after about 15 min of incubation. For higher P_{CO_2}s, higher $[HCO_3^-]_o$s may be required to prevent a change of pH_i.

By contrast, pH_i changed to a new steady value in muscles incubated in solutions with $[HCO_3^-]$s of less than 24 mM at 5 % CO_2. In every instance, the pH_i after 1 hr of incubation was significantly higher than that after 6 and 8 hr of incubation (Figs. 1 and 2). Approximately 5 hr were required for pH_i to reach a steady state. A time constant of 2·3 hr was measured for muscles incubated in 5 mM bicarbonate equilibrated with 5 % CO_2. Similar time constants were indicated for muscles incubated in solutions

with $[HCO_3^-]$ = 10 mM and equilibrated with P_{CO_2}s of 1, 5, and 13 % in oxygen. Consequently, incubation times of 7 hr were used in all cases to insure a steady-state condition.

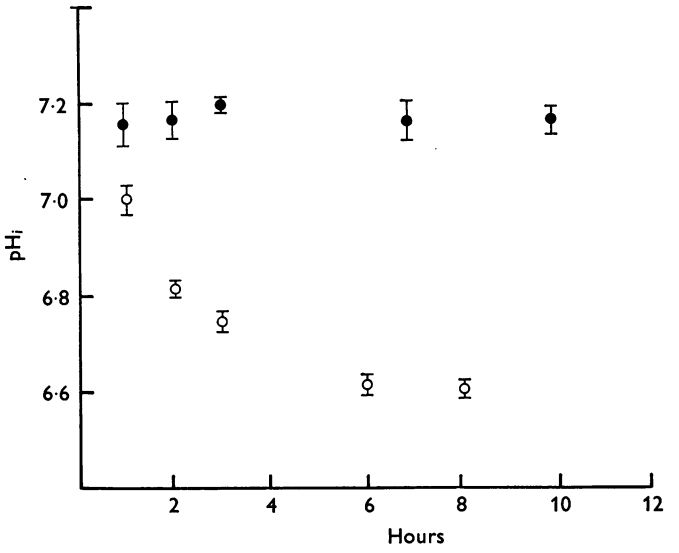

Fig. 1. The effect of the duration of incubation on the pH_i of muscles incubated under different conditions. The filled circles represent the results from muscles incubated under control conditions ($[HCO_3^-]_o$ = 24 mM, P_{CO_2} = 5 %, pH_o = 7·30). The open circles represent the results from muscles incubated under a condition of 'metabolic' acidosis ($[HCO_3^-]_o$ = 5 mM, P_{CO_2} = 5 %, pH_o = 6·64). Each point represents the mean ± S.E. of the mean of at least four muscles.

The effect of varying $[HCO_3^-]_o$ *at constant* P_{CO_2} *on* pH_i. A number of experiments were performed in which muscles were incubated in solutions with different $[HCO_3^-]_o$s equilibrated with 5 % CO_2 in oxygen. The results are shown in Fig. 3. These data also indicate that muscles exposed to a decreased $[HCO_3^-]_o$ (at constant P_{CO_2}) become acidic while muscles exposed to increased $[HCO_3^-]$s (at constant P_{CO_2}) maintain a constant pH_i.

The effect of varying P_{CO_2} *at constant* $[HCO_3^-]_o$ *on* pH_i. The results of a number of experiments in which muscles were incubated in a 24 mM-HCO_3^- solution equilibrated with different P_{CO_2}s are also shown in Fig. 3. Again pH_i changed with pH_o. The slope of the line for changes in the acid direction is the same as that obtained by varying the $[HCO_3^-]_o$ at constant P_{CO_2} (0·86). For changes in the alkaline direction, pH_i increased more slowly. This contrasts with the metabolic changes where pH_i is nearly independent of pH_o for $pH_o > 7·3$.

The effect of varying $[HCO_3^-]_o$ *and* P_{CO_2} *on* pH_i. Both $[HCO_3^-]_o$ and P_{CO_2} were varied in a final set of experiments. The results of these experiments are shown in Figs. 4 and 5. Figure 4 shows the steady-state relationship between pH_i and pH_o under various conditions. The percentages in Fig. 4 are the P_{CO_2}s for each of the curves. The figure adjacent to each point is the $[HCO_3^-]_o$ (mM). In Fig. 5, the results have been replotted on a pH–$[HCO_3^-]$ diagram; however, the plot is of external bicarbonate against internal pH with P_{CO_2} as a parameter.

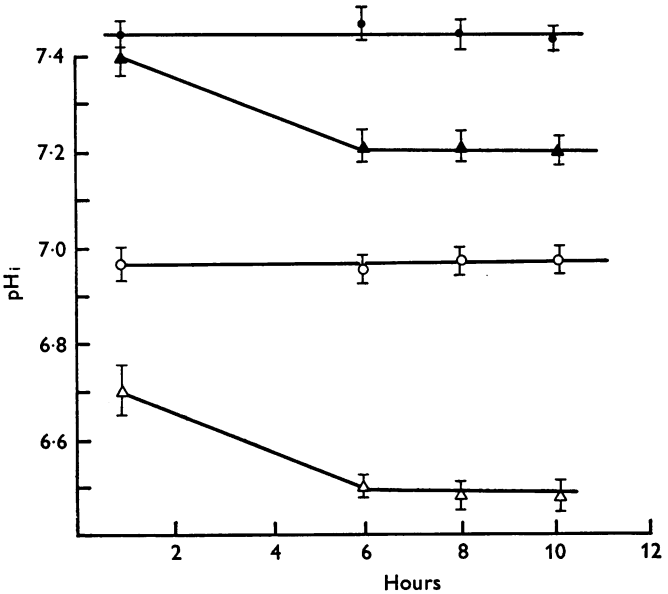

Fig. 2. The effect of the duration of incubation on the pH_i of muscles incubated under various acid–base conditions. The circles represent the results obtained with muscles incubated in a 50 mM-HCO_3^- solution. The triangles represent the results obtained with muscles incubated in a 5 mM-HCO_3^- solution. The filled symbols indicate solutions equilibrated with 1 % CO_2–99 % O_2. The open symbols indicate solutions equilibrated with 13 % CO_2–87 % O_2. Each point represents the mean ± S.E. of mean of four muscles. pH_o was 7·24 and 6·34 for the 5 mM-HCO_3^- solution equilibrated with 1 and 13 % CO_2 respectively. pH_o was 8·16 and 7·23 when the 50 mM HCO_3^- solution was equilibrated with 1 and 13 % CO_2 respectively.

A striking pattern is observed in Fig. 5. Lowering the pH of the medium at constant P_{CO_2} (lowering $[HCO_3^-]_o$) results in a decrease in pH_i. However, pH_i remains constant for an increase in $[HCO_3^-]_o$. The $[HCO_3^-]_o$ at which this plateau is attained appears to depend on the P_{CO_2} in that higher P_{CO_2}s seem to require higher $[HCO_3^-]$s.

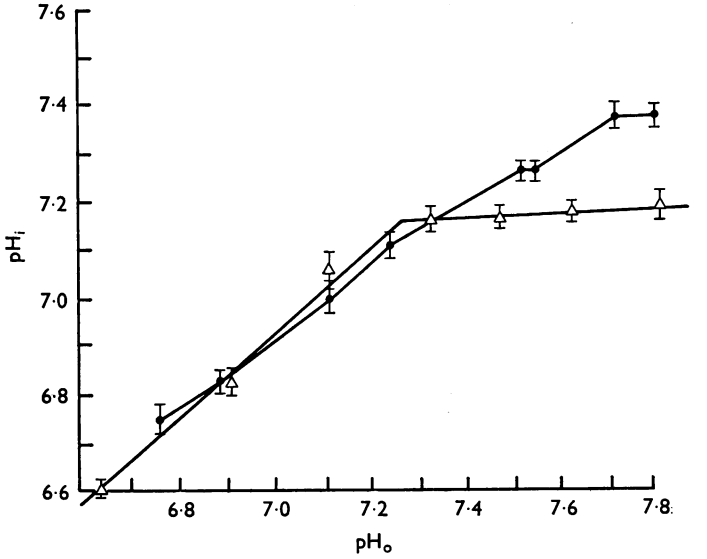

Fig. 3. A comparison of the effects of 'metabolic' and 'respiratory' changes on muscle pH_i. The triangles represent the results obtained from muscles incubated in solutions with various $[HCO_3^-]$s at constant P_{CO_2} (metabolic changes). The filled circles represent the results obtained from muscles incubated in solutions with various P_{CO_2}s at constant $[HCO_3^-]$ (respiratory changes). All points are the mean ± S.E. of mean of at least six muscles.

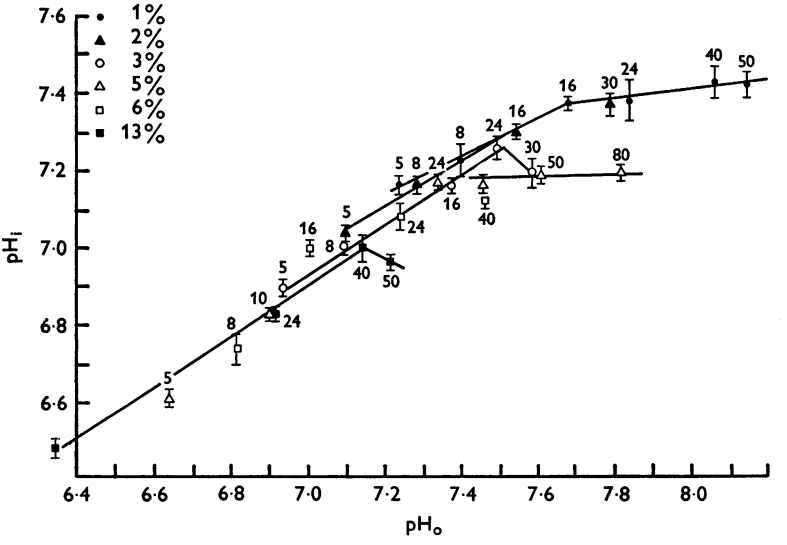

Fig. 4. The steady-state relationship between pH_i and pH_o. The key indicates the P_{CO_2}s with which the various solutions were equilibrated. The numbers above each of the points are $[HCO_3^-]_o$s (mM). Each point represents the mean ± S.E. of mean of at least six muscles.

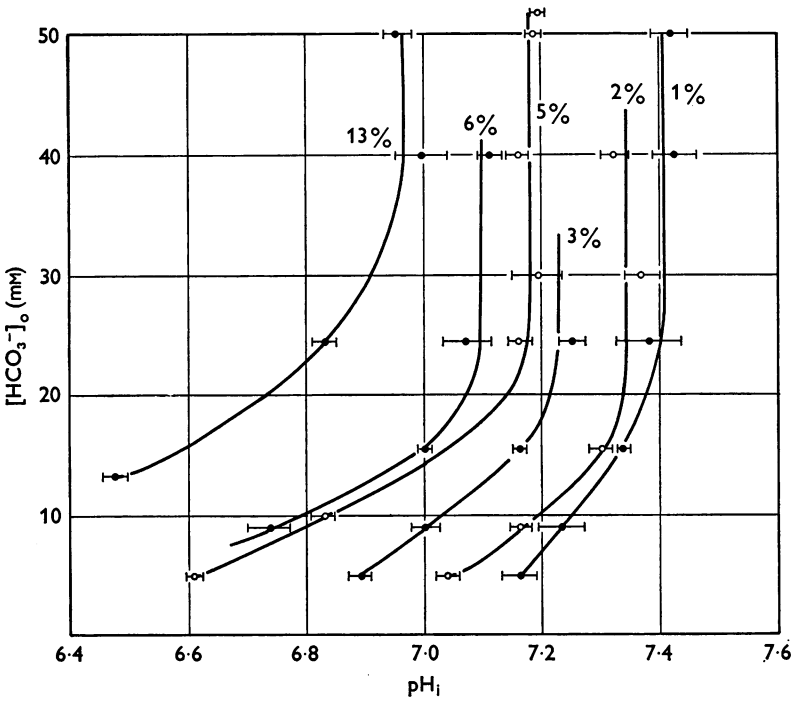

Fig. 5. The steady-state relationship between pH_i and $[HCO_3^-]_o$ at constant P_{CO_2}s. The P_{CO_2}s with which the solutions were equilibrated are indicated as percentages on the upper portion of each of the curves. Filled and open circles have been used on alternate curves so as to keep the points from becoming confused. Each point represents the mean ± S.E. of mean of at least six muscles.

DISCUSSION

Control value of pH_i. The results show that pH_i is affected by changes in the $[HCO_3^-]_o$ and by changes in P_{CO_2}. Thus in order to estimate the pH_i *in vivo*, it is necessary to know the P_{CO_2} and the $[HCO_3^-]$. If the P_{CO_2} and $[HCO_3^-]$ of frog blood *in vivo* are 5% and 24 mM respectively, then according to Fig. 5, the pH_i of frog skeletal muscle *in vivo* is 7·16. This value is in agreement with previous *in vitro* measurements of the pH_i of frog skeletal muscle using glass micro-electrodes (7·16) and the DMO (7·2) and CO_2 techniques (~ 7·0) (Kostyuk & Sorokina, 1961; Bianchi & Bolton, 1967; Fenn & Maurer, 1935; Ferguson & Irving, 1929; Root, 1933). In addition, Fenn (1928) using the CO_2 method and Kostyuk & Sorokina (1961) using micro-electrodes measured the effect of varying the $[HCO_3^-]_o$ on pH_i. Their results are in agreement with the present results in the region of overlap and thus may be taken as evidence in support of the general validity of all three techniques.

Recent as well as classical experiments indicate that the *in vivo* P_{CO_2} of the frog is of the order of 3% rather than 5% (Reeves, 1969; Fenn, 1928; Wastl & Seliskar, 1925). If this is the case, then an *in vivo* pH_i of 7·25 would be expected (Fig. 5). Thus, frog skeletal muscles appear to be slightly more alkaline than mammalian skeletal muscles.

Calculation of hydrogen and bicarbonate ion permeabilities. If it is assumed that the observed temporal changes in pH_i are due to the movement of H^+s (or HCO_3^-s) across the surface membrane, then a minimum, average membrane permeability coefficient for H^+s (or HCO_3^-s) may be calculated. Assuming that the changes in pH_i are due to a movement of H^+s, the net H^+ flux (M) may be calculated from the following expression:

$$M = \frac{(\Delta pH)\ (\text{buffer value})\ (V/A)}{\Delta t}. \tag{2}$$

The expression ΔpH represents the change in pH_i over a given time period, Δt. V/A represents the ratio of cellular volume to cellular surface area. Assuming a cylindrical cell and an average cell diameter of 40 μ yields V/A equal to 10×10^{-4} cm. The magnitude of the intracellular buffer value is not well established. Assuming a buffer value of 10 slykes yields a flux of approximately $2{\cdot}5 \times 10^{-13}$ mole/cm^2 sec for the case depicted in Fig. 1.

From the constant field and non-steady-state assumptions, the following expression may be obtained for the net passive flux:

$$M = \frac{PZ\beta E_m(C_o - C_i e^{Z\beta E_m})}{1 - e^{Z\beta E_m}}. \tag{3}$$

In this expression M is the passive flux. P is the permeability coefficient of the ion in question, C_o and C_i are the external and internal concentrations of that ion, and Z is its valence. E_m is the potential across the cell membrane. β is FZ/RT where F is the Faraday, R is the gas constant, and T is the absolute temperature.

The net flux is the sum of the passive and active fluxes (the fact that there must be an active efflux is established by the fact that the measured pH_i (7·16) is greater than the value predicted from the Nernst equation (6·0)). In the present case, the passive flux is opposite in direction to the active flux. If the active flux is ignored, then the above expression for the passive flux may be set equal to the observed flux, and the expression may be solved for the H^+ permeability (P_H). In this case, the calculated permeability will be smaller than the actual permeability since the passive flux is larger than the net (observed) flux, and P_H will be underestimated accordingly.

Solving the above expression yields $P_H \simeq 10^{-3}$ cm/sec. The potassium ion permeability of frog muscle is about 1×10^{-6} cm/sec (Hodgkin & Horowicz, 1959). Thus it can be seen that if the above pH_i changes are due to H^+ movements, then the frog muscle membrane is extremely permeable to H^+s. If the above calculation is repeated for HCO_3^-s, a permeability of approximately 3×10^{-8} cm/sec is obtained.

The above value of P_H is in agreement with the value calculated from membrane voltage and resistance changes by Woodbury (1971; Woodbury, White, Mackey & Weakly, 1968). This agreement in the results of two radically different techniques for the calculation of the hydrogen ion membrane permeability coefficient is evidence in support of the present interpretation of the results.

The concept of passive and active H^+ (or HCO_3^-) fluxes for the regulation of pH_i has been considered in the past (Caldwell, 1958; Waddell & Bates, 1969) but the present calculation of P_H from a temporal variation of pH_i during metabolic acidosis

is unique. In addition it is noted that this temporal variation in pH_i cannot be the result of changes in metabolism because the currently recognized effects of pH_o on metabolism are opposite in direction to what would be required to explain the present results (Fletcher & Hopkins, 1907; Kerly & Ronzoni, 1933; Hill, 1955; Bendall, 1960; Novotny, 1968).

Tissue CO_2 titration curve. The tissue CO_2 titration curve is defined by Davenport (1958) as the relationship between the $[HCO_3^-]_i$ and the pH_i when the muscle is titrated by varying the CO_2 tension. Fig. 6 shows my results. The slope of the line connecting the results from muscles incubated in the same $[HCO_3^-]_o$ at different P_{CO_2}s is the negative of the tissue buffer value. The line itself is the CO_2 titration curve. The different symbols represent the different $[HCO_3^-]_o$s of the incubation solutions.

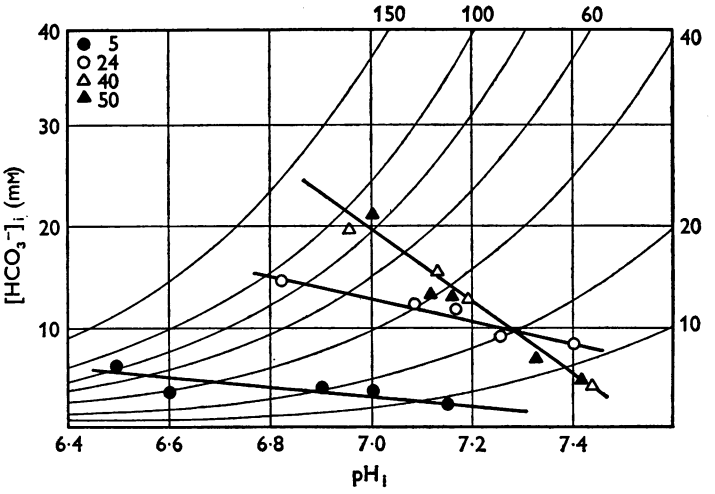

Fig. 6. The steady-state relationship between $[HCO_3^-]_i$ and pH_i following CO_2 titration with $[HCO_3^-]_o$ held constant. The various symbols represent the results obtained with the different $[HCO_3^-]_o$s (mM) indicated in the key. The negative of the slopes may be taken as the non-carbonic acid buffer values of the tissue. The lighter curves represent CO_2 isobars and are the graphical solutions of the Henderson-Hasselbach equation. The numbers along the upper and right hand margins are the CO_2 tensions expressed in millimetres of mercury for each of the isobars (after Davenport, 1958).

The results included in Fig. 6 can be divided into two groups. The one group includes all those muscles incubated in solutions with high $[HCO_3^-]$s and which maintained constant pH_i. The line with the steepest slope (that marked with triangles) in Fig. 6 represents these points. The slope of this line is approximately 38 slykes. It is experimentally indistinguishable from the theoretical titration curve of muscle as calculated by Woodbury (1965). The other group of results in Fig. 6 includes all of those points that are not on the theoretical titration line. This includes the results from those muscles incubated in solutions with $[HCO_3^-] < 38$ mM. As can be seen from Fig. 6, these points do not lie on a single line, rather a spectrum of lines is

obtained. The slopes of these lines decrease with decreasing $[HCO_3^-]_o$. Clearly, the buffer value for CO_2 titration of the muscle cannot vary with the $[HCO_3^-]_o$. Thus, this result must be due to an interaction of the pH changes caused by CO_2 titration and the pH changes caused by ionic fluxes.

DMO and nicotine. There is some question as to the meaning of the pH_i as calculated from the distribution of a weak acid such as DMO. It is agreed that because of cellular heterogeneity, a weak base would be a better indicator of cytoplasmic pH than a weak acid (Waddell & Bates, 1969). Recently nicotine has been proposed as a basic indicator for pH_i measurements (Adler, 1970). However, because nicotine causes the release of calcium ions from intracellular binding sites (Weiss, 1966), because a significant fraction of the cellular nicotine may be bound (Weiss, 1968*a*), and because nicotine may alter the normal pH_i of frog skeletal muscle (Weiss, 1968*b*); it is probably poorly suited for the task. In addition, the fact that the pH_i as indicated by nicotine is unaffected by respiratory acidosis (Adler, 1970) raises another serious doubt about the meaning of the nicotine calculation.

The fact that DMO yields an average pH_i that is higher than the arithmetic mean of the various intracellular compartments need not invalidate the above calculation of P_H. The relative volumes are such that even if the intramitochondrial pH were as high as 8·5, the cytoplasm would still make an equal contribution to the calculation of the mean. Even if this were not the case, the rate of H^+ permeation through the mitochondrial membrane is so high (Chance, Lee & Mela, 1967) that penetration of the surface membrane is probably the rate limiting step during the ionic redistribution period in metabolic acidosis. Thus the above calculation for P_H yields the surface membrane permeability coefficient as desired.

I would like to thank Dr J. W. Woodbury for his critical comments on the manuscript as well as for his efforts on my behalf during my predoctoral training. I would also like to thank Dr D. P. Simpson for his help in executing these experiments and for his critical comments on the manuscript. Special thanks to Miss G. Ward for excellent laboratory assistance, to Mrs S. Morton for assistance in preparing the manuscript, to Dr K. Magleby for assistance in writing the computer programs, to Dr C. Lenfant for providing the calibrated gas mixtures, and to Drs J. Conrad, T. Hornbein, and B. R. Fink for useful discussion and much encouragement.

This work was supported in part by USPHS grants 5 T01 GMOO 739, 5 RO1 NSO 1752, 5 PO7 FROO 374, and 1 RO1 AMO 9822.

REFERENCES

ADLER, S. (1970). Simultaneous measurement of intracellular pH with a weak acid and a weak base. *Clin. Res.* **18**, 493.

BENDALL, J. R. (1960). Post mortem changes in muscle. *The Structure and Function of Muscle*, vol. 3, ed. BOURNE, G. H. New York: Academic Press.

BIANCHI, C. P. & BOLTON, T. C. (1967). Action of local anaesthetics on coupling systems in muscle. *J. Pharmac. exp. Ther.* **157**, 388–405.

BUTLER, T. C., POOLE, D. T. & WADDELL, W. J. (1967). Acid labile carbon dioxide in muscle: its nature and relationship to intracellular pH. *Proc. Soc. exp. Biol. Med.* **125**, 972–974.

CALDWELL, P. C. (1958). Studies on the internal pH of large muscle and nerve fibres. *J. Physiol.* **142**, 22–62.

CAMPION, D. S., CARTER, N. W., RECTOR, F. C., JR. & SELDIN, D. W. (1967). Intracellular distribution of C^{14}-DMO. *Clin. Res.* **15**, 76.

CARTER, N. W., RECTOR, F. C., JR., CAMPION, D. S. & SELDIN, D. W. (1967). Measurement of intracellular pH with glass microelectrodes. *Fedn Proc.* **26**, 1322–1326.

CHANCE, B., LEE, C. P. & MELA, L. (1967). Control and conservation of energy in the cytochrome chain. *Fedn Proc.* **26**, 1341–1354.

DAVENPORT, H. W. (1958). *The ABC of Acid-Base Chemistry*, 5th edn., Chicago: Chicago Press.

FENN, W. O. (1928). The carbon dioxide dissociation curve of nerve and muscle. *Am. J. Physiol.* **85**, 207–223.

FENN, W. O. & MAURER, F. W. (1935). The pH of muscle. *Protoplasma* **24**, 337–345.

FERGUSON, J. K. W. & IRVING, L. (1929). A method to determine the carbon dioxide content of muscle. *J. biol. Chem.* **84**, 143–153.

FLETCHER, W. M. & HOPKINS, G. (1907). Lactic acid in amphibian muscle. *J. Physiol.* **35**, 247–309.

HERBERG, R. J. (1958). Phosphorescence in liquid scintillation counting of proteins. *Science, N.Y.* **128**, 199–200.

HILL, A. V. (1955). The influence of the external medium on the internal pH of muscle. *Proc. R. Soc.* B **144**, 1–22.

HODGKIN, A. L. & HOROWICZ, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.* **148**, 127–160.

IRVINE, R. O. H., SAUNDERS, S. J., MILNE, M. D. & CRAWFORD, M. A. (1960). Gradients of potassium and hydrogen ion in potassium-deficient voluntary muscle. *Clin. Sci.* **20**, 1–18.

KERLY, M. & RONZONI, E. (1933). The effect of pH on carbohydrate changes in isolated anaerobic frog muscle. *J. biol. Chem.* **103**, 161–173.

KOSTYUK, P. G. & SOROKINA, Z. A. (1961). On the mechanisms of hydrogen ion distribution between cell protoplasm and the medium, pp. 193–203. In *Membrane Transport and Metabolism*, ed. KLEINZELLER, A. & KOSTYK, A. New York: Academic Press.

NOVOTNY, I. (1968). pH changes during splitting of ATP in skeletal and cardiac muscle extracts and microsomes. *Physiologia bohemoslov.* **17**, 569–575.

PAYMASTER, N. J. & ENGELSSON, S. (1966). Calculation of pH of human erythrocyte from the distribution of DMO. *Acta anaesth. scand.* **10**, 219–224.

REEVES, R. B. (1969). Role of body temperature in determining the acid-base state in vertebrates. *Fedn Proc.* **28**, 1204–1208.

ROOT, W. S. (1933). The carbon dioxide dissociation curve of frog's skeletal muscle with special reference to the time of exposure to carbon dioxide. *J. cell. comp. Physiol.* **3**, 101–112.

STEINMETZ, P. R. (1969). Acid-base relations in epithelium of turtle bladder: site of active step in acidification and role of metabolic CO_2. *J. clin. Invest.* **48**, 1258–1265.

VAUGHAN, M., STEINBERG, D. & LOGAN, J. (1957). Liquid scintillation counting of C^{14}- and H^3-labelled amino acids and proteins. *Science, N.Y.* **126**, 446–447.

WADDELL, W. J. & BATES, R. G. (1969). Intracellular pH. *Physiol. Rev.* **49**, 285–329.

WADDELL, W. J. & BUTLER, T. C. (1959). Calculation of intracellular pH from the distribution of 5,5-dimethyl-2,4-oxazolidinedione (DMO). Application to skeletal muscle of the dog. *J. clin. Invest.* **38**, 720–729.

WASTL, H. & SELISKAR, A. (1925). Observations on the combination of CO_2 in the blood of the bullfrog (*Rana catesbeiana*). *J. Physiol.* **60**, 264–268.

WEISS, G. B. (1966). The effect of pH on nicotine-induced contracture and Ca^{45} movements in frog sartorius muscle. *J. Pharmac. exp. Ther.* **154**, 605–612.

WEISS, G. B. (1968*a*). Dependence of nicotine-C^{14} distribution and movements upon pH in frog sartorius muscle. *J. Pharmac. exp. Ther.* **160**, 135–147.

WEISS, G. B. (1968*b*). Actions of procaine on nicotine-induced effects in frog sartorius muscle. *J. Pharmac. exp. Ther.* **160**, 148–158.

WOODBURY, J. W. (1965). Regulation of pH. *Physiology and Biophysics*, 19th edn., pp. 899–983, ed. RUCH, T. C. & PATTON, H. D. Philadelphia: W. B. Saunders Co.

WOODBURY, J. W. (1971). Fluxes of H^+ and HCO_3^- across frog skeletal muscle cell membranes. *Alfred Benzon Symposium III. Acid-Base and Potassium Homeostasis of the Brain.* Copenhagen, Lund, New York: Academic Press (in the Press).

WOODBURY, J. W., WHITE, S. H., MACKEY, M. C. & WEAKLY, J. N. (1968). High membrane H^+ permeability of frog skeletal muscle. *Proc. int. Union Physiol. Sci.* **7**, 472.