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DIRECT INTRACELLULAR pH MEASUREMENT IN RAT AND CRAB MUSCLE

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SUMMARY

1. Conflicting values of intracellular pH (pH_i) were obtained by Caldwell in crab muscle and by Carter *et al.* in rat muscle. To investigate this discrepancy, double and single barrelled pH sensitive micro-electrodes as described by Carter *et al.* were used both in rat muscle (*in vivo* and *in vitro*) and in large single fibres of crab (*in vitro*).

2. The pH_i values obtained in the superficial layers of muscle cells in rat and pH_i values obtained in the superficial area of large single fibres in crab seemed in agreement with the Donnan equilibrium. Furthermore, when the membrane potential (E_m) value of extracellular pH (pH_e) value was changed, each new pH_i value was obtained instantly.

3. The pH_i values obtained in deep layers of muscle cells in rat (pH_i 6·68) and in a deep area of large single fibres in crab (pH_i 6·96) did not agree with Donnan equilibrium; these pH_i values were little influenced by modifications in E_m or pH_e values.

4. These findings could be easily explained if the length of the sensitive portion of the pH micro-electrode exceeds the diameter of rat muscle cells and is smaller than the diameter of crab large fibres. The pH_i values obtained superficially in the two preparations and which seem in agreement with the Donnan equilibrium are artificially low because the E_m values recorded by the pH micro-electrodes are partly shunted.

INTRODUCTION

Direct measurement of intracellular pH (pH_i) should be the method of choice. However, the pH_i values which have been obtained by different authors with pH sensitive micro-electrodes are conflicting.

A pH value near 7 was obtained in crab muscle (Caldwell, 1954, 1958) and in frog sartorius muscle (Kostyuk & Sorokina, 1960); this value is not in agreement with the Donnan equilibrium and is in favour of an active

transport of hydrogen ions (Caldwell, 1956); this value was little influenced by modifications in the membrane potential (E_m) or extracellular pH (pH_e). Furthermore, all the authors, except two (Conway & Fearon, 1944) using indirect methods (Fenn, 1928; Stella, 1929; Wallace & Hastings, 1942; Wallace & Lowry, 1942; Eckel, Botschner & Wood, 1959; Waddell & Butler, 1959; Irvine, Saunders, Milne & Crawford, 1960; Miller, Tyson & Relman, 1963; Adler, Roy & Relman, 1965; Burnell, 1968) found a pH_i value near 7 (values obtained either in the frog muscle or in the mammalian skeletal muscle). Recently, Carter *et al.* (1967) challenged these basic results which seemed up to now well established. Indeed these authors obtained with very sharp micro-electrodes (external tip diameter below 0·5 μ) a pH_i value near 6 in the rat leg muscle *in vivo*; this value is in agreement with the Donnan equilibrium; this value was greatly influenced by modifications in the membrane potential and remained always in agreement with the Donnan equilibrium. Each new steady-state value was obtained almost instantaneously, which is very surprising and indicates that large amounts of either hydrogen or hydroxyl or bicarbonate ions were transferred very rapidly across the muscle cell membrane (Carter *et al.* 1967); furthermore, such a transfer requires an enormous hydrogen ion permeability (Roos, 1971).

There are only two explanations for these conflicting results (Caldwell, 1968): either it is a species difference (i.e. hydrogen ion distribution is different in crab and frog muscle and in rat muscle), or there is some difference in the experimental methods using micro-electrodes.

In order to distinguish between these two hypothesis, pH sensitive micro-electrodes as described by Carter *et al.* were used to measure pH_i in rat and crab muscle. A few measurements were made *in vivo* in rat leg muscle, but most measurements were made *in vitro* in rat and in crab muscle; the *in vitro* conditions allow a systematic examination of the behaviour of muscle cells to different values of external pH or membrane potential.

A preliminary account of some of the work described in this paper was given to the XXVth International Congress of Physiological Sciences (Paillard, Sraer, Leviel & Claret, 1971).

METHODS

Animals

Rat muscle preparations. Sprague–Dawley rats weighing between 100 and 200 g were maintained on a standard laboratory diet and tap water *ad libitum*. The rats were anaesthetized by an intraperitoneal injection of sodium pentobarbitone. The *in vivo* measurements were made using the procedure described by Carter *et al.* (1967). Rat muscle preparations *in vitro* were made as follows: a flat leg muscle

(1 or 2 mm thick) was rapidly and carefully removed; the whole muscle was rinsed quickly in saline and immediately incubated in a modified Krebs–Ringer solution (in m-mole/l.): NaCl, 115; $NAHCO_3$, 23; NAH_2PO_4, 1·2; $MgSO_4$, 1·2; $CaCl_2$, 1·2; KCl, 5; glucose, 100 mg/100 ml. was added in each case.

The incubation medium was contained in a specially designed plastic box with a capacity of approximatively 150 ml. The box had a sintered glass bottom which permitted the homogeneous oxygenation of the medium. The incubation box was kept at a constant temperature which maintained the fluid in the bath at 36° C. In the experiments rat muscle was equilibrated for 4–6 hr for each modification in bicarbonate concentration, and for 1–2 hr for each modification in P_{CO_2}; 50 ml. of the incubation medium were changed every hour until the end of the experiment. In the first group of experiments, KCl was added in order to obtain a desired concentration of KCl 20 m-mole/l. Osmolality was kept constant by reciprocal changes in sodium chloride. In the second group of experiments, P_{CO_2} was kept constant at 28–33 mm Hg, and the bicarbonate concentration was set at different levels between 2 and 60 m-mole/l. to achieve the desired degree of external acidosis or alkalosis. Osmolality was kept constant by reciprocal changes in chloride. In a third group of experiments, different P_{CO_2} values were produced in the medium by varying the percentage of P_{CO_2} in the gas mixture between 1·5 and 30% while keeping the bicarbonate concentration constant at 23 m-mole/l.

Crab muscle preparations. Crab muscle preparations were obtained from specimens of *Carcinus maenas* which had been obtained from the Institut de Biologie marine, Wimereux. The preparation of flexor and extensor muscles of the carpopodite as already described (Caldwell, 1954) was used. After being exposed, the muscles were incubated at room temperature in the crab saline described by Fatt & Katz (1953). The crab saline was contained in a small long plastic box with a capacity of about 15 ml. The medium was continuously renewed in order to keep constant the pH. Several polyethylene catheters connected with the box allowed instant modifications of medium composition. In the first group of experiments a solution of 570 m-mole/l. was used. In the second group of experiments, the medium pH was changed by using adequate proportions of Tris-maleate and of NaOH. Sodium hydrogen phthalate and HCl was used to obtain a medium pH value below 4. In each case the total concentration of buffers was 20 m-mole/l. In the third group of experiments, 100% CO_2 was bubbled through the incubation medium.

Construction of the micro-electrodes

Single and double-barrelled pH sensitive micro-electrodes were manufactured exactly as described by Carter *et al.* (1967). The pH electrodes were soaked in distilled water at 4° C for 1–2 weeks before use. The asymmetry potential was then steady and not affected by modifications in ionic strength: there were no significant changes in the asymmetry potential when the pH electrodes were immersed in KCl 3 M, NaCl, 3 M, KCl 0·1 M, NaCl 0·1 M (at constant pH). Only the micro-electrodes with a slope $\geqslant$ 50 mV per pH unit at room temperature were used. Tip resistances were approximately 1000 MΩ. pH sensitive micro-electrodes coated with more than two layers of glaze (from 3 to 5) were used in some experiments; the pH_i values obtained were not different from those obtained with Carter's electrodes (coated with two layers only). On the other hand a few pH micro-electrodes not insulated (i.e. without glaze) were used.

Ling & Gerard (1949) type micro-electrodes were pulled from Pyrex glass and were filled with KCl 3 M. The reference side of double-barrelled electrodes and Pyrex electrodes were selected which had a tip resistance between 10 and 30 MΩ and a tip potential below 6 mV when tested in the modified Krebs–Ringer solution.

Application of the micro-electrodes

Micro-electrodes and indifferent electrodes (2% agar-agar Ringer or agar-agar crab saline bridge) were connected to calomel half cells. A Cary model thirty-one vibrating electrometer was connected to a Honeywell recording potentiometer. A few pH_i measurements were made in rat and crab muscle with double-barrelled micro-electrodes. The recording circuit was different from the circuit used by Carter *et al.* (1967). Only one Cary electrometer was used. In fact three measurements: E_m value obtained by the reference side, intracellular potential value (E_o) by pH sensitive side, and once again E_m value by the reference side, were made successively with regard to the indifferent extracellular electrode, while the tip of the double-barrelled micro-electrode was kept in place in the rat or crab muscle cell for the time necessary to make these three determinations. Only the E_m values $\geqslant 70$ mV in rat muscle, $\geqslant 55$ mV in crab muscle were retained.

Most measurements of pH_i were made with single-barrelled micro-electrodes. In rat muscle, several E_m values were obtained in the area when the pH electrode was inserted; the mean E_m value was compared to each intracellular potential (E_o) measured with the pH sensitive micro-electrode. In crab muscle, the E_m value and E_o value were obtained in the same area of a single fibre successively. When the E_m or pH_e value was changed, pH_i measurements were made in crab muscle fibre either continuously (with the micro-electrode kept in place in the fibre during the whole duration of the experiment), or discontinuously at appropriate intervals. In rat muscle only discontinuous measurements were possible.

In all cases, only pH_i values obtained with a pH micro-electrode whose pH slope, tip resistance, and asymmetry potential (tested in KCl 3 M and 0·1 M and in NaCl 3 M and 0·1 M at constant pH) were unchanged after each measurement, were used.

Finally the determination of the pH_i value was made by using the following equation

$$pH_i = pH_e + \frac{E_m - E_o}{K} \qquad (1)$$

K is the slope of the pH sensitive micro-electrode. E_m is membrane potential value measured by the reference side of double-barrelled micro-electrodes or by the Pyrex micro-electrode. E_o is the intracellular potential measured with pH sensitive micro-electrode: it is the algebraic sum of E_m value measured with the pH electrode and the potential difference resulting from the difference of pH inside and outside the cell.

Electrolyte determination

Determination of electrolytes and water in rat muscle were made as described by Graham, Lamb & Linton (1967), who used the corrected chloride content of muscle assuming the Donnan equilibrium for chloride ion to calculate extracellular water. Analyses of sodium and potassium in muscle and in the incubation medium were carried out with a flame photometer (Eppendorf). Chloride determination in muscle and the incubation medium was made by a potentiometric method. Actual incubation medium pH was checked at appropriate intervals during each experiment with a pH sensitive micro-electrode. P_{CO_2} in the incubation medium *in vitro* was calculated from the measured pH value and from the bicarbonate concentration known from the quantity of bicarbonate used to make the solution. CO_2 solubility coefficient used was 0·0301 m-mole/l. and per mm Hg of medium CO_2 pressure. Arterial blood pH determinations were made with a Radiometer pH meter at 37° C.

Estimation of the fibre diameter

Rough estimation of fibre diameter was made in rat and in crab muscle: muscle was fixed in Halmi's fixative solution, then embedded in paraffin wax, and transverse sections (5 μ thick) were stained with PAS.

Statistical evaluations

Statistical evaluations were made using conventional procedure. All results were expressed in mean value $\pm$ 1 S.D.

RESULTS

Viability of the rat and crab muscle preparations in vitro

The viability of the rat muscle preparation was satisfactory. Indeed, Table 1 summarizes the results obtained in a series of experiments in which muscles were incubated for periods of 6 hr in normal conditions. The total water content, potassium and sodium content, were very close to the values obtained by Relman, Gorham & Levinsky (1961) in rat diaphragm *in vitro*. The difference in value in the calculated intracellular sodium concentration probably resulted from the difference in method in determination of the extracellular water.

The viability of the crab muscle preparation was estimated from the miscroscopic aspect of the fibres. Only pH_i values obtained in fibres which remained translucent after measurements were retained.

Evidence that the pH micro-electrode tip was inside the cell

For double-barrelled micro-electrodes, the proof is given by recording a membrane potential with the reference side.

For single-barrelled micro-electrodes, the indications are the same as those described by Carter *et al.* (1967); i.e. the careful observation of the muscle surface aspect with microscope during puncture and the existence of a sudden change in the potential of pH sensitive micro-electrode. The aspect of the recording of the E_o value when the pH electrode was slowly introduced into a muscle with a Leitz micromanipulator was the same with either a single- or a double-barrelled micro-electrode. Thus single-barrelled electrodes which are much easier to make were mainly used.

Intracellular pH values in rat muscle

Normal values. In vivo, pH_i values were different near the surface of the muscle (i.e. in the more superficial cells) and in a deeper zone (i.e. in deeper layers of cells). There is no systematic variation in E_m value recorded when the reference side or Pyrex micro-electrode was introduced slowly into the muscle. And the mean E_m value was $-80{\cdot}5$ mV $\pm$ 4·5 ($n = 66$). But an E_o gradient (i.e. a pH_i gradient) was recorded as the pH

micro-electrode was slowly introduced into the muscle (Fig. 1). Steady values were obtained after passing through several layers of cells. When the mean arterial blood pH value was $7{\cdot}41 \pm 0{\cdot}02$ ($n = 11$), the mean pH_i value near the surface (i.e. superficial pH_i value) was $6{\cdot}11 \pm 0{\cdot}07$ ($n = 8$); this is in agreement with the calculated pH_i value (6·09) assuming the

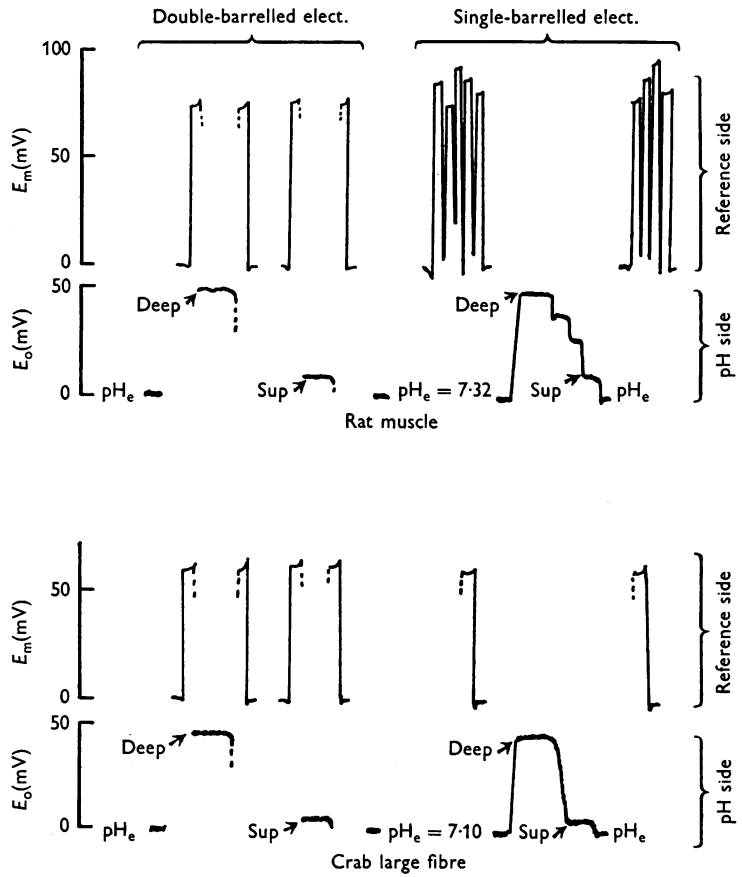

Fig. 1. Recordings obtained with double and single-barrelled micro-electrodes. Evidence of E_o (intracellular potential obtained with a pH micro-electrode) or pH_i gradient observed when a micro-electrode was progressively introduced into a rat muscle or a large single fibre of crab. (The recordings are to be read from right to left.)

Donnan equilibrium for hydrogen ions. The mean steady pH_i value in a deeper zone (i.e. deep pH_i value) was $6{\cdot}68 \pm 0{\cdot}08$ ($n = 11$); this is no longer in agreement with the Donnan equilibrium.

In vitro, pH_i measurements were carried out in muscle fibres whose measured diameter was about 20–30 μ. A pH_i gradient was also demon-

strated (Fig. 1). When the mean E_m value was $-75{\cdot}9$ mV $\pm$ 4·2 ($n = 36$), and pH_e value 7·33 $\pm$ 0·02 ($n = 7$), the mean pH_i superficial value was 6·12 $\pm$ 0·02 ($n = 6$); this is in agreement with the calculated value (6·10) assuming a Donnan equilibrium. The mean deep steady pH_i value was 6·68 $\pm$ 0·03 ($n = 7$); this is not in agreement with this.

Effect of depolarization in vitro *with KCl 20 m-mole/l.*

The mean pH_e value was 7·32 $\pm$ 0·02 ($n = 6$): the mean E_m value was 51 mV $\pm$ 2·6 ($n = 36$); the superficial E_m values were reached after only a few minutes, whereas deep E_m values were reached after incubation of muscle of about 1½ hr. After 4 hr of incubation, the mean superficial pH_i value was 6·45 $\pm$ 0·05 ($n = 6$); this is in agreement with the calculated value (6·49) assuming the Donnan equilibrium. The mean steady deep pH_i value was 6·78 $\pm$ 0·04 ($n = 6$); this is not in agreement with the Donnan equilibrium and little more alkaline than control values (6·68). The difference is significant ($P < 0{\cdot}001$).

Effect of variation in bicarbonate concentration and CO_2 pressure in vitro

Table 2 shows that the mean superficial pH_i values were always in agreement with the Donnan equilibrium. With each new pH_e value, there was instant modification of superficial pH_i value. Fig. 2 shows the relationship between superficial pH_i value and pH_e values: the pH_i values coincided approximately with the theoretical line calculated assuming the Donnan equilibrium.

However, the deep steady pH_i values were no longer in agreement with the Donnan equilibrium (Tables 3 and 4). Fig. 3 shows the effects of changes in extracellular bicarbonate concentration (P_{CO_2} kept constant at the mean value 30·3 mm Hg). The relationship between pH_i values and pH_e values was a straight line with a gentle slope; this is different from complicated DMO curve (Adler *et al.* 1965). The relationship between intracellular hydrogen ion concentration and extracellular hydrogen ion concentration was no more linear (Fig. 4). It should be noted that pH_i values became steady after 2 hr of incubation. Fig. 3 shows also the effect of variations in P_{CO_2} on pH_i values (the bicarbonate concentration kept constant at 23 m-mole/l.). The relationship between pH_i and pH_e was also a straight line, but with a steeper slope (Fig. 3). The relationship between intracellular hydrogen ion concentration and extracellular hydrogen ion concentration (or between intracellular hydrogen ion concentration and P_{CO_2}) was linear (Fig. 4). The pH_i values became steady 15 min after each modification in P_{CO_2} and remained unchanged for 2 hr. It must be pointed out that the calculated buffering capacity of muscle cells remained constant

TABLE 1. Water compartments and electrolyte composition of rat muscle *in vitro* (incubation of 6 hr)

	Medium						Membrane potential
	K^+	Na^+ (m-mole/l. H_2O)	Cl^-	HCO_3^-	p_{CO_2} (mm Hg)	pH_e	E_m mV
Mean*	5·17	143·75	118·90	23	45	7·33	80·04
S.D.	+0·73	±0·96	±3·45	—	—	—	±4·52

	Muscle								
	Total content				Extracellular	Intracellular values			
	H_2O (% wet wt.)	K^+	Na^+ (m-mole/100 g dry wt.)	Cl^-	H_2O (% wet wt.)	H_2O (ml./100 g dry wt.)	K^+	Na^+ (m-mole/l. H_2O)	Cl^-
Mean*	77·96	35·29	19·33	15·02	26·60	247·37	146·22	15·67	4·93
S.D.	±0·47	±1·22	±3·12	±4·16	±5·35	±14·9	±15·37	±2·23	±0·13

* Mean value of four determinations.

TABLE 2. Rat muscle *in vitro*. Effects of modifications of extracellular pH on superficial intracellular pH values

Extracellular pH_e	Membrane potential E_m (mV)			Intracellular values: pH_i measured			pH_i calculated*
	Mean	S.D.	*n*	Mean	S.D.	*n*	
7·93†	−84·1	2·1	24	6·61	0·02	4	6·56
7·90‡	−76·7	1·7	36	6·55	0·03	6	6·65
7·74†	−86·6	1·7	18	6·41	0·02	3	6·34
7·65‡	−78·2	4·8	60	6·29	0·03	10	6·38
7·49	−76·6	2·1	24	6·17	0·02	4	6·25
7·33‡	−75·9	4·2	36	6·12	0·02	6	6·10
7·26‡	−78·1	3·9	24	6·05	0·03	4	5·99
7·12‡	−75·0	2·3	36	5·82	0·03	6	5·90
7·04†	−77·3	2·6	24	5·87	0·03	4	5·78
6·98†	−81·2	4·4	30	5·69	0·03	5	5·64
6·96‡	−81·2	4·4	30	5·69	0·03	5	5·64

* Calculated assuming Donnan equilibrium for H^+: $pH_i = pH_e + (E_m/61·5)$.
† P_{CO_2} changes. ‡ HCO_{3e}^- changes.

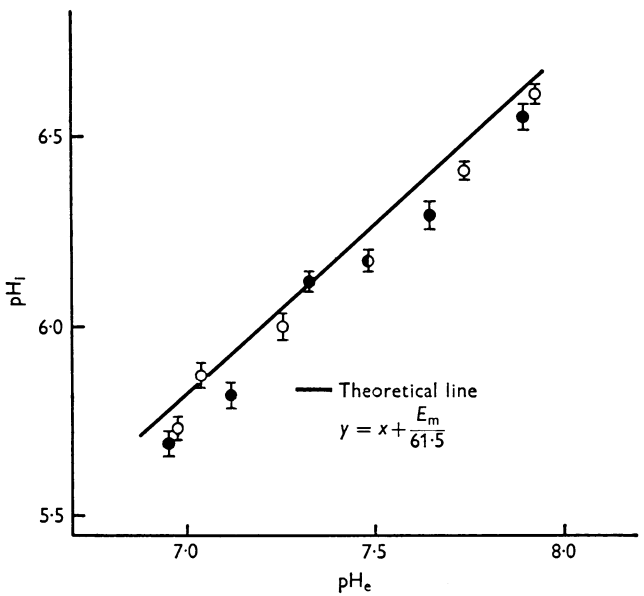

Fig. 2. Rat muscle *in vitro*. Effect of modifications in pH_e on the superficial pH_i values. ● HCO_3^- changes. ○ P_{CO_2} changes. — Theoretical line calculated assuming Donnan equilibrium for H^+ ions ($pH_i = pH_e + (E_m/61·5)$ and mean E_m value 79·04 mV).

TABLE 3. Rat muscle *in vitro*. Effects of modifications of extracellular HCO_3^- on deep intracellular pH values*

Extracellular values					Membrane potential			Intracellular values			
	pH_e				E_m (mV)			pH_i			
HCO_3^- (m-mole/l.)	Mean	S.D.	n	H_e^+† (n-mole/.)	Mean	S.D.	n	Mean	S.D.	n	H_i^+† (n-mole/l.)
60	7·88	0·03	5	13·18	−77·5	2·9	30	6·93	0·05	5	117·36
23	7·53	0·06	6	29·51	−76·3	3·5	36	6·76	0·10	6	173·79
12	7·21	0·02	8	61·69	−77·3	1·3	48	6·71	0·06	8	194·97
6	6·95	0·06	7	112·19	−75·9	2·4	42	6·59	0·05	7	257·07
5	6·81	0·01	6	158·48	−75·4	4·3	36	6·53	0·05	6	295·16
2	6·48	0·04	8	331·12	−75·3	3·5	48	6·52	0·10	8	302·02

* P_{CO_2} = 30·3 mm Hg. n, number of E_m or pH determinations. † Calculated from mean pH value.

TABLE 4. Rat muscle *in vitro*. Effects of modifications of P_{CO_2} on deep intracellular pH values*

	Extracellular values				Membrane potential			Intracellular values				
	pH_e				E_m (mV)			pH_i				
p_{CO_2} (mm Hg)	Mean	S.D.	n	H_e^+† (n-mole/l.)	Mean	S.D.	n	Mean	S.D.	n	H_i^+† (n-mole/l.)	HCO_3^-‡ (m-mole/l.)
11·6	7·92	0·05	4	12·02	−75·4	3·5	24	6·92	0·08	4	120·22	2·3
17·1	7·75	0·02	4	17·78	−74·5	2·8	24	6·87	0·05	4	134·90	3·0
28·4	7·53	0·06	6	29·51	−76·3	3·5	36	6·76	0·10	6	173·79	3·9
45·0	7·33	0·02	7	46·77	−75·9	4·2	42	6·68	0·03	7	208·94	5·1
78·2	7·09	0·03	4	81·30	−75·3	3·2	24	6·55	0·03	4	281·85	6·6
105·5	6·96	0·06	6	109·65	−76·4	3·5	36	6·45	0·08	6	354·73	7·1
179·1	6·73	0·04	4	186·22	−73·8	3·6	24	6·30	0·05	4	501·25	8·5
277·4	6·54	0·02	4	288·52	−75·7	3·4	24	6·20	0·07	4	630·91	10·5

* Extracellular HCO_3^- = 23 m-mole/l.

n number of E_m or pH determinations.

† Calculated from mean pH value.

‡ Calculated assuming P_{CO_2} to be equal in intracellular and extracellular fluid: CO_2 solubility coefficient used was 0·0301.

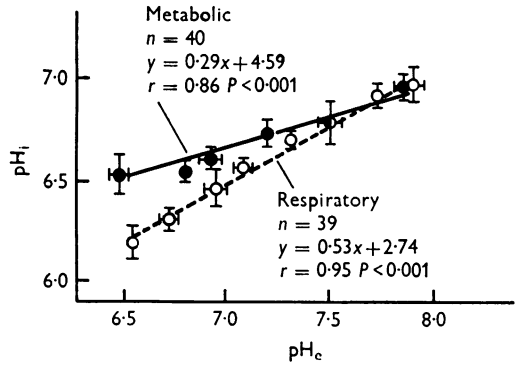

Fig. 3. Rat muscle *in vitro*. Effect of modifications in PH_e on the deep pH_i ● HCO_3^- changes. ○ P_{CO_2} changes.

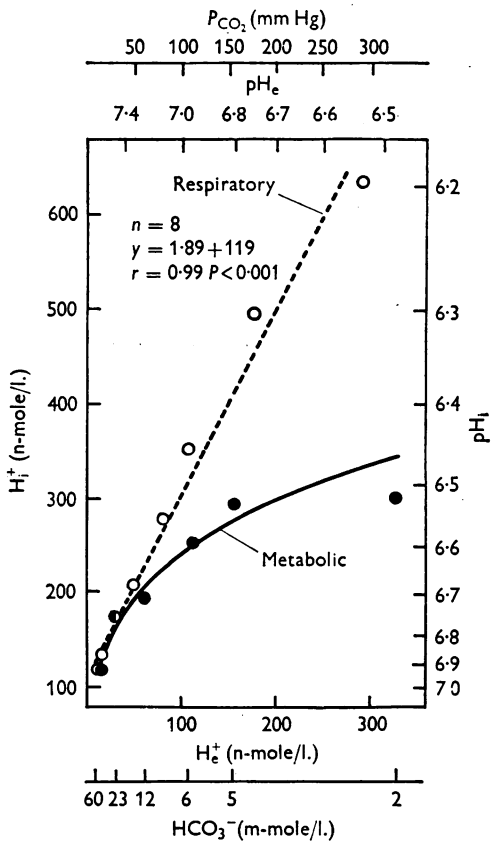

Fig. 4. Rat muscle *in vitro*. Same data as in Fig. 3, but expressed in H^+ ion concentration. The respiratory line was drawn using the experimental values. The metabolic curve was drawn from the metabolic line of Fig. 3.

(10·7 slykes) for all over P_{CO_2} values (ranging from 11 to 277 mm Hg) (Fig. 5). This buffering capacity ($\Delta HCO_3^-/\Delta pH_i$) was calculated as described by van Slyke (1922), and assuming P_{CO_2} to be equal in intra- and extracellular fluid.

Intracellular pH values in crab muscle in vitro

Normal values. The pH_i measurements were made in large single fibres having a diameter of 400–700 μ. The size of the fibres allowed pH_i measurement near the surface (i.e. superficial pH_i value) and more deeply (i.e. deep pH_i value) into a single fibre. When a single or double-barrelled micro-electrode was introduced gently into a large fibre, a E_o gradient was obtained, i.e. pH_i value increased progressively, until a steady value was obtained (Fig. 1). When the mean pH_e value was $7{\cdot}65 \pm 0{\cdot}05$ ($n = 8$) (unbuffered crab saline), the pH_e value just near the surface of the fibre (i.e. contact pH) was $6{\cdot}96 \pm 0{\cdot}04$ ($n = 8$). When the mean E_m value was $55{\cdot}75$ mV $\pm 1{\cdot}58$ ($n = 8$), the mean superficial pH_i value was $6{\cdot}05 \pm 0{\cdot}08$ ($n = 8$); this is in agreement with the value calculated (6·00) assuming the Donnan equilibrium (using the contact pH in place of the medium pH). The mean steady deep pH_i value was $6{\cdot}96 \pm 0{\cdot}10$ ($n = 8$); this is not in agreement with the Donnan equilibrium. When the mean pH_e value was $7{\cdot}81 \pm 0{\cdot}02$ ($n = 5$) (buffered crab saline), the contact pH value was identical with the medium pH. In this case, when the mean E_m value was $59{\cdot}6$ mV $\pm 1{\cdot}31$($n = 5$), the mean superficial pH_i value was $6{\cdot}91 \pm 0{\cdot}56$ ($n = 5$); this is approximately in agreement with the value calculated (6·78) assuming the Donnan equilibrium. The mean steady deep pH_i value was identical, but it is probably fortuitous.

It must be pointed out that when reference side of a double-barrelled micro-electrode or a Pyrex micro-electrode was introduced gently into a single fibre of crab, there were two types of responses: either only one steady value E_m was obtained, or several (two to five) E_m values of the same magnitude were obtained.

Effect of depolarization with KCl 570 m-mole/l.

The mean steady E_m values obtained in 10–20 min was 0 mV. When the mean pH_e value was $7{\cdot}80 \pm 0{\cdot}0$ ($n = 8$) (buffered crab saline), the mean steady deep pH_i value was $7{\cdot}01 \pm 0{\cdot}02$ ($n = 8$); this is not in agreement with the value calculated (7·80) assuming Donnan equilibrium. It should be noted that deep pH_i values were little more alkaline than normal values. This difference is significant ($P < 0{\cdot}01$).

Fig. 6 shows one experiment in which E_m values and pH_i values (superficial and deep) were recorded at different times after KCl was added. In the same fibre, the superficial pH_i values were at all times in agreement

with the Donnan equilibrium calculated with regard to E_m values. On the contrary, the deep pH_i values remained close to control values over 2 hr.

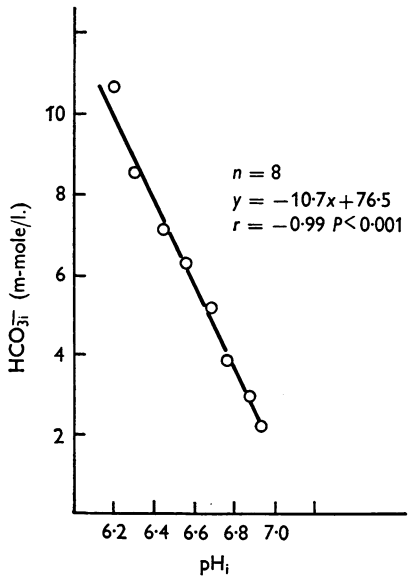

Fig. 5.

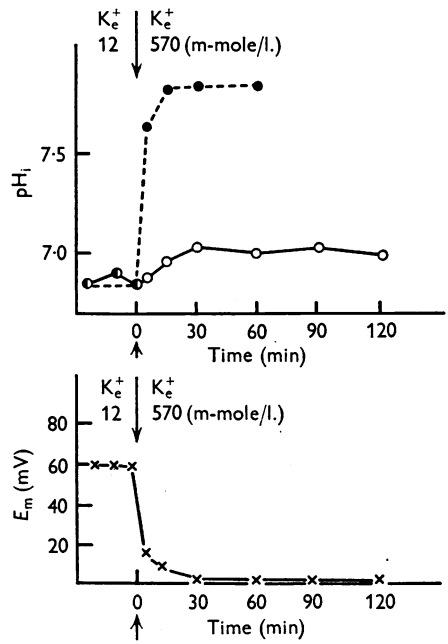

Fig. 6.

Fig. 5. Rat muscle *in vitro*. Buffering capacity. HCO_{3i}^- concentrations were calculated from the mean deep pH_i and the P_{CO_2} values.

Fig. 6. Large fibre of crab muscle *in vitro*. pH_i values plotted against time when the fibre was completely depolarized with KCl 570 m-mole/l. (at constant pH_e 7·8). ●—● superficial pH_i values. ○—○ deep pH_i values. - - - - pH_i values calculated assuming Donnan equilibrium $pH_i = pH_e + (E_m/58)$. ×—× E_m values.

Effect of variations in pH_e values

When the pH_e values were changed from 8·60 to 3·64, the mean steady deep pH_i values were not in agreement with the Donnan equilibrium (Table 5). Fig. 7 shows that the relationship between pH_i and pH_e values is linear and close to that obtained in the rat muscle. The relationship between extracellular hydrogen ion concentration and intracellular hydrogen ion concentration is no more linear (Fig. 8).

Fig. 9 shows two experiments in which the pH_i values (superficial and deep) were recorded at different time into the same large single fibre, after modifications in the pH_e values. When pH_e values were changed from 7·19 to 8·60 or from 7·5 to 6·2, the steady superficial pH_i values were instantly

TABLE 5. Crab muscle fibre *in vitro*. Effects of modifications of extracellular pH on deep intracellular pH values

Extracellular values				Membrane potential			Intracellular values			
pH_e			H_e^+*	E_m (mV)			pH_i			H_i^+*
Mean	S.D.	*n*	(*n*-mole/l.)	Mean	S.D.	*n*	Mean	S.D.	*n*	(*n*-mole/l.)
8·60	0·00	7	2·51	−57·3	3·6	7	6·93	0·05	7	116·71
7·81	0·02	5	15·56	−59·6	1·3	5	6·91	0·06	5	122·05
7·47	0·03	5	33·87	−63·6	2·8	6	6·93	0·05	6	117·83
7·20	0·02	9	62·77	−64·3	0·7	9	6·78	0·07	9	167·11
7·11	0·03	12	77·63	−63·2	4·6	12	6·68	0·07	12	206·91
6·90	0·00	4	125·90	−62·0	0·0	4	6·56	0·02	4	271·66
6·22	0·05	5	604·23	−59·0	2·2	5	6·43	0·11	5	371·88
5·88	0·09	6	1318·22	−65·0	2·4	6	6·21	0·16	6	615·76
5·14	0·00	5	7246·37	−65·0	0·0	5	6·25	0·07	5	563·38
3·64	0·13	5	22805·01	−62·6	3·4	5	5·88	0·12	5	1320·48

n, number of E_m or pH determinations. * Calculated from mean pH value.

obtained and in agreement with the value calculated assuming the Donnan equilibrium. On the contrary, the deep pH_i values remained steady for the first 5 min, and then were slowly modified but they never reached the value calculated assuming the Donnan equilibrium. Deep values were steady after about 1 hr.

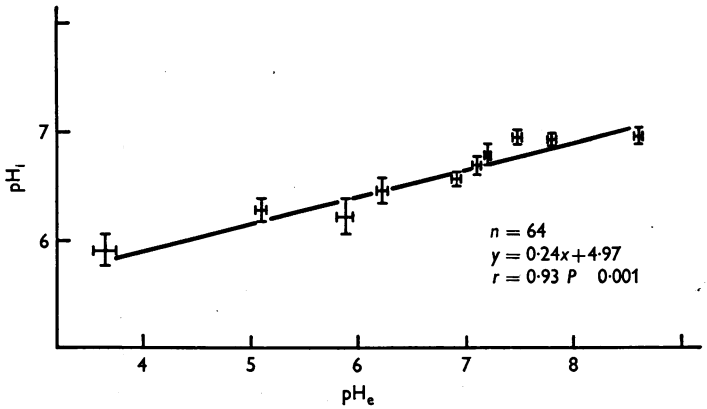

Fig. 7. Large fibres of crab muscle *in vitro*. Effect of modifications in pH_e on the deep pH_i values. The medium was buffered with Tris maleate-NaOH or with sodium hydrogen phthalate.

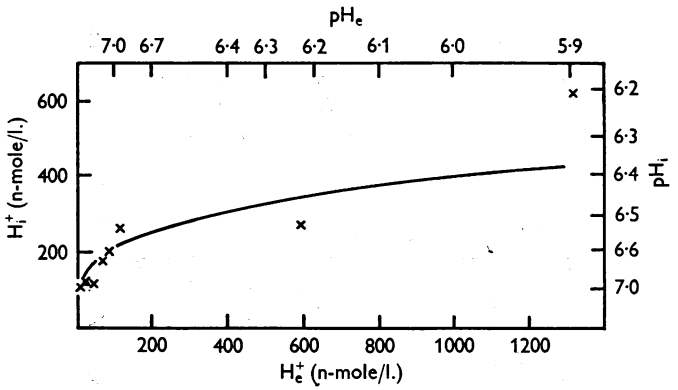

Fig. 8. Large fibres of crab muscle *in vitro*. Same data as in Fig. 7, but expressed in H^+ ion concentration. The curve was drawn from the line of Fig. 7.

In two experiments, the pH_e value was changed from 7·3 to 5·9 with bubbling 100 % CO_2. The mean E_m value was 64 mV (66 and 62 mV). The mean steady superficial pH_i value was immediately obtained and was 4·85 (4·8 and 4·9); this is in agreement with value calculated (4·8) assuming Donnan equilibrium. The mean deep pH_i value was rapidly (within 5 min) changed to 6·15 (6·06 and 6·24) and remained steady for 2 hr; this is not in agreement with Donnan equilibrium.

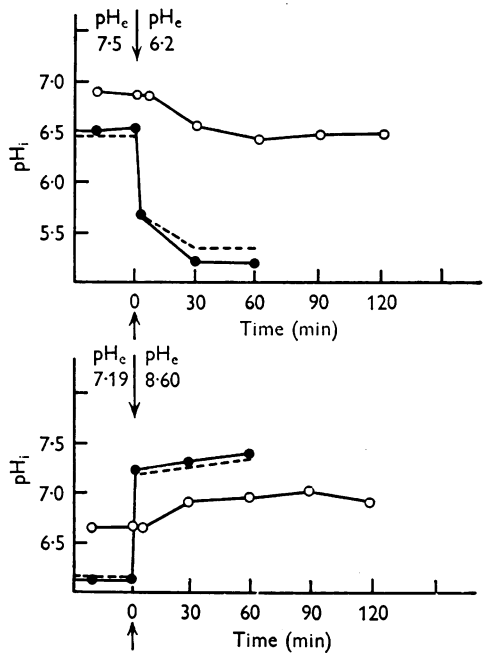

Fig. 9. Large fibres of crab muscle *in vitro*. pH_i values plotted against time when the pH_e values were changed from 7·5 to 6·2 and from 7·19 to 8·6 (the mean E_m values were respectively $59·9 \pm 4·7$ and $60·4 \pm 2·3$ mV). ●—● superficial pH_i values. ○—○ deep pH_i values. - - - pH_i values calculated assuming Donnan equilibrium, with regard to the contact pH_e values.

pH_i values obtained in rat and crab muscle with non-insulated pH micro-electrode

The superficial pH_i values obtained in rat and crab muscle were not different from the superficial pH_i values obtained with Carter's electrodes; in rat muscle, when the mean pH_e value was $7·30 \pm 0·03$ ($n = 4$) and E_m value 76·2 mV $\pm$ 3·5 ($n = 15$), the mean pH_i value was $6·10 \pm 0·03$ ($n = 4$), which is in agreement with the calculated value assuming Donnan equilibrium (6·06); in crab single fibre, when the mean pH_e value is $7·80 \pm 0·02$ ($n = 4$) (buffered saline) and E_m value 61 mV $\pm$ 2·4 ($n = 4$), the mean pH_i value was $6·82 \pm 0·04$ ($n = 4$) which is in agreement with the calculated value (6·75).

DISCUSSION

Both in rat and in crab muscle, the superficial pH_i values obtained with Carter's electrodes were in agreement with the calculated values assuming Donnan equilibrium, and the deep pH_i values were not. Therefore the discrepancies found between the pH_i values obtained by Carter *et al.*

(1967) in rat muscle, and the pH_i values obtained by Caldwell (1954, 1958) in crab muscle are not a species difference. Thus, the discrepancies found must be due to differences in the experimental methods. Hence the problem is to find which values are correct: the superficial pH_i values or the deep pH_i values.

Significance of superficial and deep pH_i values in rat and in crab muscle

These results, apparently conflicting between the superficial and deep pH_i values, both in rat and in crab muscle, can be explained only in two ways:

First, the superficial pH_i values obtained in both preparations are correct, i.e. hydrogen ions are passively distributed across the cell membrane. This interpretation would agree with Carter's results (1967) obtained *in vivo*. But in this hypothesis, what is the significance of the deep pH_i values? Are these values artifacts or not? Indeed artifacts could arise from the progressive penetration of the micro-electrode into the muscle. A modification of the tip resistance, pH slope, or asymmetry potential of the pH micro-electrode could produce such artifacts, when the closed tip was broken during the puncture; however, the absence of such modification was checked for each penetration of the electrodes and therefore this possibility is ruled out. On the other hand the absence of tip potential > 6 mV for the reference side of double-barrelled micro-electrodes or of Pyrex micro-electrode rules out another course of error in the pH measurement. Finally deep values cannot result from systematic artifact. So another interpretation of deep pH_i values could be the following: in rat muscle *in vitro* the pH_i gradient could be explained by the presence of a similar pH gradient in the interstitial fluid, resulting from the lack of equilibration between it and the incubation medium, as an isolated muscle is not perfused: in this case the Donnan equilibrium for hydrogen ions would be respected for each cell and the interstitial fluid in immediate contract with the cell. However, the demonstration of pH_i gradient in rat muscle *in vivo* (which is perfused) is a strong argument against this interpretation. This interpretation cannot be applied to the crab muscle where both superficial and deep values were recorded into a single fibre, unless it is assumed that there is a true pH_i gradient within the fibre. Finally the low superficial pH_i values obtained both in rat and crab muscle with non-insulated pH micro-electrodes raised a further doubt on the validity of the identical values obtained with Carter's electrodes (see below).

Secondly, the superficial pH_i values are not correct and therefore are artifacts, i.e. hydrogen ions are not distributed according to the Donnan

equilibrium. In this hypothesis, the superficial pH_i values could easily be explained by an inadequate insulation of Carter's electrodes, i.e. that the sensitive portion of the micro-electrode tip must exceed 20–30 μ (the diameter of rat muscle cell); in this case the intracellular potential measured by the pH electrode would be expressed

$$E'_o = E'_m + K(pH_e - pH'_i), \tag{2}$$

E'_m and pH'_i are the values given by a pH micro-electrode inadequately insulated (i.e. the sensitive portion of the micro-electrode is both in and outside the cell). The pH'_i value must then be intermediate between the true pH_i value (for instance 6·7–6·9) and the pH_e value. E'_m value is artificially low compared with the true E_m value because of the shunt created between the extra and intracellular fluid. The fact that superficial E'_o values are identical when measured with Carter's electrodes or with non-insulated electrodes (i.e. when almost all the part of the sensitive portion is outside the cell) is in favour of a pH'_i value very close to the pH_e value, i.e. that K $(pH_e - pH'_i)$ becomes negligible. Then the E'_o value would be only the result of the E_m shunted value. The artificially low value of the superficial pH_i would then be explained because the pH_i values are calculated according to the equation which uses the true E_m value (eqn. (1)). In this case the agreement with the Donnan equilibrium would be purely fortuitous. That interpretation is not in agreement with the data of Carter (1967) who found that a not adequately insulated micro-electrode gave a E'_m value very close to the true E_m value. However, the existence of low superficial pH_i values in agreement with the Donnan equilibrium obtained with non-insulated micro-electrode, both in rat and in crab muscle, is a strong argument against Carter's findings (1967). Furthermore, the immediate modifications in the superficial pH_i values following modifications in the E_m values (Carter, 1967) or the pH_e values (present work) would easily be explained by the use of inadequately insulated micro-electrode. A rough estimation of the length of the sensitive portion of micro-electrode (about 90–100 μ) may be calculated from the number of rat muscle cells that the electrode must cross until the pH_i value is steady.

What is the significance of the steady deep pH_i values in this hypothesis? In the large single fibre of the crab, the pH sensitive portion of the micro-electrode tip is entirely within the single fibre; the lack of instant variations in the pH_i values when the pH_e value or E_m value was changed seems to confirm this. Furthermore, the results are very close to those obtained by Caldwell (1954, 1958). However, the complicated structure of the crab muscle fibre must be taken into account to interpret the pH_i measurement obtained with Carter's or Caldwell's electrodes. Indeed the fact of frequently obtaining several peaks of E_m values while penetrating

a single fibre and the presence of the sarcolemmal invaginations seen in microscopic examination (Selverston, 1967) are in favour of the existence of micro-expansions of extracellular fluid within the fibre. Thus it is possible that there are micro-bands of the sensitive portion of micro-electrodes in contact with one or several expansions of extracellular fluid (Fig. 10). However, the homogeneous pH_i values obtained for each pH_e value seem to indicate that the peculiar structure of the crab muscle fibre does not introduce any artifact in the pH_i measurement (i.e. that there is no significant shunt in E_m values measured by a pH micro-electrode

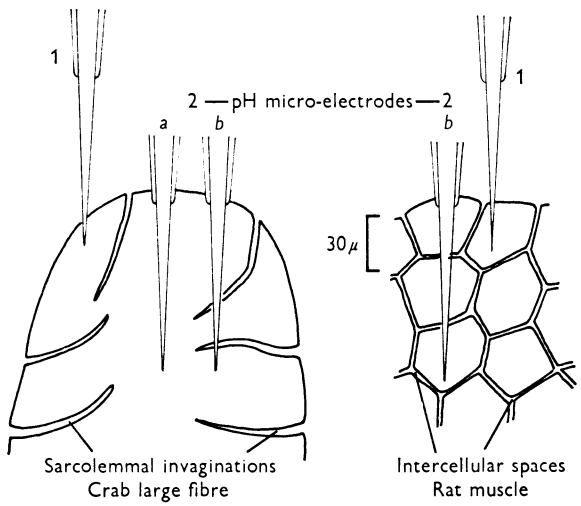

Fig. 10. Interpretation of the pH_i values obtained with Carter's electrodes in rat muscle and in crab muscle fibre. (1) Electrodes superficially inserted; the sensitive portion is both inside and outside the cell: the pH_i values are in agreement with the Donnan equilibrium. (2) Electrodes deeply inserted; the sensitive portion is totally within the rat muscle or the crab large fibre: the pH_i values are not in agreement with the Doonan equilibrium; the sensitive portion is *b* or is not *a* in contact with sarcolemmal invaginations or interstitial spaces.

deeply inserted). Thus it can be argued that the deep steady pH_i values recorded in the rat muscle have the same significance as those obtained in crab fibre (Fig. 10); when the pH micro-electrodes are deeply inserted through several layers of cells, there are micro-bands of the sensitive portion of the micro-electrodes tip in contact with micro-expansions of the extracellular fluid which are situated in the interstitial spaces. Thus making the three assumptions: that the micro-electrode tip is sufficiently sharp not to depolarize the cells into which it is inserted; that the pH_i values are identical in adjacent cells in a steady state; and that the interstitial spaces in contact with the sensitive portion of the micro-electrode do not create

a significant shunt in the E_m value (as proved in the crab fibre) it can be postulated that the deep pH_i values in rat muscle are as correct as the deep values obtained in the crab muscle fibre. The similar pH_i–pH_e relationship in both the crab and the rat muscle could be further argument.

Comparison between pH_i values obtained with micro-electrodes and those obtained with DMO method in rat muscle cells in vitro

The micro-electrode method of measuring pH_i in rat muscle cells can be accepted as correct providing the above mentioned assumptions to be true. Furthermore, they are the only direct measurements made up to now in mammalian skeletal muscle, except for Carter's experiments (1967). The indirect method using DMO is generally accepted as being accurate; the recent evidence of an active transport of DMO (Dietschy & Carter, 1965) and of an intracellular protein binding (Campion, Carter, Rector & Seldin, 1967) are not conclusive.

The values of pH_i obtained with the micro-electrodes were always lower than those obtained using the DMO method. An explanation which may account for this discrepancy is that there is a significant pH heterogeneity within the cell. Previous data support this possibility; the mitochondrial pH determined with DMO (Addanki, Cahill & Sotos, 1967) or with bromothymol blue (Chance & Mela, 1966) is higher than that of the surrounding medium. According to the existence of a pH heterogeneity within the cell, the significance of pH_i values obtained with indirect method must be discussed. Indeed, the indirect method using a weak acid such as DMO would measure the mean hydroxyl ion concentration in the whole cell (Caldwell, 1956). The calculated pH_i value would be intermediate between the value of the region of highest pH and the value of the region of lowest pH, but much closer to the highest pH value (Waddell & Bates, 1969). The alkaline region in the cell could be the mitochondria (Addanki *et al.* 1967). The micro-electrodes would measure the mean hydrogen ion concentration in the cytoplasmic water. It may be pointed out that the pH_i values obtained recently in the rat diaphragm *in vitro* (Adler, 1970), using the nicotine method, are very close to the micro-electrodes values both in the normal conditions (pH_i 6·7 with nicotine) and with modifications of the pH_e except in respiratory acidosis. Nicotine which is a weak base would measure the mean hydrogen ion concentration of the whole cell (Caldwell, 1956). The calculated pH_i values should be much closer to the lowest pH values of the cell (Waddell & Bates, 1969). In this case, the region of lowest pH value in the cell would be the pH of the cytoplasmic water.

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