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Measurement of Intracellular pH of Skeletal Muscle with pH-sensitive Glass Microelectrodes *

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Summary. We used three methods to examine the relationship among intracellular pH, transmembrane potential, and extracellular pH. Single-barreled electrodes permitted the determination of resting potential and intracellular pH with a minimum of cellular injury. Double-barreled electrodes, which incorporated a reference as well as a pH-sensitive electrode in a single tip, facilitated the direct measurement of intracellular pH without the interposition of the transmembrane potential. Triple-barreled electrodes permitted measurement of intracellular pH during the controlled hyperpolarization or depolarization of the cell membrane.

The results of all three methods were in close agreement and disclosed that the H^+ activity of intracellular and extracellular fluid is in electrochemical equilibrium at any given transmembrane potential. This implies that the determinants of intracellular pH are the transmembrane potential and the blood pH. The actual pH of the normal resting muscle cell is 5.99, as estimated from the normal transmembrane potential and blood pH, or as determined by direct measurements of intracellular pH.

Introduction

The measurement of intracellular pH (pH_i) of skeletal muscle has to a large extent involved indirect techniques that depend on the differential distribution of weak acids and bases between intracellular and extracellular fluids. Conway and Fearon (1), in early studies utilizing the CO_2–HCO_3^- buffer system, found pH_i to be approximately 6.0, with intracellular and extracellular hydrogen ion activity (H^+_a) in Donnan equilibrium. Other investigators (2–15), however, using either the CO_2–HCO_3^- system or the weak acid, 5,5-dimethyl-2,4-oxazolidinedione (DMO), have found much higher values for pH_i, ranging from 6.9 to 7.1.

Attempts to measure pH_i directly with pH-sensitive glass microelectrodes have been hampered by the technical difficulties in manufacturing suitable electrodes. Two separate studies on pH_i of skeletal muscle, however, have been published. Caldwell (13, 14), using relatively large microelectrodes with tip diameters of 50 to 100 μ, found that pH_i in crab skeletal muscle was approximately 7.0 and more or less independent of extracellular pH and transmembrane potentials (Em). Kostyuk and Sorokina (15), utilizing smaller electrodes (tip diameters of less than 1.0 μ) that were insulated either by shellac or by an outer shell of pH-insensitive glass, found pH_i in frog skeletal muscle also to be in the range of 7.1.

In preliminary studies in this laboratory, pH_i

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in rat skeletal muscle, measured with pH–sensitive microelectrodes, was found to be different from that obtained by Caldwell (14) and Kostyuk and Sorokina (15). Therefore, to determine pH_i and its relation to Em more precisely, we devised three methods. First, double–barreled electrodes were constructed, consisting of an integral pH–sensitive and reference electrode with a combined tip diameter less than 1 μ and insulation down to the terminal 5 to 20 μ of the electrode tip. This electrode had the great advantage of obviating the effects of interposed Em on pH_i measurements, since both electrodes were in the cell cytoplasm, and of permitting the simultaneous measurement of Em. Second, single–barreled pH–sensitive microelectrodes were constructed with tip diameters less than 0.5 μ. The extremely small tip size of these electrodes minimized disruption of the cell membrane in the course of puncture. Finally, triple–barreled electrodes were constructed, consisting of pH–sensitive and reference electrodes, as well as a third electrode through which current could be passed. The combined tip diameter was approximately 1 μ. This method provided a means of changing Em experimentally while simultaneously measuring pH_i and Em. In all of these methods the electrodes were insulated with a special glaze, which greatly decreased insulation leaks at the junction of the cell membrane and electrode. In addition, the effects of inadequate electrode insulation were specifically investigated

The results obtained with all three methods were in complete agreement and indicated that H^+_a of intracellular and extracellular fluid was in electrochemical equilibrium at all levels of Em. In the normal resting skeletal muscle fiber the Em was -89 mv and the pH_i approximately 6.0. These results are at great variance with those obtained by others utilizing either direct or indirect techniques. Only with inadequately insulated electrodes were we able to duplicate the results obtained by Caldwell (14) and Kostyuk and Sorokina (15).

Methods

Intracellular pH of skeletal muscle was measured in Sprague–Dawley rats weighing between 250 and 300 g. Before study, the rats were maintained on a standard laboratory chow diet and tap water ad libitum. The rats were anesthetized by an intraperitoneal injection of sodium pentobarbital; and the thigh muscles were exposed by removing the skin and subcutaneous tissues of one hind leg. Fascia was carefully dissected from the surface of these muscles and care was taken to disturb muscle fibers as little as possible. The muscle surface was continuously perfused with castor oil, preheated to maintain the temperature of the muscle at 37° C and to minimize loss of CO_2. Mineral oil was found to be entirely unsatisfactory for use with these micro pH electrodes. The siliconized surface of the electrodes avidly holds mineral oil and precludes any measurement of pH as a result of very high impedance of the layer of mineral oil. This does not occur with silicone oils or castor oil. In early studies, however, it was found that silicone oil eventually made micropuncture of muscle fibers difficult because of progressive hardening of the cell membrane. For this reason, castor oil was used.

Construction and testing of pH–sensitive microelectrodes. In manufacturing either single–, double–, or triple–barreled electrodes the pH–sensitive barrel must be adequately insulated so that only the portion of tip that is inside the muscle cell during puncture is sensitive to pH. In addition, the transition from the insulated to the uninsulated part of the electrode must be extremely smooth so that a portion of the insulated area can also be inserted into the cell without unduly disrupting the cell membrane. This assures that there are no insulation leaks at the junction of the cell membrane and the electrode.

The principle of the insulation technique was to coat Corning no. 0150 pH–sensitive capillary glass with a compatible glaze, Pemco no. TR-514-A,[1] which when heated fluxed to the surface of the glass capillary and completely blocked pH sensitivity. When this coated capillary was heated and pulled into a microelectrode, the insulating glaze extended almost to the tip. However, a small portion of uninsulated glass was pulled from beneath the glaze as the glass was drawn into a long taper, so that the terminal 5 to 20 μ of the electrode tip consisted of uninsulated pH–sensitive glass.

The length of the pH–sensitive tip was a function of the following three variables: *1*) diameter of the capillary tubing; *2*) thickness of the glaze; and *3*) length of the taper. The optimal dimension of the Corning no. 0150 capillary was found to be 0.8 ± 0.05 mm o.d. The proper thickness of glaze was obtained by closing one end of the capillary with soft paraffin and dipping in diluted glaze (200 g of glaze diluted with 450 ml of distilled turpentine). The dipped capillaries were air dried. The capillaries were dipped in glaze and air dried a second time and then heated at 600° C for 6 minutes. To prevent heat distortion, we sealed small copper wires into the unglazed end of the capillary so that the capillaries could be suspended in a vertical position during the period of heating. The ideal length of taper was found to be 9 to 11 mm from the beginning of the taper to the end of the tip.

[1] The glaze was supplied in no. 34 oil from Pemco, Division of Glidden Co., Baltimore, Md.

Single-barreled electrodes were pulled by heating glazed capillaries in a Scientific Instruments pipette puller. Those electrodes which on subsequent testing were shown to have closed tips were selected for use. Double-barreled electrodes, one side pH-sensitive, the other serving as a reference electrode, were prepared by cementing a glazed pH capillary and a slightly larger capillary (1.0 ± 0.05 mm o.d.) of Corning no. 0129 lead glass together with epoxy resin. Triple-barreled electrodes, consisting of one pH side and two reference sides, were prepared in a similar fashion using two pieces of the Corning no. 0129 capillary. The double-barreled or triple-barreled capillaries were then heated in the pipette puller until the glass was soft. The double capillary then was rotated 360° and the triple capillary was rotated 180°; the pipette puller was then released, pulling the components into either double- or triple-barreled tips. With lead glass capillary slightly larger in diameter than the pH-sensitive glass, the tip of the reference side pulled out slightly farther than the pH side. This resulted in the pH side being pulled closed while the reference side remained open. All of the electrodes were then filled with distilled water while heating under vacuum.

The process of pulling the electrodes altered the glass so that the tip resistances were very high (10^{11} ohms) and the pH sensitivity was poor. However, after the electrodes were soaked in distilled water at 4° C for approximately 1 week, the tip resistances fell to approximately 10^9 ohms and pH sensitivity was regained.

The pH-sensitive side of the electrode was used with distilled water as the internal reference solution. Although distilled water ordinarily has a very low conductivity and hence is a poor reference solution, sufficient electrolyte was leached from the pH glass within 1 to 2 days to raise the conductivity of the water far above that of the glass, so that the water functioned as a perfectly satisfactory reference solution.[2] The reference sides of the double- and triple-barreled electrodes were filled with 2.5 M KCl–0.5 M KNO_3 by threading small polyethylene tubing almost to the tip and displacing the water. A 34 (B and S)-gauge Ag–AgCl electrode was inserted into the single reference side of the double electrodes and into both reference sides of the triple electrodes and sealed with Silastic cement. Before use each electrode was siliconized by dipping in a 1:4 dilution of General Electric Dri-Film in toluene.

Either the single pH electrode or the pH side of the double- or triple-barreled electrodes was placed in a Teflon electrode holder filled with 2.5 M KCl–0.5 M KNO_3; an Ag–AgCl electrode in contact with the electrolyte solution in the holder was connected with the input of a Cary model 31 vibrating reed electrometer. The output of the electrometer was connected to a Leeds–Northrup recording potentiometer. When single-barreled electrodes were used, a Beckman calomel electrode served as the reference. With both double- and triple-barreled electrodes, one of the reference sides of the electrode was connected to the low-impedance side of the electrometer.

The micro pH electrodes were calibrated in standard buffers at room temperature (potassium phthalate pH 4.0, sodium–potassium phosphate pH 6.8, potassium phosphate pH 7.0, sodium–phosphate pH 7.41, potassium borate pH 10).[3] In all electrodes used the relation between electrode voltage and pH was linear over a range from pH 4.0 to 10. Electrodes reading less than 50 mv per pH U were discarded. However, in a few instances electrodes with lower slopes were specifically selected for special purposes. The initial calibration and testing of electrodes were performed in buffers at room temperature. Once the electrodes were selected for use in measuring pH_i they were then recalibrated in buffers maintained at 37° C.

To make certain that variations in tip potential of the reference side due to differences in the ionic strength of the standard buffers and the cytoplasm of the cell would not cause errors in pH measurement, we tested electrodes in a potassium phosphate buffer simulating the internal environment of the cell. This buffer had an osmolality of 330 mOsm per kg, contained 200 mEq K^+ per L, and had a pH of 6.92 at 37° C. All of the electrodes used read the pH of this buffer within ± 0.02 pH U of 6.92. In addition, the effect of a variety of solutions (buffers pH 4.0, 6.8, and 7.0; 0.15 M NaCl; 0.15 M KCl; and muscle homogenate) on the tip potential of the reference side of the electrode was evaluated by measuring the voltage between the reference side and a Beckman calomel electrode. Those electrodes in which the tip potential of the reference side varied by more than 5 mv among the various solutions were discarded.

Initially, we were unsuccessful in developing an *in vitro* method of testing the length of the pH-sensitive area of the microelectrodes. For this reason, we ascertained the adequacy of the insulation of electrodes used in the early experiments presented in this paper by using the rat renal tubule as previously described (16). When testing the single-barreled electrodes we placed the reference calomel electrode in a small beaker of saline into which the clipped end of the rat's tail was inserted; the pH sides of the double-and triple-barreled electrodes were read against their integral reference electrodes. Three small cups containing pH buffer standards (pH 4.0, 6.8, and 7.4) in 3% agar were placed in the peritoneal cavity of the rat; each cup was in electrical contact with the peritoneal surface through an opening in the bottom of the cup. We standardized the pH electrodes by reading the voltage in each of these buffers.

After calibration of the electrode, a surface tubule of

[2] In preliminary tests electrodes filled with distilled water were shown to have the same stability and pH sensitivity as did microelectrodes filled with more conventional reference solutions, such as buffered sodium citrate or 0.1 N HCl. However, the more conventional reference solutions rapidly dissolved the electrode tips (within 8 to 12 hours). For this reason distilled water was used as the internal reference solution.

[3] Beckman Instruments, South Pasadena, Calif.

the kidney was punctured by a double-barreled injection pipette. One side of this pipette was filled with silicone oil and the other with an isotonic buffer solution. The tubule was filled with oil and then punctured with the pH electrode. The oil drop was then split and the buffer solution (pH 6.8 or 7.4) was perfused past the electrode tip; the occasional electrode that did not read within 0.1 of the known pH of the buffer was discarded. As a final test of the electrode insulation, a second buffer (pH 6.0, potassium phosphate), differing in pH from the intratubular buffer, was layered over the surface of the kidney; if the procedure caused a permanent shift in the reading greater than 0.1 pH U, the electrode was discarded.

Recently, we have been successful in developing a satisfactory *in vitro* method of testing the length of the pH-sensitive area of the microelectrodes. A variety of difficulties had to be overcome in developing the *in vitro* test system. For example, it was found that with buffers solidified with 3% agar into which the microelectrodes could be inserted a measured distance was entirely unsatisfactory, owing to the fact that when a buffer of a different pH was overlaid on the surface of the agar buffer, the new buffer rapidly seeped down the side of the electrode and caused a shift in the pH reading that eventually approached the value of the overlaid buffer. We tried several different membranes to obviate the seepage of buffer along the surface of the electrode, including cellophane, Mylar, silicone rubber, Teflon, and paraffin. All proved to be too tough to puncture with the fragile microelectrodes.

It was possible, however, to make very thin latex membranes that prevented the seepage of buffer and could be easily punctured by the microelectrodes. These membranes were made by placing a very small drop of undiluted latex injection compound [4] over a 3-mm hole in a 2- × 2-cm square of Parafilm. When dried, these membranes were approximately 1.0 μ thick. The test system was prepared by placing warm pH 6.0 buffer (potassium phosphate) containing 3% agar into a glass chamber that contained an Ag–AgCl reference electrode. After the buffer cooled, one of the latex test membranes was placed over the surface of hardened buffer.

The sequence for testing the microelectrodes was as follows: *Step 1.* Under microscopic visualization the microelectrode tip was advanced through the latex membrane into the agar buffer for a measured distance (5 to 15 μ). The depth of penetration was measured with an eyepiece micrometer. The voltage in the pH 6.0 buffer was read between the pH and reference sides in the double-barreled electrodes, and between the pH electrode and an Ag–AgCl reference electrode in the agar buffer when single-barreled electrodes were used. *Step 2.* pH 7.4 buffer (potassium phosphate) was layered over the surface of the latex membrane. If the voltage reading was unaltered, the electrode was considered insulated for that depth of penetration. However, if the voltage changed, the electrode was considered inadequately insulated. If the electrode tested uninsulated, it was then progressively advanced into the agar buffer until the correct reading for pH 6.0 buffer was obtained. The measurement of the depth of penetration with the eyepiece micrometer gave the length of the uninsulated portion of the electrode with an accuracy of ± 1.0 μ. *Step 3.* The possible effect of the muscle Em on the measurement of intracellular pH was simulated by imposing a 90-mv potential across the latex membrane by means of reference electrodes in the agar buffer and pH 7.4 buffer and a calibrated potentiometer. With an insulated electrode, no effect of the imposed membrane potential was seen in the pH electrometer reading.

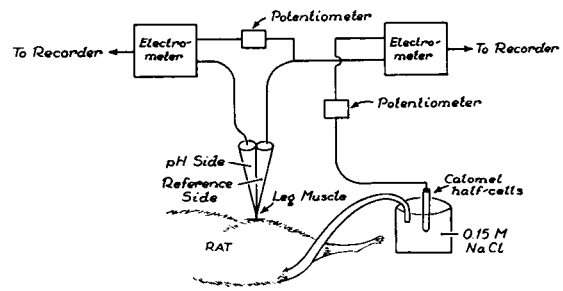

FIG. 1. TECHNIQUE FOR SIMULTANEOUS MEASUREMENT OF INTRACELLULAR pH AND TRANSMEMBRANE POTENTIAL WITH DOUBLE-BARRELED ELECTRODES.

Simultaneous measurement of pH_i and Em with double-barreled electrodes. Figure 1 depicts the circuit used with the double-barreled microelectrodes. By using two Cary model 31 electrometers with separate recorders it was possible to record intracellular pH and transmembrane potential simultaneously. A potentiometer was placed on the low impedance side of each electrometer so that the tip potential of the reference side could be adjusted to zero on the surface of the muscle and the voltage reading of the pH side could be adjusted to appropriate readings in standard buffers. The transmembrane potential was determined by the voltage difference between the reference side of the double electrode inside the muscle and a calomel half cell in electrical contact with the extracellular fluid of the rat through the severed tail. It was found that a voltage of ± 500 mv applied between the reference side of the electrode and the calomel half cell did not affect the pH reading of the glass electrode in buffer solutions. This test showed that there was no electrical feedback between the two electrometers and that the voltage difference between reference side of the electrode and a calomel half cell did not influence the potential between the reference and pH sides of the double electrode.

Simultaneous measurement of pH_i and Em while experimentally varying Em with triple-barreled electrodes. In order to examine the relationship of intracellular pH and transmembrane potential over a wide range of membrane potentials, we used triple-barreled electrodes (one pH barrel and two reference barrels). The second reference barrel was used to apply current across the muscle

[4] General Biological Supply House, Chicago, Ill.

membrane in order to vary the transmembrane potential.

Each triple-barreled electrode had to be carefully selected and tested. It was necessary to find the maximal current that could pass through the second reference without resulting in resistive coupling between the pH side and the first reference electrode. This was easily accomplished by placing the electrode in a buffer of known pH together with two additional calomel reference electrodes, one of which was used to read the tip potential of the first reference electrode and the other to complete the current circuit between the second reference electrode and the solution. By passing current between the second reference electrode and its calomel electrode and simultaneously measuring the tip potential of the first reference and the pH of the buffer solution, it was possible to increase the current progressively until the tip potential of the first reference electrode and the pH reading began to vary. This was defined as the maximal tolerated current. During the *in vivo* experiments, the current applied to the second reference electrode was always kept well below the level that had been shown to influence the other two sides of the electrode. In general, the maximal tolerated current for most triple-barreled electrodes was in the order of 5×10^{-7} amp.

Measurement of Em in normal resting skeletal muscle. Since the simultaneously measured Em with both the double- and triple-barreled pH electrodes frequently yielded varying degrees of membrane depolarization, the precise Em of normal resting skeletal muscle was measured with single-barreled Ling-type microelectrodes (17). These were pulled from borosilicate glass having an outside diameter of 0.9 mm and were filled with 2.5 M KCl-0.5 M KNO_3. Electrodes were selected which had tip resistances between 10 and 50 megohms.

In all experiments, arterial blood pH was determined with a Beckman anaerobic electrode at 37° C and a Vibron or Beckman expanded scale pH meter.

Results

Testing of electrodes. When the double-barreled electrodes were tested in various standard buffers, the relation between pH and the voltage reading was linear over a range from pH 4.0 to 10.0. The calibration curve for each electrode was highly reproducible during the course of an experiment and from day to day.

The results of the *in vitro* test for adequacy of insulation are shown in Table I for typical insulated and inadequately insulated electrodes. The voltage readings in standard buffers pH 6.0 and 7.4 were obtained first. The electrode was then advanced through the latex membrane into the agar buffer, pH 6.0, to a depth of 10 μ. The voltage reading in the agar buffer was always approximately the same as that obtained in the standard pH 6.0 buffer. While the tip was still in the agar buffer, the latex membrane was overlaid with pH 7.4 buffer. This had no effect on the voltage reading of the insulated electrode, which continued to read pH 6.0. With the inadequately insulated electrode the voltage shifted to 86 mv, indicating an apparent pH of 7.24. The next step in the test was to impose a potential difference of 90 mv across the latex membrane with the agar buffer being negative relative to the superficial buffer. The imposition of the transmembrane potential had no effect on the voltage reading of the insulated electrode, but shifted the voltage of the inadequately insulated electrode from + 86 to + 101 mv. This caused a shift in the apparent pH from 7.24 to 6.97. Inadequately insulated electrodes, therefore, are subject to two sources of error: *1*) they are influenced by the pH of the fluid on both sides of the membrane, and *2*) they are partially affected by the transmembrane potential. Only electrodes with uninsulated tips less than 20 μ long were used for the measurement of pH_i.

We examined the ability of these well-insulated

TABLE I

In vitro test of double-barreled microelectrodes

	Insulated electrode			Uninsulated electrode		
	Depth of penetration	Electrometer reading	Apparent pH of agar buffer	Depth of penetration	Electrometer reading	Apparent pH of agar buffer
	μ	mv		μ	mv	
Buffer pH 6.0		+157 } 58 mv/pH U			+155 } 55 mv/pH U	
Buffer pH 7.4		+ 76 }			+ 78 }	
Agar buffer pH 6.0	10	+155	6.04	42	+157	5.96
Overlaid buffer pH 7.4	10	+155	6.04	42	+ 86	7.24
Transmembrane potential of 90 mv imposed	10	+155	6.04	42	+101	6.97

double-barreled microelectrodes to measure pH of biologic fluids by comparing the pH of blood and muscle homogenates obtained with these electrodes with that obtained with Beckman macroelectrodes. As shown in Table II, the measurement of blood pH with the microelectrode agreed very closely with that obtained with the Beckman electrode. The greatest discrepancy between the two readings was 0.16 pH U; the average difference was 0.02. The results obtained in muscle homogenates are also listed in Table II. Again, the results obtained with the microelectrode closely agreed with those obtained with the Beckman electrode. These results indicate that the microelectrodes are nearly as accurate as the Beckman electrode in measuring the pH of biologic fluids and that these electrodes are not subject to any unique artifacts in the presence of proteins or other macromolecules.

Measurements with the double-barreled electrodes. Simultaneous measurements of blood pH, pH_i, and Em of skeletal muscle were obtained in 57 normal rats. The blood pH was 7.41 ± 0.06 (SD). The pH_i varied widely from 5.77 to 7.14, with an average of 6.48. The Em ranged from − 30 to − 92 mv.

TABLE II

Comparison of pH measurements of blood and muscle homogenate made with double-barreled electrodes and with Beckman glass electrodes

Experiment	Double-barreled microelectrodes*	Beckman electrode	Δ pH
		Blood	
1	7.37	7.37	0.00
2	7.33	7.38	−0.05
3	7.39	7.35	+0.04
4	7.50	7.45	+0.05
5	7.46	7.48	−0.02
6	7.76	7.60	+0.16
7	7.38	7.32	+0.06
8	7.39	7.47	−0.08
9	7.39	7.39	0.00
10	7.34	7.34	0.00
11	7.48	7.44	+0.04
12	7.45	7.42	+0.03
13	7.36	7.42	−0.06
14	7.42	7.42	0.00
15	7.44	7.44	0.00
16	7.40	7.40	0.00
			Mean +0.02 ± 0.04
		Muscle homogenates	
1	7.02	7.10	−0.08
2	7.15	7.14	+0.01
3	7.10	7.20	−0.10
4	6.97	7.02	−0.05

* Each value represents a single measurement obtained with a different double-barreled electrode.

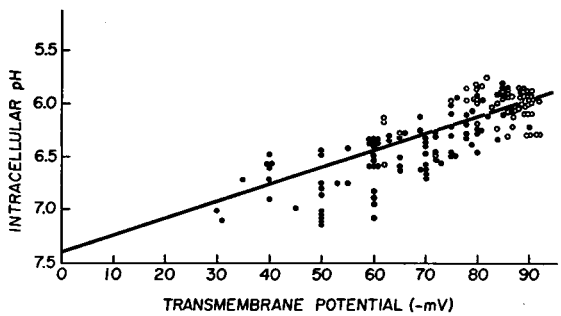

FIG. 2. RELATION BETWEEN INTRACELLULAR pH (pH_i) AND TRANSMEMBRANE POTENTIAL (Em) AS MEASURED WITH DOUBLE-BARRELED ELECTRODES. The solid line is calculated from the Nernst equation, $Em = 61.5\ (pH_i - pH_{blood})$, assuming electrochemical equilibrium of H^+ in intracellular and extracellular fluid and a blood pH of 7.40. The solid dots represent values obtained with electrodes tested in the renal tubule. The open circles represent values obtained with electrodes tested in the *in vitro* system.

In Figure 2, the relation between Em and pH_i is plotted. The solid line represents the relation between pH_i and Em, estimated from the Nernst equation, assuming H^+_a in intracellular and extracellular fluid to be in thermodynamic equilibrium. The solid dots are results obtained in earlier experiments with electrodes tested in the renal tubule, whereas the open circles represent values obtained with electrodes whose insulation was checked in the *in vitro* system. It is evident that the points scatter about the solid line, suggesting that H^+_a is in fact in electrochemical equilibrium.

The wide scatter in Em from − 30 to − 92 mv indicates that micropuncture with these microelectrodes tends to cause an electrical leak, thus depolarizing the membrane. If H^+_a were in equilibrium across the cell membrane, any artifactual change in Em might in itself alter pH_i. The true pH_i, therefore, can only be ascertained in those measurements where Em was normal. To determine the normal Em of resting skeletal muscle, we next made measurements using Ling-type microelectrodes. The average Em in 259 measurements in 12 rats was − 88.9 ± 3.9 mv, a value very similar to that obtained by others (18, 19).

In view of the value of − 89 mv for the normal

TABLE III

Intracellular pH and transmembrane potential of normal rat skeletal muscle measured with double-barreled electrodes

Rat no.	Blood pH (pH_b)	Electrode no.	Membrane potential (Em)	Intracellular pH		
				Measured	Calculated*	Difference†
			mv			
1	7.39	1	86	6.29	5.99	+0.30
		2	90	5.92	5.93	−0.01
		3	90	5.94	5.93	+0.01
			90	6.08	5.93	+0.15
			90	5.96	5.93	+0.03
			92	6.28	5.90	+0.38
2	7.42	4	86	5.87	6.02	−0.15
			88	5.80	5.99	−0.19
		5	85	5.92	6.04	−0.12
			92	5.77	5.92	−0.15
		6	90	5.86	5.96	−0.10
			89	6.02	5.97	+0.05
3	7.41	7	89	5.97	5.96	+0.01
		8	85	5.90	6.03	−0.13
		9	85	5.88	6.03	−0.15
4	7.41	10	88	5.82	5.98	−0.16
			87	6.00	6.00	0.0
5	7.38	11	89	5.92	5.93	−0.01
			90	5.97	5.92	+0.05
			87	6.05	5.97	+0.08
6	7.44	12	92	5.95	5.94	+0.01
			90	5.94	5.99	−0.04
			90	6.08	5.98	+0.10
		13	87	6.20	6.03	+0.17
			90	5.94	5.98	−0.04
			90	6.28	5.98	+0.30
7	7.42	14	90	6.08	5.96	+0.12
			90	5.86	5.96	−0.10
			85	6.03	6.04	−0.01
			90	5.96	5.96	0.00
		15	90	6.28	5.96	+0.32
			90	6.28	5.96	+0.32
		16	90	5.96	5.96	0.00
			90	5.92	5.96	−0.04
			89	5.94	5.97	−0.03
			87	6.10	6.01	+0.09
		17	85	5.85	6.04	−0.19
			85	5.80	6.04	−0.24
Mean ± SD			88.7 ± 2.2	5.99 ± 0.14	5.97 ± 0.04	+0.02 ± 0.15

* Calculated equilibrium intracellular pH = pH_b − (Em/61.5).
† Difference = measured pH − calculated pH.

Em, only those measurements of pH_i in which the simultaneous Em was between − 85 and − 93 mv were used to establish the normal pH_i. Thirty-eight measurements that were found to fit these criteria are listed in Table III. pH_i ranged from 5.8 to 6.3, with an average of 5.99 ± 0.14 (SD). The calculated equilibrium pH_i for the average Em of − 88.9 mv and blood pH of 7.41 is 5.97. The close agreement between the observed pH_i and the calculated equilibrium value strongly suggests that H^+ is in electrochemical equilibrium across the muscle cell membrane.

Since these results are at such marked variance with those of Caldwell (14) and Kostyuk and Sorokina (15), the question of inadequate electrode insulation was specifically investigated. On the basis of the *in vitro* test, electrodes with pH-sensitive tips ranging from 5 to 80 μ in length were selected. The values of pH_i obtained with these various electrodes were plotted against the

length of the pH–sensitive tip as shown in Figure 3. Only those values of pH_i in which the simultaneous Em was − 85 mv or greater are plotted. Those electrodes with pH–sensitive tips of 20 μ or less gave pH_i of approximately 5.9. Those electrodes with pH–sensitive tips greater than 50 μ gave pH_i of approximately 7.0. Those electrodes with sensitive tip lengths between 20 and 50 μ gave intermediate pH_i between 6.0 and 7.0. The measured value of pH_i, therefore, is clearly a function of the adequacy of electrode insulation. Those electrodes shown to have adequate insulation by *in vitro* testing yielded a pH_i value of approximately 5.9, whereas those shown to be inadequately insulated yielded a value for pH_i more closely related to that reported by Caldwell (14) and Kostyuk and Sorokina (15).

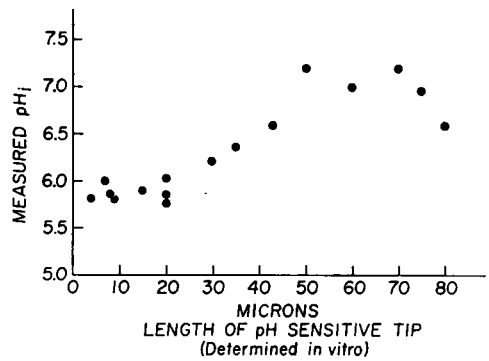

FIG. 3. RELATIONSHIP OF MEASURED INTRACELLULAR pH TO THE MEASURED LENGTH OF THE pH–SENSITIVE TIP OF DOUBLE–BARRELED ELECTRODES. All measurements of pH_i were accompanied by simultaneously measured transmembrane potentials of − 85 mv or higher.

Measurements with single–barreled electrodes. The low membrane potential obtained in many of the punctures with the double-barreled electrodes is undoubtedly the consequence of an electrical leak due to the comparatively large size of the electrode tip (0.5 to 1.0 μ). To minimize this source of error, we measured pH_i separately with single–barreled electrodes whose tip size was minute (0.1 μ).

The experiments with the single–barreled pH–sensitive electrodes were specifically designed to determine if H^+_a is in electrochemical equilibrium across the muscle cell membrane. The potential difference between the single–barreled pH electrode and the extracellular reference electrode was measured first while both were in extracellular fluid and then after the microelectrode had been advanced into the muscle cell. If the microelectrode had a theoretic slope of 61.5 mv per pH U and if H^+_a was in electrochemical equilibrium, then there should have been no change in the potential reading between the two when the microelectrode was advanced from the extracellular into the intracellular fluid. However, the microelectrodes used in these experiments had somewhat less than theoretic slope. This means that even if H^+_a was in electrochemical equilibrium, a small potential change would have occurred when the microelectrode was advanced into the cell; this change can be predicted from the electrode slope and the normal resting Em of − 89 mv (Table IV, column 4). On the other hand, if H^+_a was not in electrochemical equilibrium and pH_i was 7.0 as reported by others, the observed voltage change would have been much higher. This value can be calculated from an assumed pH_i of 7.0, the normal resting Em of − 89 mv, and the electrode slope (Table IV, column 5).

The observed potential changes with eight different electrodes are shown in Table IV, column 3. In every instance the observed potential change closely approximated the predicted value for electrochemical equilibrium (Table IV, column 4) and was markedly different from the potential change predicted from a pH_i of 7.0 (Table IV, column 5).

One of the possible difficulties in single–barreled electrode experiments, where potential change is small, is determining whether or not the electrode tip is inside the cell. We used two techniques to obviate this difficulty. First, the muscle surface was carefully observed microscopically during the puncture procedure. Almost invariably the cell membrane could be seen to dimple and then return to its normal position as the electrode entered the cell. This type of microscopically observed puncture with the Ling–type microelectrode invariably gave a high sustained Em. The second means of surmounting this difficulty was to select pH–sensitive electrodes with a low slope and thus amplify the predicted voltage change. As shown in Table IV, electrode number 4 had a slope of 45 mv and the predicted voltage change was 22.3 mv. When the muscle was punctured, sudden changes in the potential occurred as the membrane was penetrated, and the observed potential changes were

TABLE IV

Single-barreled electrode measurements

Electrode number and slope	Number of measurements	Measured Δ E* (mean ± SD)	Calculated Δ E† assuming H_a^+ equilibrium	Calculated ΔE‡ assuming non-equilibrium of H_a^+
		Insulated electrodes		
1 56.5 mv	3	16.3 Range 5–25	7.1	66.4
2 50.3 mv	10	14.2 ± 3.2	16.1	68.9
3 58.0 mv	10	3.7 ± 4.7	4.9	65.8
4 45.0 mv	11	17.7 ± 4.1	23.7	71.0
5 52.0 mv	6	7.7 ± 3.9	13.6	68.2
6 55.5 mv	3	8.8 Range 7.5–10	8.5	66.8
7 49.0 mv	7	18.9 ± 5.1	18.0	69.4
8 61.0 mv	8	1.5 ± 1.2	0.5	64.6
		Poorly insulated electrodes		
9 44 mv	8	68.6 ± 2.5	25.2	71.4
10 50 mv	9	82.7 ± 4.9	16.5	69.0

* Δ E is the difference between the potential of the single-barreled electrode in extracellular fluid (ECF) and that measured within the muscle fiber.

† Δ E = 89 mv − (electrode slope × 1.45) where 89 mv is the assumed Em and 1.45 is equivalent pH units at 37° C (89/61.5 = 1.45). H_a^+ = intra- and extracellular hydrogen ion activity.

‡ Δ E = 89 mv − (electrode slope × 0.4) where 89 mv is the assumed Em and 0.4 is equal to the difference between pH of ECF and an assumed intracellular pH (pH_i) of 7.0 (see text).

again equal to the value predicted for electrochemical equilibrium.

The effect of inadequate electrode insulation was also tested in these experiments. The results from two inadequately insulated electrodes are shown at the bottom of Table IV. In contrast to the results obtained with the insulated electrodes, there was a marked change in voltage when the inadequately insulated electrodes penetrated the muscle membrane. It is not immediately apparent why a poorly insulated electrode, which should be reading predominantly pH of the extracellular fluid, should record such a large voltage change when the cell is punctured. Although the reason is not completely clear, it was repeatedly demonstrated in the *in vitro* test system that inadequately insulated electrodes read most of the transmembrane potential (70 to 80 mv out of 90), even though the electrode was predominantly detecting the pH of the overlaid buffer. Thus, the poorly insulated electrodes behaved the same in the *in vitro* test system as during muscle puncture. If the voltage change obtained when puncturing muscle with these electrodes is corrected for an Em of − 89 mv, the calculated pH_i is approximately 7.0. Thus, the inadequately insulated single electrodes gave results very similar to the inadequately insulated double-barreled electrodes.

Measurements with the triple-barreled electrodes. The experiments with double-barreled electrodes summarized in Figure 2 suggested that electrochemical equilibrium of H^+_a existed over a very wide range of Em. However, in these studies Em fell because of an electrical leak incident to the micropuncture. To determine whether, in fact, thermodynamic equilibrium of H^+_a obtained over a wide range of Em independent of membrane injury, we conducted experiments with triple-barreled electrodes through which a current could be passed to alter Em. After puncturing a single

TABLE V

Effect of changing transmembrane potential on intracellular pH as determined with a triple-barreled electrode (study 3)

Initial pH_i	Initial Em	Current	New Em	New pH_i	Theoretical cell pH_i*	Theoretical electrode pH†
	mv	*amp*	*mv*			
6.15	−73	0				
		10^{-8}	−19	7.02	7.03	7.41
6.15	−73	0				
		6×10^{-9}	−43	6.71	6.65	6.85
		9×10^{-9}	−23	7.13	7.00	7.36
6.15	−75	0				
		10^{-8}	−27	7.06	6.94	7.25
		10^{-7}	+15	7.69	7.62	8.24
6.22	−71	0				
		-2×10^{-8}	−149	4.74	4.94	4.41
		-4×10^{-8}	−233	3.25	3.57	2.45
		0 (Recovery)	−47	6.64	6.61	6.78

* Calculated on the basis of equilibrium distribution of H^+ across cell membrane.
† Calculated on the basis that the change in Em causes an electrical artifact in the pH electrode; the electrode slope was used in these calculations (see text).

muscle fiber and obtaining a stable Em greater than − 70 mv, current was applied through the third barrel of the electrode so as to either raise or lower Em. In these experiments, care was taken to select triple-barreled electrodes whose voltage change per unit pH change was less than the theoretical value of 61.5 mv. This greatly facilitated the differentiation of electrical artifacts from true changes in pH_i. For example, if H^+_a rapidly achieved electrochemical equilibrium, a 61.5-mv change in Em would cause a 1.0-U change in pH_i. If the pH electrode had a perfect slope, an electrical artifact of 61.5 mv would also read as a 1.0-U change in pH_i. However, if the slope of the electrode were significantly less than the theoretical value, for example 50 mv per pH U, then an electrical artifact of 61.5 mv would be read as a 1.2-U change in pH_i.

The data from a representative triple-barreled electrode experiment are presented in Table V. The initial Em was − 73 mv and pH_i was 6.15. The cell membrane was both depolarized and hyperpolarized by the passage of current through the electrode. Em was varied between + 15 and − 233 mv. When the membrane potential was changed to a new value, the pH_i shifted to a new steady state almost instantaneously. The exact rate at which pH_i changed could not be ascertained because the limiting response time of the high resistance electrodes and the recording system was of the order of 10 to 15 seconds. The pH_i ranged from 7.69 in the maximally depolarized state to 3.25 in the maximally hyperpolarized state. In every instance, the measured pH_i was close to the equilibrium pH_i calculated from the observed Em and was different from the value that would have been anticipated from an electrical artifact affecting the micro pH electrode. This can be seen more clearly in Figure 4, where the actual pH_i falls closer to the line predicted from the Em

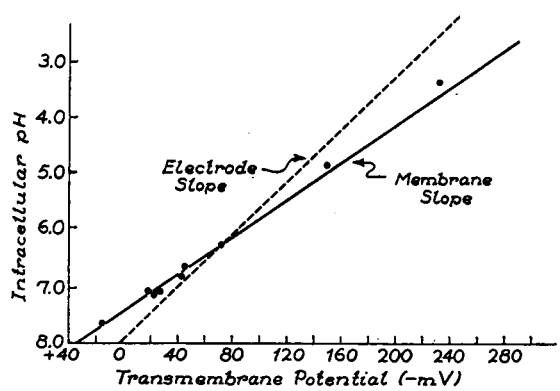

FIG. 4. EFFECT OF CHANGING TRANSMEMBRANE POTENTIAL ON INTRACELLULAR pH AS DETERMINED WITH TRIPLE-BARRELED ELECTRODES. The solid line is calculated on the basis of electrochemical equilibrium across the cell membrane, which would give a slope of 1 pH U per 61.5-mv change. The dotted line is calculated on the basis of the electrode slope (see text). The electrode used in this study had a slope of 43 mv per pH U.

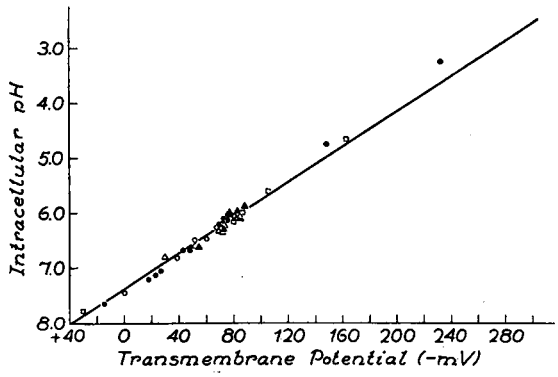

FIG. 5. RELATIONSHIP BETWEEN INTRACELLULAR pH AND TRANSMEMBRANE POTENTIAL WHEN Em IS EXPERIMENTALLY VARIED WITH TRIPLE-BARRELED ELECTRODES. Data from five experiments are plotted, each with a different symbol.

than to the line predicted from the electrical characteristics of the micro pH electrode. This relation suggests that the observed changes in pH_i are true changes in pH rather than electrode artifacts. The data from five similar experiments are plotted in Figure 5. The solid line represents theoretical pH_i at each Em, assuming that H^+_a in intracellular and extracellular fluid is in electrochemical equilibrium and assuming an extracellular pH of 7.4. The experimental points are closely clustered along the theoretical line. From these data we concluded that H^+_a is in rapid electrochemical equilibrium across the muscle cell membrane and that, consequently, the pH_i is determined by both the Em and the pH of extracellular fluid.

Discussion

The results of our studies support Conway's hypothesis that in resting skeletal muscle intracellular H^+_a is in electrochemical equilibrium with extracellular H^+_a (20). Moreover, variations in Em produce predictable changes in pH_i. It follows, therefore, that with the knowledge of blood pH and Em, pH_i of muscle can be calculated. In a separate series of experiments utilizing Ling-type electrodes the resting Em of normal rat muscle was found to be − 89 mv. With this value of Em and a blood pH of 7.4, the calculated pH_i would be 5.97. Direct measurement of pH_i obtained with double-barreled electrodes in which the membrane was not significantly depolarized (as indicated by Em between − 85 and − 93 mv, Table III) disclosed an average pH_i of 5.99 ± 0.14, which is in excellent agreement with the value of 5.97 predicted for thermodynamic equilibrium.

The value of 5.99 for the pH_i of normal rat skeletal muscle obtained in the present studies is far below the value of approximately 7.0 reported in other studies that utilized either glass electrodes or the partition of weak acids or bases (21). In a preliminary report from our laboratory, pH_i was given as 6.79 (22). The validity of those earlier measurements, however, is questionable for the following three reasons: first, the adequacy of electrode insulation could not be sufficiently established; second, the tip diameters were greater than 1.0 μ; and third, the simultaneous Em was not recorded.

It is important, therefore, to consider the possible sources of error in our present method. The first is a loss of selectivity of the micro pH electrode. Although the Corning 0150 glass has been shown to be sensitive only to H^+ when used within the physiologic pH range (23), it is possible that some selectivity is lost in the process of manufacturing the microelectrodes. This seems unlikely, however, since it was shown that these electrodes accurately measured pH of buffer solutions despite wide variations in ionic composition. A second possibility is that the high protein content of intracellular fluid in some way influences the behavior of the micro pH electrodes. This also appears unlikely, since these electrodes gave the same pH readings of blood and muscle homogenates (Table II) as did Beckman macro pH electrodes. A third possibility is that the electrodes were inadequately insulated. This can be excluded by the fact that those electrodes which were shown to be adequately insulated by *in vitro* testing gave a pH_i of approximately 6.0, whereas only those electrodes shown by the *in vitro* test to be inadequately insulated gave higher values, which were similar to those reported by others. A fourth possibility is that there were electrical artifacts due either to changes in the tip potential of the reference electrode or to an effect of Em on the over-all behavior of the double-barreled electrode. We minimized errors in tip potential by selecting electrodes with small tip potentials that remained constant in different test solutions

of varying ionic compositions. The fact that the double-barreled microelectrodes gave the same pH reading of blood and of muscle homogenates as did Beckman macro pH electrodes is evidence that the tip potential was not in any important way altered by biologic solutions. It is unlikely that the normal Em of skeletal muscle influences pH readings obtained with the microelectrodes, since a transmembrane potential of − 90 mv was demonstrated to have no effect on the behavior of well-insulated electrodes in the *in vitro* test system (Table I).

A final possibility is some type of artifact resulting from cellular injury. Micropuncture of cells may cause two types of injury. First, the disruption of the integrity of the cell membrane may give rise to an electrical leak, which lowers Em. If an electrical leak is the only disturbance created by the micropuncture, this would not necessarily alter pH_i unless the latter were, in part at least, determined by potential. If, in fact, it could be shown that H^+ were in electrochemical equilibrium across the cell membrane, than a fall in Em as a result of an electrical leak would raise pH_i but should not alter the relationship among pH_i, extracellular pH, and Em. The results with both single- (Table IV) and double-barreled electrodes (Figure 2, Table III) suggest that H^+_a is in electrochemical equilibrium at all values of Em. This point was much more securely established by experiments with triple-barreled electrodes in which the cell membrane was not only depolarized but also hyperpolarized. This was accomplished not as a result of fortuitous electrical leaks incident to micropuncture, but rather by the controlled passage of current through the electrode (Figures 4 and 5, Table V). An induced variation in Em from + 30 to − 230 mv was invariably followed by a rapid restoration of electrochemical equilibrium of H^+.

Micropuncture might cause a second form of injury resulting in intracellular acid-base changes that could alter the relation between pH_i and blood pH. Acute cellular injury might alkalinize the cell by accelerating hydrolysis of creatine phosphate (24) and by permitting seepage of extracellular fluid into the cell. On the other hand, injury might result in localized acidification around the electrode tip, perhaps by increased production of lactic acid. It is highly unlikely, however, that in the presence of significant acid-base changes due to cellular injury, pH_i would respond in such a rapid and sensitive way to alterations in Em as was observed with the triple-barreled electrodes. Moreover, studies with single-barreled electrodes in which the minute tip diameter minimized cellular injury also indicated electrochemical equilibrium (Table IV).

In summary, none of these possible sources of error account for the lower pH_i obtained in our studies or for the fact that pH_i is in electrochemical equilibrium with pH of extracellular fluid.

The question may then be asked why other investigators using either glass microelectrodes or buffer partition techniques found a pH_i of approximately 7.0, which was not in electrochemical equilibrium with extracellular pH (pH_e). In view of the fact that we obtained a pH_i of approximately 7.0 with electrodes proven to be inadequately insulated by our *in vitro* test, it is tempting to speculate that the electrodes used by both Caldwell (14) and Kostyuk and Sorokina (15) were also uninsulated. However, their published results showing that the electrodes were not affected by alterations in pH_e (other than those changes produced by variations in Pco_2) militate somewhat against this conclusion.

In attempting to evaluate the difference in pH_i obtained by others using buffer partition techniques and by us using glass microelectrodes, it is helpful to consider the nature of pH_i. As discussed by Caldwell (24), it is extremely unlikely that there is any single homogeneous value for pH_i in a complex, highly organized structure such as a skeletal muscle cell. First, the interior of subcellular particles (e.g., nuclei, mitochondria, microsomes) might have a pH entirely different from that of the cell cytoplasm due to metabolic processes, Donnan effects, or possible other factors. Second, the cell cytoplasm itself, which consists of a high concentration of polyelectrolytes, most likely has no uniform pH. The pH in the micro-environment surrounding charged macromolecules, which has been termed surface pH (pH_s), may differ markedly from the pH of the bulk solution. According to Danielli (25), pH_s is a function of the isoelectric point of the molecule, the ionic strength of the solution, and the pH of the bulk phase. If the isoelectric point of the polyelectrolytes is higher than the bulk phase

pH, pH_s will be alkaline relative to the bulk solution, whereas pH_s will be relatively acid if the isoelectric point is lower than the bulk phase pH. Therefore, in a solution containing many different polyelectrolytes with differing isoelectric points there will be a single value for the pH of the aqueous bulk phase but many different values for pH_s.

The measured value for pH_i will depend on the method employed. For example, in protein solutions the pH obtained with the glass electrode may differ markedly, sometimes as much as 1 to 2 U, from that obtained with various acid–base indicators (25). Since the volume of water surrounding polyelectrolytes is of molecular dimension, both macro and microglass electrodes measure bulk phase pH, whereas the acid–base indicators may measure some integrated pH, which is perhaps disproportionately influenced by pH_s. Acid–base indicators that are positively charged in the ionized form will be attracted by Donnan forces to negatively charged polyelectrolytes and will give a pH value more acid than the bulk phase pH; indicators that are negatively charged in the ionized form (e.g., the CO_2–HCO_3^- and DMO systems) will be preferentially attracted to positively charged polyelectrolytes and will give a pH more alkaline than the bulk phase pH. Although the bulk of muscle protein, consisting of actin and myosin, has isoelectric points below 6.0 and would thus be negatively charged in a solution with a bulk phase pH of 6.0, electrophoretic studies show that a significant portion of the protein is positively charged (26). Whether the concentration of these basic proteins is sufficiently high to account for the difference in the bulk phase pH of 6.0 measured with our glass electrodes and the pH 7.0 obtained with CO_2–HCO_3^- and DMO methods is not known. However, the fact that some of these positively charged proteins that migrate towards the cathode are extremely basic, having isoelectric points as high as 11.0 (e.g., histones), makes it likely that the intracellular pH measured with negatively charged acid–base indicators would be significantly more alkaline than either the bulk phase pH or the average pH_s.[5]

It is reasonable to assume, therefore, that in the cell cytoplasm each different protein has a different pH_s depending upon its isoelectric point, and that each pH_s is in Donnan equilibrium with the single bulk phase pH. The glass microelectrodes measure only the bulk phase pH, whereas acid–base indicators, such as the CO_2–HCO_3^- system or DMO, measure neither the bulk phase pH nor the average pH_s. Although the glass electrode measures only the bulk phase pH rather than the average or integrated cell pH, the question as to whether or not H^+ ions are in electrochemical equilibrium across the cell membrane can be answered only by knowing the H^+_a in the bulk phase and not on the basis of any average H^+_a throughout the cell cytoplasm. The results of our studies indicate that the bulk phase pH, measured with the microelectrodes, is indeed in electrochemical equilibrium with extracellular pH, and that when Em is altered there is a rapid change in measured pH_i with reestablishment of electrochemical equilibrium.

The speed with which electrochemical equilibrium was restored after a transient disequilibrium produced by changing Em with triple-barreled electrodes was surprising and unexpected. In those studies the change in pH_i after a change in Em was almost instantaneous. Any delay observed between the change in Em and pH_i could by fully accounted for by the response time of the micro pH electrode and the recording equipment. This rapid change in pH_i indicates that large amounts of either H^+ or OH^- or HCO_3^- ions can be transferred across the muscle cell membrane in a very short time.

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[5] In a complex system of polyelectrolytes acid–base indicators such as CO_2–HCO_3^- and DMO do not measure either the bulk phase pH or the average cell pH. However, as pointed out by Caldwell (24) and by Adler, Roy, and Relman (12), they do measure the average HCO_3^- and OH^- concentrations in the cell.

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