The Simultaneous Determination of Muscle Cell pH Using a Weak Acid and Weak Base

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ABSTRACT Should significant pH heterogeneity exist within cells then the simultaneous calculation of intracellular pH from the distribution of a weak acid will give a value closest to the highest pH in the system, whereas calculation from the distribution of a weak base will give a value closer to the lowest pH. These two values should then differ significantly. Intact rat diaphragms were exposed in vitro to varying bicarbonate concentrations (pure metabolic) and CO_2 tensions (pure respiratory), and steady-state cell pH was measured simultaneously either by distribution of the weak acid 5,5-dimethyloxazolidine-2,4-dione-^{14}C (pH DMO) or by distribution of the weak base nicotine-^{14}C (pH nicotine). The latter compound was found suitable to measure cell pH since it was neither metabolized nor bound by rat diaphragms.

At an external pH of 7.40, pH DMO was 7.17 while pH nicotine was 6.69—a pH difference of 0.48 pH units ($P < 0.001$). In either respiratory or metabolic alkalosis both DMO and pH nicotine rose so that differences between them remained essentially constant. Metabolic acidosis induced a decrease in both values though they fell more slowly than did extracellular pH. In contradistinction, in respiratory acidosis, decreasing extracellular pH from 7.40 to 6.80 resulted in 0.35 pH unit drop in pH DMO while pH nicotine remained constant. In every experiment, under all external conditions, pH DMO exceeded pH nicotine.

These results indicate that there is significant pH heterogeneity within diaphragm muscle, but the degree of heterogeneity may vary under different external conditions. The metabolic implications of these findings are discussed. In addition, the data show that true overall cell pH is between 6.69 and 7.17—a full pH higher than would be expected from thermodynamic considerations alone. This implies the presence of active processes to maintain cell pH.

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INTRODUCTION

Change in hydrogen ion activity is a primitive and simple means by which organisms may alter metabolic reaction rates (1–3). This seems rather obvious when one considers that almost all enzymes exhibit pH optima and that most naturally occurring catabolic processes release protons (4, 5). It is not surprising, therefore, that the organism has evolved sophisticated pulmonary and renal compensatory responses to prevent rapid and possibly fatal changes in extracellular acidity (6, 7). Though much information is available concerning how extracellular acidity is maintained constant, much less is known concerning intracellular pH. There is a practical reason for this neglect since the measurement of cell pH is difficult, and the methods presently employed are open to many theoretical objections (8, 9). Nevertheless, in recent years the distribution of a weak acid, 5,5-dimethyloxazolidine-2,4-dione (DMO)[1] has been extensively employed to measure cell pH, and much useful information has been obtained (10–13).

Unfortunately, changes in cell metabolism induced by variations in extracellular pH or the CO_2-bicarbonate buffer system do not always seem to correlate with either extracellular or intracellular pH changes. Longmore, Niethe, and McDaniel (14), using a perfused rat liver preparation, demonstrated that even when the cell PH, measured using DMO, is kept constant, alterations in the extracellular bicarbonate concentration can markedly affect glycogen synthesis. Adler (15) demonstrated a similar phenomenon in rat diaphragm

[1] *Abbreviation used in this paper:* DMO, 5,5-dimethyloxazolidine-2,4-dione.

using citrate accumulation as a measure of cell metabolism. It is possible that the DMO technique is unable to distinguish between changes in the pH of the portion of the cell involved in the metabolic reaction studied and changes in the over-all cell pH. This would require that a significant degree of pH heterogeneity be present within the cell.

To investigate whether such heterogeneity exists and if it varies under different extracellular conditions, the pH of intact rat diaphragm was measured simultaneously using the weak acid, DMO, and a weak base, nicotine. Differences between the pH calculated from these two methods should, theoretically, give a measure of cell pH heterogeneity (8, 9, 11). The experiments reported in this paper not only indicate that the pH within cells is heterogeneous but also show that this degree of heterogeneity differs under varying external pH conditions.

METHODS

Intact diaphragms from 75–90 g Sprague-Dawley rats were incubated simultaneously in two incubation chambers in a modified Krebs-Ringer bicarbonate solution at 37°C as previously described (16). Glucose, 100 mg/100 ml, was employed as a substrate, and the potassium concentration was maintained between 5.3 and 5.9 mEq/liter. Both chambers were attached to a single CO_2-O_2 gas tank. In this manner the P_{CO_2}, pH, bicarbonate concentration, osmolality, and electrolyte concentration of the media of the two chambers were identical. In every experiment 12 diaphragms were incubated in each chamber for 4 hr at constant external conditions to assure the attainment of steady-state condition. 2 mM nicotine, nonradioactive (cold nicotine), was present in the bathing media of each chamber throughout the experiment unless otherwise indicated. At the beginning of the final hour of incubation, inulin, 800 mg/100 ml, was also added to each of the bathing media. In addition, for the last hour, 25 μCi of DMO-^{14}C [2] was added to the medium in one chamber while 25 μCi of nicotine-^{14}C [3] was added to the other for calculation of intracellular pH.

Each analysis was carried out on a pool of 2 diaphragms, and each experimental value, therefore, unless otherwise indicated, represents the mean of 6 analyses on the 12 diaphragms. The radioactive substances were extracted from the tissue by grinding in distilled water in a glass homogenizer as previously described (17). Inulin distribution was used as a measure of extracellular space. Intracellular pH, calculated from either DMO-^{14}C or nicotine-^{14}C distribution, was derived from the standard equations previously employed (11, 18).

Metabolic experiments. In this series of experiments both chambers were attached to a single 5.5% CO_2 tank, balance oxygen, maintaining the bath CO_2 tension between 30 and 38 mm of mercury. Bicarbonate concentration was varied between 3.0 and 51.7 mEq/liter to achieve the desired degree of external acidosis or alkalosis. Iso-osmolality was maintained by reciprocal changes in bicarbonate and chloride. The pH and CO_2 content of the medium in each box remained constant throughout the incubation with a variance of no more than 0.03 units and 4 mm of mercury, respectively.

Respiratory experiments. In this group of experiments different CO_2 tensions were obtained by altering the percentage of CO_2 in the gas mixture between 1.5 and 32.0%. Both chambers were again attached to a single tank. Bicarbonate concentration of the Ringer's solution was held constant between 19.8 and 22.6 mEq/liter. Variations in pH and P_{CO_2} throughout the experiment were, as in the previous group of experiments, no greater than 0.03 units and 4 mm of mercury, respectively.

Determination of the pK of nicotine. To determine the pK of nicotine a 100 mM nicotine hydrochloride and 150 mM sodium chloride solution was titrated at 37°C with sodium hydroxide. The value obtained was 7.95; a result similar to that obtained by Effros and Chinard (19). It was then assumed, as with DMO, that the pK of nicotine in the medium and within the cell was identical.

Analytic methods. Bath pH was measured with a Radiometer pH meter at 37°C, and the CO_2 content of the medium was determined manometrically. CO_2 tension was calculated from the pH and CO_2 content. Sodium and potassium were measured on a Baird KY-2 flame photometer. Cold nicotine was measured using the method of Hucker, Gillette, and Brodie (20). The ^{14}C compounds were counted in a Packard liquid scintillation counter. In each experiment quenching was determined using an external standard. No significant differences were found between medium and tissue counts so no quench correction had to be employed in any experiment.

RESULTS

Completeness of extraction of nicotine. To determine the completeness of the extraction of nicotine-^{14}C into distilled water during homogenization, a hyamine digest was performed on the precipitated protein phase. Less than 3% total radioactivity was found in the hyamine. This small amount is easily accounted for as the trapped water in the precipitate. The relative amounts of nicotine-^{14}C and DMO-^{14}C remaining in digests were identical demonstrating that extraction of either compound is essentially complete when the tissue is ground in water.

Evidence for nonmetabolism of nicotine by rat diaphragms. Despite some evidence from the literature that the diaphragm does not metabolize nicotine (21), experiments were performed in our system to confirm this point. Nicotine is soluble in heptane, while its major metabolites, particularly cotinine, are not (22). The latter are soluble, however, in chloroform. Tissue was incubated for 4 hr in a medium containing 2 mM cold nicotine and nicotine-^{14}C. External pH in the two experiments was 7.37 and 7.40. After incubation the tissue was removed and ground in distilled water. The homogenate was then shaken with a heptane-chloroform mixture. Radioactivity obtained from tissue homogenates in the heptane phase was 319 $\pm$452 dpm/min (mean $\pm$SD with an $n = 6$), while they were 63 $\pm$11

[2] New England Nuclear Corp., Boston, Mass.
[3] Mallinkrodt Chemical Works, St. Louis, Mo.

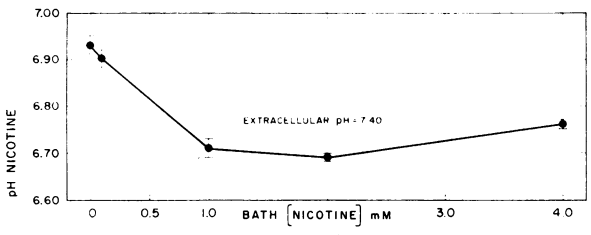

FIGURE 1 Effect of bath (nicotine) on calculated pH nicotine at an extracellular pH of 7.40. Each point represents the mean ±SEM of six determinations.

dpm/min in the chloroform phase. A similar result was found for the incubating medium in these experiments with 8006 ±56 dpm/min in heptane and only 124 ±19 dpm/min in chloroform. Thus, less than 1% of the label is present in the chloroform phase indicating that no significant metabolism of nicotine-^{14}C had occurred during the 4 hr of incubation.

Evidence for the ^{14}C label remaining attached to nicotine. Though the partition experiments indicate that no metabolism of the nicotine takes place, it was still possible that the ^{14}C label, which is present on an *N*-terminal methyl group, might come off the parent compound by transmethylation and attach to another substance also soluble in heptane. If this happened, cell pH calculated from nicotine-^{14}C distribution would be significantly different from that derived from the actual measurement of nicotine itself. To determine whether such a difference occurs, diaphragms were incubated for 4 hr at an external pH of 7.40, and intracellular pH was calculated from either nicotine-^{14}C or cold nicotine distribution. Cold nicotine (2 mM) was added to each of the bathing media of each chamber so that the action of the compound would be identical. In three separate experiments (18 analyses) intracellular pH calculated using cold nicotine distribution was 6.63 ±0.05 SEM. The recovery of cold nicotine from bath and tissue was equal and ranged from 92 to 104% in the three experiments. Intracellular pH calculated from nicotine-^{14}C distribution was 6.69 ±0.04 SEM. The difference between the two results is not statistically significant at the 0.05 level. These experiments suggest that the ^{14}C label is not removed from the nicotine during the four hr of incubation and also support the partition experiments in indicating that no metabolism of the nicotine occurs.

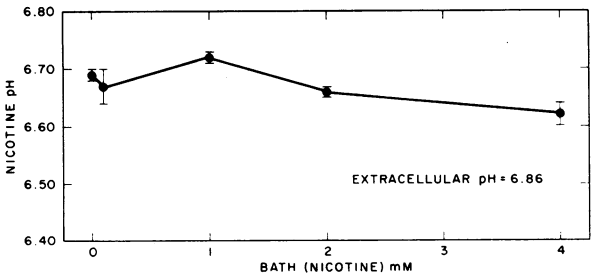

FIGURE 2 Effect of bath (nicotine) on calculated pH nicotine at an extracellular pH of 6.86. Each point represents the mean ±SEM of six determinations.

Evidence against protein binding of nicotine by rat diaphragm muscle. To be suitable for the measurement of cell pH, a compound must not be bound to cellular proteins. Though there is some disagreement (23, 24), most investigators feel that it has been well demonstrated that DMO is not so bound (17, 25). The following two types of experiments were carried out to demonstrate whether nicotine is bound to cell proteins of rat diaphragms.

Ultrafiltration experiments. 12 rat diaphragms were incubated for 4 hr with nicotine-^{14}C. Tissue was then removed and homogenized in distilled water. The homogenate was placed in a centriflo ultrafiltration apparatus,[4] and the percentage of radioactivity passing through the membrane was measured. Any nicotine-^{14}C bound to protein would not pass through since this filter retains molecules of molecular weight greater than 50,000. The filtrate was counted after ultrafiltration and was compared with the supernate before filtration. The counts in the two groups, respectively, were 6024 ±113 and 6027 ±142 per 200 mg of tissue indicating no detectable protein binding of nicotine-^{14}C.

Carrier experiments. As another test of possible significant binding of nicotine by rat diaphragm muscle, incubations were carried out at an external pH of 7.40 in the presence of varying concentrations of cold nicotine. Should nicotine be bound within the tissue then as the cold nicotine concentration is progressively increased, nicotine-^{14}C will be displaced from its binding sites and calculated cell pH will increase. The results are shown in Fig. 1. As this figure clearly shows, when nicotine concentration is raised from 0 to 1 mM, there is a fall, not a rise in calculated cell pH. Further increases in cold nicotine concentration to 4 mM result in no further change in calculated cell pH. It seems, therefore, that at physiologic pH no significant binding of nicotine takes place.

This lack of binding is also demonstrable at an abnormal extracellular pH value. Fig. 2 shows the effect of varying cold nicotine concentration on calculated intracellular pH at a medium pH of 6.86. The latter was achieved by raising the CO_2 tension of the medium to 120 mm of mercury while the bicarbonate concentration was held at 21 mEq/liter. As can be seen there is no significant change in intracellular pH as the cold nicotine concentration is progressively raised from

[4] Amicon Corp., Lexington, Mass.

0 to 4 mM. Thus, no significant binding of nicotine takes place at an extracellular pH of either 7.40 or 6.86.

Effect of nicotine on calculated cell pH using DMO as its measure. Weiss (26) has shown that in amphibian muscle nicotine may alter DMO distribution and hence calculated cell pH using the DMO method. Furthermore, in the carrier experiments performed at an external pH of 7.40, cell pH calculated from nicotine distribution (pH nicotine) fell as the cold nicotine concentration was raised from 0 to 1 mM. This could be due to a decrease in the actual over-all cell pH. Diaphragms were, therefore, incubated for 4 hr in the presence of varying concentrations of cold nicotine while DMO-^{14}C was employed to calculate cell pH. The results are shown in Fig. 3. Progressive increases in nicotine concentration had no effect on cell pH measured using DMO distribution (pH DMO). Since there is no statistically significant difference between any of the points on the curve, it appears that nicotine, at least in the concentration used in these experiments, does not affect the pH of rat diaphragm muscle.

Effect of nicotine on membrane permeability. In a series of three experiments, diaphragms were incubated for 4 hr in the presence and absence of 2 mM nicotine. Water content, inulin space, sodium, and and potassium were compared in the two groups. No statistically significant differences were found between them. In the case of potassium, a primarily intracellular cation, intracellular potassium concentration was 153 ±5 mEq/liter in the nicotine group compared with 151 ±6 mEq/liter in the nonnicotine diaphragms. External potassium concentration for both groups averaged 5.6 mEq/liter. Thus, in the presence of nicotine, diaphragm muscle was able to maintain a 28:1 intracellular: extracellular K^+ gradient demonstrating the maintenance of membrane selectivity for the duration of the experiment.

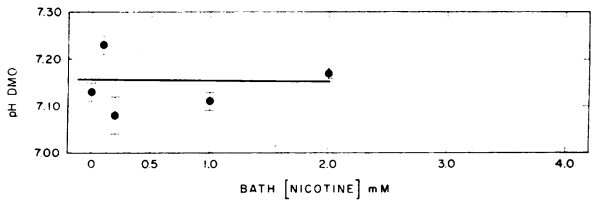

FIGURE 3 Effect of bath (nicotine) on calculated pH DMO at an extracellular pH of 7.40. Each point represents the mean ±SEM of six determinations. The horizontal line was calculated using the method of least squares.

Effect of external bicarbonate concentration on intracellular acidity. Table I shows the effect of varying the external bicarbonate concentration at a constant CO_2 tension on pH DMO and pH nicotine. The external concentrations of bicarbonate, hydrogen ion, and pH in the medium are given in the second, third, and fourth columns while the last two columns list the muscle pH measured by each of the two methods. The

TABLE I

*Effect of Progressive Changes of Extracellular Bicarbonate Concentration on Muscle pH**

No. of analyses	Medium‡			Muscle			
	(HCO_3^-)	(H^+)	pH	(H^+) DMO	(H^+) nicotine	pH DMO§	pH nicotine§
	mEq/liter	*nmoles/liter*		*nmoles/liter*			
6	3.2	244	6.61	178	398	6.75 ±0.02	6.40 ±0.04
6	3.6	251	6.60	186	339	6.73 ±0.02	6.47 ±0.05
6	5.8	135	6.87	126	275	6.90 ±0.05	6.56 ±0.04
6	5.8	132	6.88	112	269	6.95 ±0.06	6.57 ±0.06
6	8.3	92.2	7.04	89.1	302	7.05 ±0.09	6.52 ±0.04
6	8.8	91.2	7.04	74.1	295	7.13 ±0.02	6.53 ±0.05
6	10.2	76.7	7.12	87.1	245	7.06 ±0.04	6.61 ±0.04
6	12.8	57.5	7.24	79.4	204	7.10 ±0.03	6.69 ±0.02
35‖	20.5	40.7	7.39	67.6	204	7.17 ±0.06	6.69 ±0.07
6	32.8	26.0	7.59	60.3	132	7.22 ±0.08	6.88 ±0.09
6	32.8	21.9	7.66	51.3	138	7.29 ±0.06	6.86 ±0.04
6	50.2	16.8	7.78	43.7	138	7.36 ±0.04	6.86 ±0.06
5	51.2	16.4	7.78	38.9	135	7.41 ±0.03	6.87 ±0.06

* All experiments carried out at a constant P_{CO_2} of 30–38 mm Hg.

‡ Values for the medium represent the mean of the value for the two chambers. In no instance did pH in the two boxes differ by more than 0.03 U.

§ Represents the mean ±SD.

‖ Number of analyses for pH nicotine was 41.

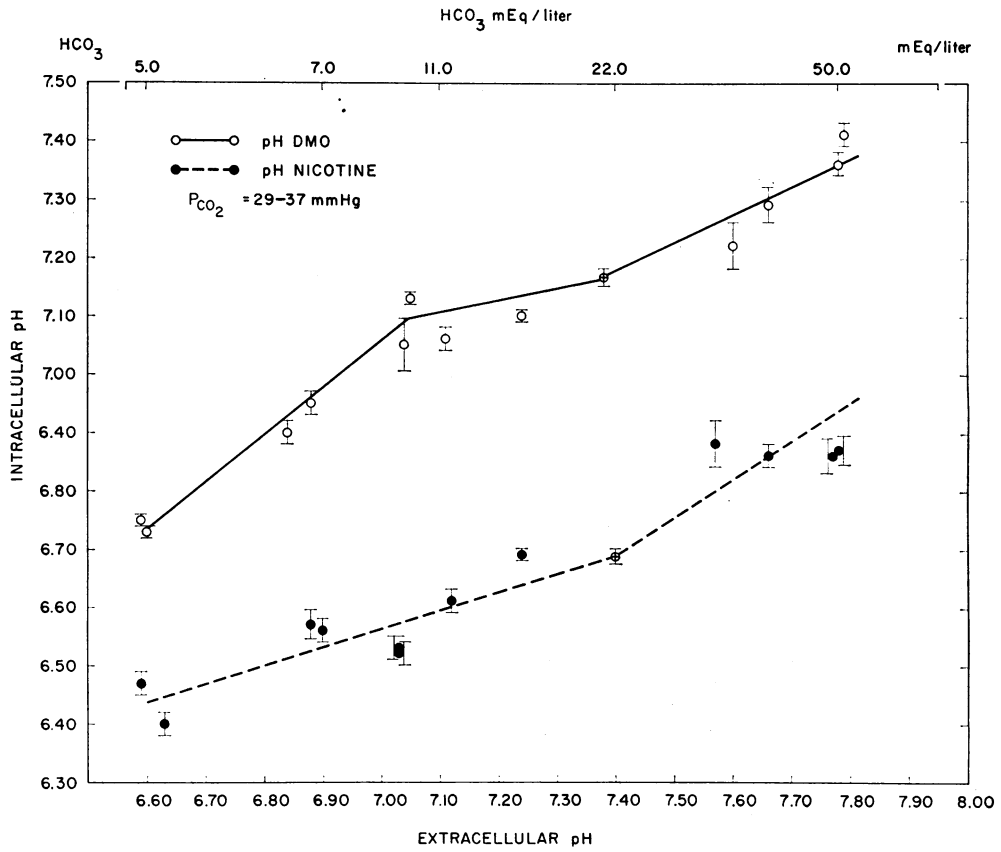

FIGURE 4 A comparison between pH DMO and pH nicotine in metabolic acidosis and alkalosis. Except for cross-hatched normal value each point represents the mean ±SEM of six analyses. Lines were drawn from the calculated equations derived using the least squares method.

results of these experiments are shown graphically in Fig. 4 and demonstrate the relationship between external and internal acidity over an extracellular pH range of 6.60–7.78 for each of the methods. Except for the normal value at an external pH of 7.40, each point represents the mean ±SE of the mean of six determinations. The "normal point", represented by a cross-hatched circle, is the mean ±SE of the mean of 35 determinations.

It is apparent that under the physiologic conditions of an extracellular pH of 7.40, a CO_2 tension of 33–36 mm of mercury and a bicarbonate concentration of 21 mEq/liter, intracellular pH determined by DMO is 7.17 while it is 6.69 using nicotine—a difference of 0.48 pH units. Raising extracellular pH, by raising the bicarbonate concentration, causes an almost equal and progressive increase in both pH DMO and pH nicotine. Cell pH appears to be more resistant to change during extracellular metabolic acidosis. Large decreases in extracellular pH are accompanied by much smaller variations in either pH DMO or pH nicotine and confirms early reports that the cell resists changes in its pH when faced with extracellular metabolic acidosis (10, 11, 27). It should also be noted from Fig. 4, that pH DMO is always higher than pH nicotine. This occurs at every experimental point in both acidosis and alkalosis.

Effect of CO_2 tension on intracellular acidity. Table II shows the data obtained when extracellular pH is varied by changing the CO_2 tension of the medium at a constant external bicarbonate concentration of 20–23 mEq/liter. Fig. 5 shows the relationship between extracellular and intracellular acidity in these experiments plotted in the same manner as Fig. 4.

From both Table II and Fig. 5 it is apparent that increasing extracellular alkalosis is associated with increasing cell pH measured using either the DMO or nicotine method. However, when extracellular pH is lowered from 7.40 to 6.80 by progressively raising the CO_2 tension from 36 to 148 mm of mercury, cell pH, as determined by the two methods, varies in quite different ways. Over this range of CO_2 tension, pH DMO

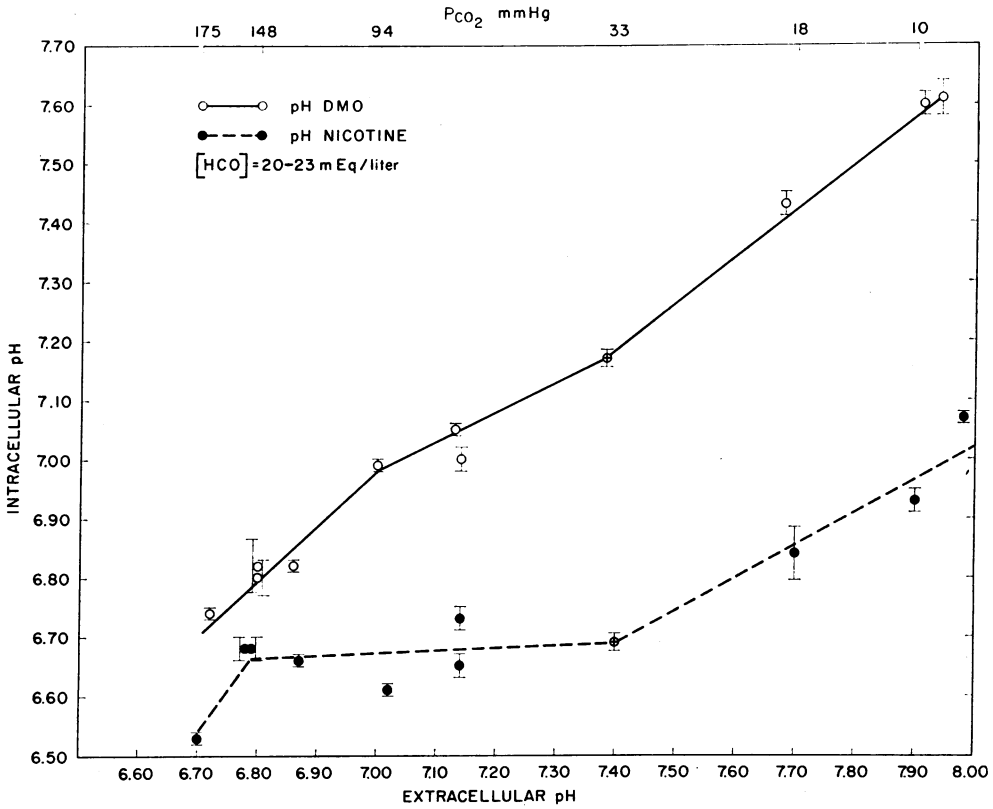

FIGURE 5 A comparison between pH DMO and pH nicotine in respiratory acidosis and alkalosis. Except for the cross-hatched normal value each point represents the mean ±SEM of six analyses. Lines were drawn from the calculated equations derived using the least squares method.

TABLE II

*Effects of Progressive Changes in CO_2 Tension on Muscle pH**

No. of analyses	Medium‡			Muscle			
	P_{CO_2}	(H^+)	pH	(H^+) DMO	(H^+) nicotine	pH DMO§	pH nicotine§
6	177	195	6.71	182	295	6.74 ±0.03	6.53 ±0.03
6	148	160	6.80	158	209	6.80 ±0.08	6.68 ±0.04
6	146	163	6.79	151	209	6.82 ±0.10	6.68 ±0.05
6	125	136	6.86	151	219	6.82 ±0.03	6.66 ±0.03
6	94	98.0	7.01	102	245	6.99 ±0.02	6.61 ±0.02
6	62	73.2	7.14	89.1	224	7.05 ±0.02	6.65 ±0.04
6	61	72.4	7.14	100	186	7.00 ±0.04	6.73 ±0.04
35‖	36	40.7	7.39	67.6	204	7.17 ±0.06	6.69 ±0.07
6	18	20.4	7.69	37.2	144	7.43 ±0.04	6.84 ±0.10
6	10	12.0	7.92	24.5	117	7.61 ±0.07	6.93 ±0.03
6	10	11.6	7.94	25.1	85	7.60 ±0.05	7.07 ±0.02

* All experiments carried out at a constant extracellular (HCO_3^-) of 19.8–22.6 mEq/liter.

‡ Values for the medium represent the mean of the value for the two chambers. In no instance did pH in the two boxes differ by more than 0.04 U.

§ Represents the mean ±SD.

‖ Number of analyses for pH nicotine was 41.

shows a decrease of 0.35 pH units while no significant change in pH nicotine occurs. Thus, pH inside the cell as measured by nicotine remains constant over an extracellular pH range of 0.60 pH units. At an external pH of 6.80, the difference between pH DMO and pH nicotine is now only 0.13 pH units contrasted with the 0.48 pH unit difference found at an external pH of 7.40. Further raising of the CO_2 tension above 148 mm of mercury and lowering of pH beyond an external pH of 6.80 results in a decrease in cell pH measured by either method. As was the case in the metabolic experiments, it should be noted that pH nicotine is lower than pH DMO in all experimental situations.

DISCUSSION

Most work has shown that muscle cell pH lies between 6.80 and 6.90 (8, 10, 11, 17). Recently, however, a group of investigators employing a pH-sensitive microelectrode produced data showing that the pH of resting rat skeletal muscle is approximately 6.00 (23). They stated that the large discrepancy between their results and earlier determinations could be due to two factors. In those studies which employed microelectrodes they felt that technical artifacts, such as junction potentials or injury currents, might give a falsely high pH reading. In experiments which employed the distribution of weak acids such as DMO or carbonic acid (11, 28), criticism was leveled on the basis that there could be electrostatic binding of charged anionic portions of the acids to cellular proteins leading to a falsely elevated calculated intracellular pH value (24). Since ionic distribution in intact diaphragms remains normal (29) and transmembrane potential has been measured in rat skeletal muscle (23), use of the standard Nernst equation allows one to determine what the pH inside cells would be if hydrogen ions were distributed according to thermodynamic equilibrium. These measurements predict that cellular pH would be approximately 5.90 at an extracellular pH of 7.40. A pH of 6.90, on the other hand, would imply active transport of hydrogen ions out of the cell or hydroxyl and/or bicarbonate ions into cells (11).

The present study was designed to avoid the criticism mentioned above. The anionic portions of DMO, if bound, would, as mentioned previously, yield a falsely high calculated cellular pH value. Use of the weak base nicotine, however, obviates this difficulty since if the cationic portions of this substance are bound, then a falsely low value for cell pH would be calculated. The present experiments show that the pH obtained from distribution of the weak acid, DMO, was 7.17 while the pH obtained using the distribution of the weak base, nicotine, was 6.69 at an external pH of 7.40. Therefore, true over-all cell pH must lie somewhere between these two readings or in the region of 6.90 and shows that the pH of diaphragm muscle lies outside of thermodynamic equilibrium indicating active transport of hydrogen ion and/or hydroxyl and bicarbonate ions across muscle cell membranes.

The difference found between cell pH calculated from the DMO and nicotine distributions may be explained in one of three ways. First, there could be binding of the substances by the diaphragm. Early work with DMO indicates that this is unlikely (10), though electrostatic binding cannot be ruled out (23, 24). The data presented in this paper demonstrate that nicotine also is not bound to cellular constitutents since binding could not be demonstrated either by ultrafiltration or carrier experiments. A second explanation for the differences found between the two methods may lie in the value of the dissociation constants of the weak acid or base. An assumption is made that the pK of nicotine and DMO within cells is identical with that measured in extracellular fluid (10, 11, 18). Though this assumption is not open to experimental verification, it is unlikely that errors in this assumption could explain the experimental results. The difference in pK would have to be at least 0.5 units to account for the marked differences between the two determinations found at an external pH of 7.40. Furthermore, during the respiratory acidosis experiments pH DMO and pH nicotine approached one another at very high P_{CO_2} values. If the pK of one of these substances was incorrect, then at low pH ranges one would again see marked differences between the two methods. Interestingly, Effros and Chinard, measuring the extravascular space of the lung, used a similar pK for nicotine and found the pH value of the space to be identical with the pH nicotine of muscle determined in this study (19). For these reasons, therefore, it seems unlikely that errors in the dissociation constant of the acid or base could account for the differences between the two methods. A third explanation which may account for the experimental results is that there is significant pH heterogeneity within the cell. This explanation seems most likely to account for the data for the reasons given below.

Theoretically, the pH determined from the distribution of a weak acid yields a value which is close to the mean hydroxyl ion concentration of a multicompartment system while the distribution of a weak base should give a value closer to the mean hydrogen ion concentration (8, 11). However, given the modern operational definition of pH (9), so precise a statement cannot be made. What can be said is that if there is significant heterogeneity within the system being studied, then the pH derived from the distribution of a weak acid will yield a value closer to the highest pH within that system while the pH derived from distribution of a weak base

will yield a value closer to the lowest pH within that system. This follows directly from the mathematical analyses of Caldwell (8) and Adler, Roy, and Relman (11) and is in exact accord with the results of this study. At an external pH of 7.40, pH DMO was 0.48 pH units higher than pH nicotine; a statistically significant difference in which the pH derived from the distribution of the acid is higher. Furthermore, at every experimental point pH DMO exceeded pH nicotine—a result to be expected if significant heterogeneity exists over the entire external pH range studied.

Nonhomogeneity of pH within cells is compatible with other reported data. Addanki, Cahill, and Sotos, employing the weak acid DMO, incubated mitochondria and found the calculated pH within them to be higher than that of the surrounding medium (30). This work is substantiated by other investigators who used bromothymol blue as a pH indicator and determined that mitochondrial pH indeed seems to be alkaline when compared with that of the surrounding medium (31). In addition Struyvenberg, Morrison, and Relman (32) calculated the pH of isolated renal tubules and using DMO as a measure of pH found it to be significantly higher than muscle cell pH measured in the same laboratory (11). This result could be due to the abundant mitochondria found within renal tubular cells which in turn could have contributed to a higher over-all calculated cell pH. The presence of many protein surfaces within the cell, such as ribosomes, will also induce Donnan effects and possible pH gradients.

Cellular metabolism, in many tissues, has been shown not to be a simple function of extracellular pH (3, 33, 34), but seems rather to be dependent on the particular CO_2/bicarbonate combination employed to achieve any one extracellular pH value. It is possible that the poor correlation between extracellular pH and metabolism is due to the absolute level of total CO_2, i.e., the algebraic sum of the bicarbonate concentration and alpha P_{CO_2} which could directly affect individual biochemical reactions. There is evidence that this does in fact occur since CO_2 has been shown to be directly involved in constituent enzyme systems (35). An additional possibility is that changes in extracellular pH are unaccompanied by similar variations in cellular acidity. Adler, Roy, and Relman have indeed shown this to be true (11, 36). Thus, a study of extracellular pH conditions would not necessarily give a true measure of what was happening to pH within the cell. There is other evidence, however, which indicates that this second explanation alone is insufficient. In a study by Longmore et al. (14) changes in glycogen synthesis in a perfused rat liver preparation could not be correlated with cell pH measured using the DMO method. Furthermore, Adler (15) has demonstrated a similar phenomenon for citrate accumulation in intact rat diaphragms. This lack of correlation between cell pH and metabolism might be a reflection of the methods employed to measure cell pH. Thus, in the present study, when CO_2 tensions were progressively raised from 40 to 148 mm of mercury and extracellular pH was decreased from 7.40 to 6.80, pH DMO decreased while pH nicotine remained essentially constant. The true state of cellular acidity was not, therefore, apparent from either of these methods alone. This could explain the aforementioned apparent lack of correlation between pH DMO and either glycogen synthesis or citrate metabolism in liver and rat diaphragm, respectively. Progressive decreases in the difference between pH DMO and pH nicotine imply a progressive lessening of pH heterogeneity within the cells. Since pH DMO yields a value closer to the higher pH within a heterogeneous system, this may be a reflection of increasing acidity within an organelle such as mitochondria leading to possible profound metabolic effects despite little or no change in pH in other portions of the cell or even changes occurring in an opposite direction.

The presence of significant pH heterogeneity has many implications for the study of acid-base conditions. Attempts to define changes in potassium or other ionic movements on the basis of intracellular to extracellular hydrogen ion ratios (37) may be fruitless since there probably are, in reality, an infinitude of these gradients within any one tissue. Also, experiments in which whole body pH is measured become even more difficult to evaluate since not only is there variation from tissue to tissue within the body (9, 38) but also within individual cells themselves multiple areas of pH exist. The presence of these gradients within the cell may, of themselves, be important metabolically. Mitchell has proposed his chemi-osmotic theory for energy transfer in mitochondria (39) which depends on just such a hydrogen ion gradient being present between mitochondria and cytoplasm. This study would support that notion since pH heterogeneity seems to exist within the cell itself and not only in an vitro mitochondrial system.

The present study did not address itself to the means by which these gradients are maintained. The fact that true over-all cell pH lies well outside thermodynamic equilibrium implies that metabolic energy is being expended to maintain cell pH and would further suggest that changes in metabolism could affect cell pH heterogeneity as well as the converse. Further experiments are necessary to study this point as well as to determine what effect compensation of metabolic and respiratory acid-base disturbances has on cell pH heterogeneity. It is interesting, however, that changes in heterogeneity and resistance to cell pH change occur in acidosis to a much greater extent than in alkalosis. This is con-

sistent with previous speculations (11, 40) that the muscle cell is more frequently faced with the threat of acidosis and has, therefore, evolved appropriate responses to deal with this threat. Clarification of this and the other speculations raised by this paper await methods for measuring changes within individual portions of the cell and further studies on the relationship between cell pH, cell metabolism, and external acidity.

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