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Research Article

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The Role of pH, P_{CO_2}, and Bicarbonate in Regulating Rat Diaphragm Citrate Content

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ABSTRACT Intact rat diaphragms were exposed in vitro to varying CO_2 tensions and bicarbonate concentrations, and the steady-state citrate content of diaphragm muscle was measured to investigate the relationship between metabolism and extracellular pH, P_{CO_2}, and (HCO_3^-). In addition, rat hemidiaphragms were incubated with 1,5-citrate-^{14}C under different acid-base conditions, and $^{14}CO_2$ production was determined as a measure of citrate oxidation.

Acidification of the bathing medium achieved by raising CO_2 tension or lowering (HCO_3^-) was associated with a decrease in muscle citrate content. On the other hand, alkalinization of the medium induced by lowering CO_2 tension or raising (HCO_3^-) caused tissue citrate content to rise. At a physiologic extracellular pH value of approximately 7.40, citrate content was decreased or normal depending on the CO_2/HCO_3^- combination employed to attain the pH. Under low bicarbonate and low P_{CO_2} conditions, citrate content was reduced. A similar result was found at external pH values of 7.15, implying that at these two extracellular pH levels (HCO_3^-) primarily determines citrate content. When changes in citrate content were compared with intracellular pH data reported earlier using the same intact diaphragm preparation, no simple relation between citrate content and intracellular pH was found. The effect of acidity on citrate content seems related to a change in citrate oxidation since the latter increased progressively with increasing degrees of medium acidity.

These results show that cellular metabolism is not a simple function of extracellular pH but is dependent on the particular combination of P_{CO_2} and bicarbonate employed to achieve the pH value. These studies also suggest that accumulation or disposal of organic acids, such as citric acid, helps to regulate cellular acidity thereby contributing to the cells' defense against external acid-base disorders.

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INTRODUCTION

Recently, as newer methods of measuring intracellular pH have become available (1, 2), investigators have been able to determine the effect of extracellular acidity on cell pH (3–5). Three interesting facts have emerged from these studies. First, extracellular bicarbonate concentration was shown to affect cell pH (3–5), a result at variance with earlier reports which appeared to show that the cell was indifferent to extracellular bicarbonate (6–9). Second, cell pH was found not to have a simple relationship to extracellular pH but appeared to be a complex function dependent on the particular combination of P_{CO_2} and bicarbonate used to achieve a particular extracellular pH value (4). Third, the cell was able to maintain its pH constant over a wide range of extracellular acidity (3). Adler, Roy, and Relman speculated that this remarkable ability of the cell to maintain its pH constant could be due either to changes in the flux rates of hydrogen, hydroxyl, or bicarbonate ions or to changes in the internal metabolism of acids or bases (3).

This study was undertaken to determine if the content of a key intermediary metabolite, citrate, varied in intact diaphragm when extracellular pH was systematically altered, and whether any changes found were due to extracellular pH alone or were dependent on the CO_2 tension or bicarbonate concentration of the extracellular fluid. Citrate was chosen since its metabolism has been shown to vary directly with pH in renal cortical slices (10). In addition, the citrate content of rat gluteal muscle changes markedly when an extracellular acidosis is induced by acute intraperitoneal injections of rubidium chloride (11). Changes in citrate content were also compared with the intracellular pH data reported earlier, using the same intact diaphragm preparation (3, 4).

The data reported in this study show that extracellular bicarbonate does indeed influence the citrate content of diaphragm muscle and show further that bicarbonate, extracellular pH, P_{CO_2}, or intracellular pH does not

alone determine muscle citrate content. The changes in citrate content may be due to an alteration in the oxidation of citrate to CO_2, a response to pH change which appears similar to the one operating in the renal cortex.

METHODS

Intact diaphragms from 75–90-g male Sprague-Dawley rats were incubated in a modified Krebs-Ringer bicarbonate solution at 37°C as previously described (3). Glucose, 100 mg/100 ml, was employed as the substrate, and 62.5 mg of chloromycetin was added to each liter of solution to prevent bacterial contamination. A control system was used consisting of two separate incubating boxes employed simultaneously, allowing one box to serve as a control while the medium in the other was altered to fit the experimental conditions (12). Unless otherwise specified, control conditions were always pH 7.35–7.42 P_{CO_2} 34–42 mm of mercury, bicarbonate concentration 19.9–22.9 mEq/liter. In every experiment 16 diaphragms in each box were equilibrated for 4 hr at constant external conditions. At the end of the final hour of incubation, the intact diaphragms were taken sequentially from the two boxes, the muscles were rapidly removed from their rib attachments, frozen immediately in liquid nitrogen, and then weighed in the frozen state. Eight diaphragms were combined, placed in glass homogenizing tubes containing iced 6% perchloric acid, and ground while immersed in an ice bath. The homogenate was centrifuged at 2500 rpm for 10 min, and the supernatant was titrated with KOH to pH 7.0 and saved for citrate analysis. Bath samples were checked at appropriate intervals for pH and total CO_2 content. Each experimental value represents the average of two groups of eight diaphragms each.

Metabolic experiments. In one group of experiments both boxes were attached to a single 5.5% CO_2 tank (balance O_2) which maintained the bath CO_2 tension constant between 34 and 42 mm mercury. Bicarbonate concentration in the control box was 19.9–22.5 mEq/liter, while the bicarbonate concentration in the experimental box was varied between 3.5 and 67 mEq/liter to achieve the desired degree of external acidosis or alkalosis. Iso-osmolarity was maintained by reciprocal changes in bicarbonate and chloride. The pH and CO_2 content of the medium in each box remained constant throughout incubation with a variance of no more than 0.04 U and 3 mm Hg, respectively.

Respiratory experiments. In another group of experiments the control box was identical with that of the previous experiments, but different CO_2 tensions were produced in the experimental box by altering the percentage of CO_2 in the gas mixture between 1.6% and 25%. Each box, therefore, was attached to a different gas tank, but both contained the same Ringer's solution, with a bicarbonate concentration of 19.9–22.9 mEq/liter. Variations in pH and P_{CO_2} throughout the experiment were no greater than 0.03 U or 3 mm of mercury, respectively, in either box.

Isohydric experiments. In this series of experiments pH was equal in the two boxes, but the pH was achieved in different ways by altering both bicarbonate and CO_2 tension. Three groups of experiments were performed one at a physiologic bath pH of 7.33–7.42, a second group at low external pH values, and a third at elevated ones. In no instance did the pH of the two boxes differ from each other by more than 0.05 U.

Citrate oxidation experiments. Specially constructed 25-ml Erlenmeyer flasks with a center well containing a small glass tube were gassed for 1 min with a CO_2-oxygen gas mixture selected for the particular experiment. Before passing through the flasks, gas mixtures were saturated with water vapor. The flasks were rapidly sealed with a sleeve-type rubber stopper, 4 ml of previously CO_2-O_2 saturated Krebs-Ringer bicarbonate solution containing 1 mM of malate, 1 mM of citrate, and 0.5 μCi of 1,5-citrate-^{14}C were added to each flask, and the flasks were gassed again for 20 sec and immediately restoppered. Four weighed hemidiaphragms, obtained from 75–90-g Sprague-Dawley rats, were quickly added to the prepared flask which was then resealed and placed in a metabolic shaker where the temperature was kept at 37°C. The experiment was stopped after 90 min incubation by the addition of 1.0 ml of 1.5 N hydrochloric acid delivered into the flask through a 3 in. 22 gauge needle penetrating the sleeve-type stopper. 30 sec later this same method was employed to add 0.5 ml of Hyamine to the tube in the center well. Shaking of the stoppered flask was continued further for 2 hr to facilitate absorption of the CO_2. The tube containing the hyamine and absorbed CO_2 was removed and added directly to a counting vial containing 15 ml of Liquifluor (New England Nuclear Corp., Boston, Mass.) counting medium, and the $^{14}CO_2$ was counted in a Packard liquid scintillation counter. Blanks prepared in exactly the same manner, except for omission of tissue, were counted in each experiment.

To ascertain the constancy of the pH of the medium, similar flasks were prepared in each experiment except for omission of radioactive citrate. Four hemidiaphragms were again added to each flask, and incubation was carried out as before at 37°C. Medium from the flasks was removed at 90 min, and the pH and total CO_2 content were measured and compared to the pH and total CO_2 content of flasks containing no tissue.

The analytic methods used are as follows. Bath pH was measured with a radiometer pH meter at 37°C, and CO_2 content of the medium was determined manometrically. CO_2 tension was calculated from the pH and CO_2 content. Potassium was measured on a Baird KY 2 flame photometer (Baird-Atomic, Inc., Cambridge, Mass.). Citrate was determined enzymatically by employing citrate lyase and reading the color change of NADH at 366 mμ on a Zeiss PMQ spectrophotometer (13). The results were corrected for quenching by use of an external standard.

RESULTS

Time experiments. It has previously been demonstrated that intracellular pH varies with changes in P_{CO_2}, reaching a new steady state in 1 hr, while changes in external bicarbonate concentration induce a new steady-state intracellular pH value after 2 hr (3). To determine whether a similar situation exists for citrate content, intact diaphragms were exposed either to an elevated external bicarbonate concentration of 67.2 mEq/liter or to an elevated P_{CO_2} of 131 mm Hg, and after varying periods of time muscle citrate content was measured. The results are shown in Fig. 1 which plots the ratio of citrate content in the experimental diaphragms to the content in simultaneously incubated controls against the duration of incubation. Each point is the mean of four determinations, except for the 6 hr respiratory experiment which represents the mean of only two experiments. The lower half of the figure re-

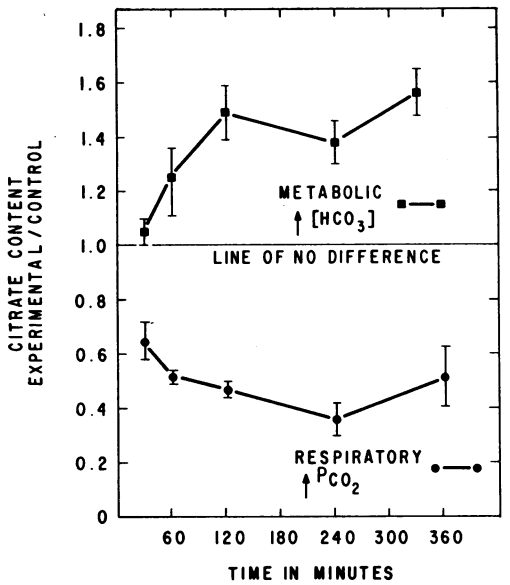

FIGURE 1 Demonstration of the approximate steady state in muscle. Changes in citrate content expressed as the ratio of experimental to control citrate are plotted against time of incubation for two separate acid-base conditions. Each point represents the ±SEM of four determinations except for the 6 hr respiratory point which is the average of two determinations.

veals that markedly elevated P_{CO_2} rapidly lowers citrate content. After 1 hr of incubation, no significant further changes in citrate content occurred over the next 5 hr of the experiment. The effect of external bicarbonate is not as rapid, but after 2 hr of incubation citrate content showed no significant change for the next 4 hr. In all subsequent experiments incubation was, therefore, carried out for a 4 hr period to assure attainment of steady-state conditions.

Effect of external bicarbonate concentration on the citrate content of intact diaphragms. Table I shows the effect of external bicarbonate concentration on diaphragm citrate content. Progressive decrease in external bicarbonate concentration from 66.8 mEq/liter to 3.5 mEq/liter was associated with a decrease in muscle citrate content from 151 to 43% of control values. The variation in control citate content demonstrates the necessity of using simultaneously incubated controls, since control values varied from 0.205 to 0.325 μmoles/g when diaphragms were incubated under identical conditions, a variation of 50%.

In each of the seven experiments, metabolic acidosis was accompanied by a decrease in citrate content, while at elevated bicarbonate values muscle citrate rose in each experiment. The citrate content of diaphragms incubated in alkaline media differed significantly from those incubated in acid media, with a P value of less than 0.001. This group of experiments could not, therefore, differentiate between the effect of extracellular bicarbonate or hydrogen ion concentrations on muscle citrate.

Effect of changes in P_{CO_2} on the citrate content of intact diaphragms. Diaphragms were incubated in a bathing medium in which bicarbonate concentration was held constant, while the CO_2 tension of the medium was varied by altering the concentration of carbon dioxide in the gas mixture. The results are shown in Table II. Raising P_{CO_2} progressively from 11.1 to 124.2 mm Hg was associated with a fall in citrate content from 134 to 57% of controls. As in the previous experiment, acidosis was always accompanied by a decrease in muscle citrate, while alkalosis in each experiment was associated with an increase in citrate content. The citrate content of diaphragms incubated at an elevated pH differed significantly from those incubated in an acid medium, the P value being < 0.001.

To facilitate comparison of the effects of P_{CO_2} and bicarbonate concentration more directly Fig. 2 shows both respiratory and metabolic experiments as a function of extracellular pH. The vertical line in this figure defines a normal extracellular (H^+) of 40 nmoles/liter or a pH of 7.40. All values to the left of the line indicate external alkalosis, while those to the right represent external acidosis. At any value of extracellular pH

TABLE I

Effect of External Bicarbonate Concentration on Diaphragm Citrate Content‡*

Experimental medium			Muscle		
(HCO^-_3)	(H^+)	pH	Control citrate§	Exp. citrate	Citrate exp.‖ / Citrate control
mEq/liter	*nmoles/liter*		*μmoles/g*	*μmoles/g*	
66.8	14.5	7.84	0.234	0.354	1.51
66.2	14.8	7.83	0.256	0.373	1.46
54.5	17.4	7.76	0.290	0.379	1.31
54.4	17.4	7.76	0.266	0.322	1.21
37.7	26.3	7.58	0.205	0.271	1.32
15.1	60.3	7.22	0.273	0.176	0.64
9.0	104.7	6.98	0.325	0.266	0.82
9.0	104.7	6.98	0.304	0.208	0.68
6.4	151.4	6.82	0.237	0.140	0.59
6.1	162.2	6.79	0.206	0.126	0.61
3.5	245.5	6.61	0.287	0.124	0.43
3.4	251.3	6.60	0.264	0.114	0.43

* All experiments carried out at a constant P_{CO_2} of 34–42 mm Hg.

‡ All values are the mean of two groups of eight diaphragms each.

§ Medium P_{CO_2} 34–42 mm Hg and (HCO_3^-) 19.9–22.5 mEq/liter.

‖ Differences between alkaline and control or acid and control significant $P < 0.001$.

TABLE II

*Effect of Changes in P_{CO_2} on Diaphragm Citrate Content**‡*

Experimental medium			Muscle		
P_{CO_2}	H^+	pH	Control citrate§	Exp. citrate	Citrate exp.‖ / Citrate control
mm Hg	*nmoles/liter*		*μmoles/g*	*μmoles/g*	
11.1	12.6	7.90	0.211	0.273	1.29
11.7	12.6	7.90	0.190	0.254	1.34
20.0	20.9	7.68	0.178	0.197	1.11
20.6	22.4	7.65	0.212	0.272	1.28
20.6	24.0	7.62	0.274	0.330	1.20
64.9	70.8	7.15	0.246	0.186	0.76
70.8	75.8	7.12	0.274	0.226	0.82
86.4	97.7	7.01	0.244	0.187	0.77
91.0	100.0	7.00	0.251	0.178	0.71
118.0	125.9	6.90	0.212	0.129	0.61
124.2	173.9	6.76	0.258	0.148	0.57

* All experiments carried out at a constant extracellular (HCO_3^-) of 19.9–22.9 mEq/liter.

‡ All values are the mean of two groups of eight diaphragms each.

§ Medium P_{CO_2} 34–41 mm Hg and (HCO_3^-) 19.9–22.9 mEq/liter.

‖ Differences between alkaline and control or acid and control significant $P < 0.001$.

there is no real difference between metabolic and respiratory points. Thus, in pure metabolic and respiratory acid-base states, acidosis is always accompanied by a decreased citrate content while alkalosis causes an increase in muscle citrate.

Incubation of diaphragms under isohydric conditions. To ascertain whether P_{CO_2} or extracellular bicarbonate concentration, independent of pH, could influence muscle citrate content, intact diaphragms were incubated at a single extracellular pH value achieved by using different combinations of bicarbonate and P_{CO_2}. In this manner it could be shown whether citrate content could be predicted from extracellular pH alone.

External pH in the experimental boxes ranged between 7.33 and 7.42 and did not differ in any single experiment more than 0.05 pH U from the pH value in the control box. Control conditions were as before with the P_{CO_2} held constant between 36 and 42 mm Hg and the bicarbonate concentration kept at 21–23 mEq/liter. The results are shown in Table III. From the first and last columns it is apparent that when external bicarbonate concentration was low, diaphragm citrate content was decreased in each of four experiments. When bicarbonate concentration was elevated above normal, the content of citrate in experimental and control tissue was identical. Thus, at physiologic extracellular pH values, muscle citrate could be reduced or normal depending on the particular combination of P_{CO_2} and bicarbonate employed. The previous data show that lowering P_{CO_2} alone causes an increase in muscle citrate content. From the decrease in muscle citrate it is apparent that, at low external bicarbonate concentrations and low P_{CO_2}, it was the bicarbonate, not the more permeable species carbon dioxide, which seemed to exert the predominant effect on muscle citrate.

To determine whether muscle citrate content could be predicted from extracellular pH at abnormal external acidities the experiments shown in Table IV were per-

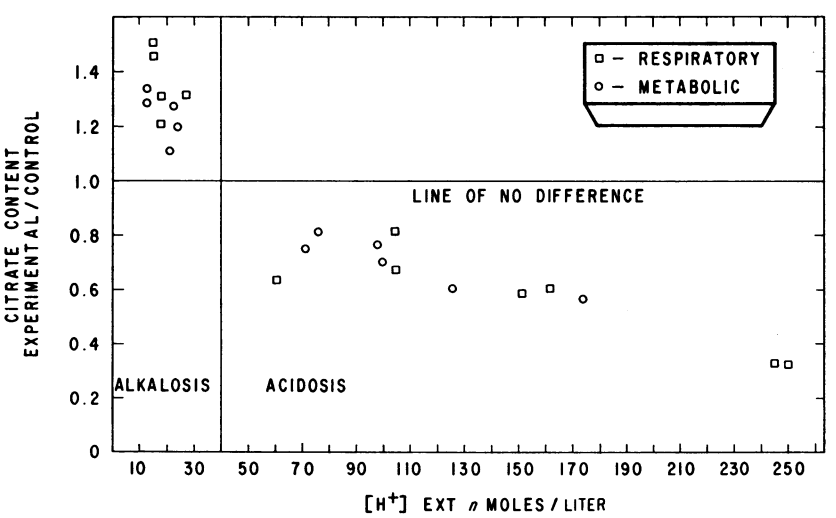

FIGURE 2 The relationship between external acidity and citrate content. The points labeled metabolic are found in Table I and those labeled respiratory in Table II.

formed. At an identical acid pH of either 7.15 or 7.05, diaphragms incubated in the bath containing the lower bicarbonate concentration and P_{CO_2} were found to have a lesser amount of citrate. These results are identical with those shown previously at physiologic pH values, i.e., extracellular bicarbonate seemed to exert the major influence since a low P_{CO_2} alone increases citrate content. At elevated extracellular pH values, however, a quite different result was found. When the external pH was between 7.58 and 7.66, diaphragms incubated at external bicarbonate concentrations of 11.3 or 11.6 mEq/liter contained the same amount of citrate as did diaphragms incubated at 66.5 and 67.9 mEq/liter. Thus, in an alkaline environment, citrate content seemed to be related primarily to extracellular pH rather than to P_{CO_2} or bicarbonate concentration.

Citrate oxidation experiments. The increased content of citrate within the tissue during alkalosis could be due to a decrease in the conversion of citrate to CO_2, the response which seems to be operative in the renal cortex (10). In order to determine the conversion of 1, 5-citrate-^{14}C to $^{14}CO_2$, hemidiaphragms were incubated in flasks containing 1, 5-citrate-^{14}C, and the $^{14}CO_2$ evolved was trapped and counted. This measurement will henceforth be referred to as citrate oxidation.

Initially, to make certain that the system was stable, flasks containing no tissue were incubated for 3 hr. The pH, total CO_2 content, and P_{CO_2} remained constant, demonstrating that no significant loss of CO_2 from the flasks occurred. Since tissue produces both carbon dioxide and hydrogen ions the constancy of the pH in the incubating flasks over a 90 min period of incubation was measured. Table V shows the pH, bicarbonate concentration, and P_{CO_2} before and after 90 min of incubation. Though some intragroup changes in these variables did occur, they were relatively small, and the differences between the six incubated groups remained quite large. This was true even if medium pH was altered by metabolic or respiratory means. The following data on citrate oxidation were obtained by using the average of the initial and final pH, bicarbonate, and P_{CO_2} values.

TABLE III

*Citrate Content of Diaphragms Incubated at Physiologic Extracellular pH Values Achieved by Different Combinations of HCO_3^- and P_{CO_2}**

Experimental medium			Muscle		
(HCO_3^-)	P_{CO_2}	pH	Citrate control‡	Citrate exp.	Citrate exp.§‖ / Citrate control
mEq/liter	*mm Hg*		*μmoles/g*	*μmoles/g*	
6.1	11.3	7.35	0.206	0.170	0.82
6.3	12.0	7.39	0.254	0.148	0.58
9.1	17.7	7.33	0.268	0.163	0.61
10.3	17.9	7.36	0.287	0.162	0.56
57.3	91.1	7.42	0.228	0.237	1.04
59.1	98.5	7.40	0.246	0.253	1.03
78.5	125.0	7.42	0.240	0.238	0.99
79.7	130.0	7.41	0.279	0.278	1.00

* All values are the mean of two groups of eight diaphragms each.

‡ Medium P_{CO_2} 35–41 mm Hg and (HCO_3^-) 20.0–22.4 mEq/liter.

§ Difference between low bicarbonate and control significant $P < 0.001$.

‖ Difference between high bicarbonate and control not significant $P > 0.5$.

Fig. 3a shows the effect of bicarbonate concentration on the conversion of radioactive citrate to $^{14}CO_2$. Increasing the bicarbonate concentration of the medium from 6.2 to 51.4 mEq/liter caused a progressive decrease in citrate oxidation. Thus, at an external pH of 6.79, citrate oxidation was almost 2½ times as great as that seen at a pH of 7.68. Each point differed significantly from the others, with a P value of less than 0.001. As shown in Fig. 3 *b*, when the P_{CO_2} of the medium was altered, keeping bicarbonate concentration constant at

TABLE IV

Citrate Content of Diaphragms Incubated at Abnormal Medium pH Achieved by Different Combinations of HCO_3^- and P_{CO_2}

Medium						Muscle		
(HCO_3^-)		P_{CO_2}		pH		Citrate content		Citrate Group 1
Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Citrate Group 2
mEq/liter		*mm Hg*				*μM/g*		
41.9*	5.0	121.2	15.0	7.16	7.14	0.226	0.164	1.38
36.5	5.0	133.2	18.9	7.06	7.04	0.223	0.130	1.72
66.5	11.6	60.9	12.0	7.66	7.61	0.240	0.222	1.08
67.9	11.3	68.0	12.5	7.62	7.58	0.247	0.282	0.88

* All values are the mean of two groups of eight diaphragms each.

TABLE V

Constancy of the pH, Bicarbonate Concentration, and P_{CO_2} of the Incubation Medium After 90 min of Incubation

	pH		(HCO^-_3)		P_{CO_2}	
			mEq/liter		*mm Hg*	
Time in minutes	0	90	0	90	0	90
Group 1	6.84	6.74	6.5	5.9	39	45
Group 2	7.19	7.04	14.7	12.0	39	46
Group 3	7.38	7.21	22.3	20.0	39	50
Group 4	7.75	7.62	52.4	50.4	39	50
Group 5	7.05	6.96	22.3	20.8	82	91
Group 6	7.68	7.52	22.3	20.0	20	25

22 mEq/liter, an increase in P_{CO_2} was accompanied by a decrease in citrate oxidation. The differences between groups was significant, with a P value of < 0.01.

Similar changes in extracellular acidity, induced by either metabolic or respiratory means, produced similar effects on citrate oxidation. Examination of the data in Fig. 3 show that when the pH was altered by metabolic means 0.56 U, from 7.68 to 7.12, the rate of citrate oxidation increased from 1.03 to 1.80. An almost identical pH change of 0.55 U, from 7.57 to 7.02, caused by varying the CO_2 tension of the medium increased citrate oxidation from 1.22 to 1.88. Differences between the metabolic and respiratory groups were not significant. Thus, increasing acidity of the media causes increasing oxidation of radioactive citrate by diaphragm muscle, a result quite similar to that seen with renal cortical slices (10).

DISCUSSION

In both metabolic and respiratory acidosis, extracellular pH may be returned to near normal values but at abnormal P_{CO_2} and bicarbonate concentrations (14, 15). The consequences to the body of this compensation are not completely understood. There is a large body of work which shows that even when extracellular pH is maintained, constant changes in bicarbonate concentration or P_{CO_2} may affect cellular metabolism. Craig (16) found in 1944 that rat cortex or medulla incubated in vitro produces less lactate as the CO_2 tension of the medium is increased in an isobicarbonate system, but that in an iso-pH system raising the P_{CO_2} increases lactate production. Katzman, Villee, and Beecher (17) incubated hemisected diaphragms as well as liver, kidney, and heart muscle slices, and they found differences in lactic acid production between isobicarbonate and iso-pH systems. They postulated an important regulatory role of Krebs cycle enzymes, since blockage of this cycle markedly altered their findings. Other investigators working on rat liver slices demonstrated that changes in P_{CO_2} affect glycogen synthesis even when extracellular pH is held constant (18). Further work with liver slices showed that it is the extracellular bicarbonate concentration, and not the pH, which is rate limiting in fatty acid synthesis, whereas extracellular pH changes account for variations in the conversion of acetate to CO_2 (19). Kamm, Fuisz, Goodman, and Cahill (20), studying gluconeogenesis in rat renal cortical slices, found that at a single extracellular pH value of approximately

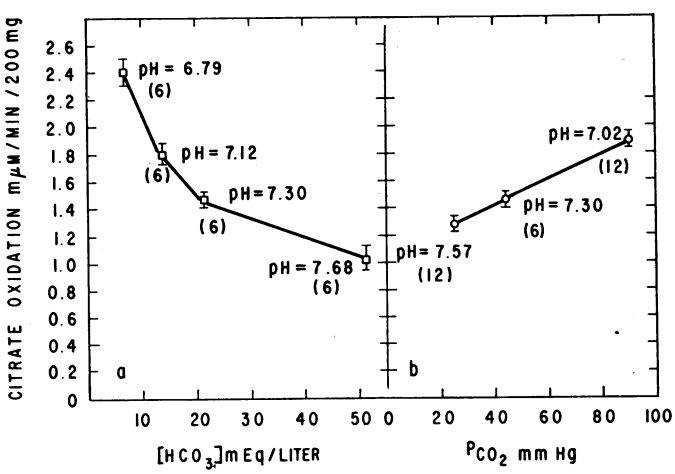

FIGURE 3 The effects of variation in external pH on citrate oxidation of rat hemidiaphragms. (*a*) Each point represents the ±SEM of six determinations at the average medium pH indicated. The pH of the medium was altered by using different bicarbonate concentrations at a single P_{CO_2} value. (*b*) Each point represents the ±SEM of 6 or 12 determinations at the average medium pH indicated. The pH of the medium was altered by using different concentrations of CO_2 in the gas phase at a single bicarbonate concentration.

7.40, glucose production varies in a linear fashion with the bicarbonate concentration of the medium. In rat epididymal fat pads, on the other hand, glucose metabolism is regulated primarily by extracellular pH and not by the bicarbonate concentration or CO_2 tension of the medium (21).

The studies enumerated above confirm that it is impossible to predict from the extracellular pH, bicarbonate concentration, or P_{CO_2} alone the cellular metabolic changes which may occur in tissue and substantiate the metabolic role of CO_2 in constituent enzyme systems (22). Almost all the studies cited were performed on nonintact tissue, and it is possible that in the presence of intact cellular membranes the results obtained would be quite different, since cell membranes are more permeable to a nonionized species such as molecular CO_2 than to an ionized species like bicarbonate (23). In agreement with this idea, numerous authors have shown that cellular acidity is not markedly altered by variations in external bicarbonate concentration (6–8). In contradistinction to this Adler, Roy, and Relman (3) demonstrated that although intact diaphragms maintained intracellular pH constant when extracellular bicarbonate concentration was lowered from 25 to 10 mEq/liter, lowering bicarbonate concentration further or raising it above 30 mEq/liter caused marked changes in intracellular acidity. They concluded that the cell was not indifferent to external bicarbonate.

The data reported in this study seem to uphold this conclusion. Lowering extracellular pH by either raising P_{CO_2} or decreasing bicarbonate concentration caused a progressive decrease in citrate content, while raising pH by either respiratory or metabolic means increased tissue citrate. The tissue system used in these experiments has previously been shown to have functionally intact membranes (24) and in spot studies on electrolytes in this study intracellular potassium concentration averaged 150 mEq/liter when the extracellular potassium concentration was 5 mEq/liter. Intracellular sodium concentration also remained low, proving physiologic membrane selectivity. Thus, in intact tissue the extracellular bicarbonate concentration not only affects intracellular pH, but as shown by the changes in citrate content it also affects cellular metabolism. Furthermore, in the isohydric state, at either normal or acid pH values, when the external bicarbonate concentration was low, tissue citrate content decreased. The decrease in citrate seems to be due to the bicarbonate ion since the lowering of P_{CO_2} by itself, as the previous data showed, would have raised the citrate content. This further demonstrates that a charged species such as bicarbonate can indeed alter the metabolic state of the cell even though external pH remains unchanged.

Since Adler, Roy, and Relman have shown that cellular pH is a complex function of both P_{CO_2} and external bicarbonate concentration, not predictable from extracellular pH alone (4), it is possible that the changes in citrate content found in the isohydric experiments can be explained by changes in intracellular pH or cellular bicarbonate concentration. They found that at an extracellular pH of 7.16 or 7.06 intracellular pH could be normal, high, or low depending upon the particular combination of P_{CO_2} and bicarbonate employed. At low P_{CO_2} values intracellular pH rose, while at elevated P_{CO_2} levels intracellular pH fell. Similar results transpired when the extracellular pH was maintained at 7.40. The citrate results do not correlate well with these findings. At lower bicarbonate and P_{CO_2} values, a situation in which the muscle cell was found to be more alkaline, citrate content not only did not rise, it fell. Thus, intracellular acidosis induced by pure respiratory or metabolic means or intracellular alkalosis achieved by the mixed means just enumerated can lead to a decrease in cell citrate content. In extracellular alkalosis, however, intracellular pH always rises and so does the citrate content. The data also show that at lowered or normal external pH values the citrate content is primarily responsive to changes in external bicarbonate.

A similar conclusion regarding citrate control has been reached by Simpson (10). He studied citrate utilization and oxidation both in rat renal cortical slices and in mitochondria and found that changing the bicarbonate concentration in an isohydric system has no effect on citrate oxidation by slices, while changing bicarbonate concentration at one extracellular pH in a mitochondrial system profoundly alters the citrate oxidation rate. He postulated that it was the intracellular bicarbonate concentration which controls citrate oxidation. However, when intracellular bicarbonate concentration in the diaphragm is calculated from the intracellular pH and P_{CO_2} values, no correlation exists between it and the citrate content. Citrate metabolism may be controlled differently in kidney and diaphragm, and Simpson's theory regarding intracellular bicarbonate could still be correct for renal cortex. This seems unlikely for the following two reasons. First, in both tissues citrate content rises in alkalosis and decreases in acidosis in the acute experiments already cited as well as in rats made chronically acidotic or alkalotic (25). Second, this study demonstrates that hemidiaphragms incubated in acidic media converted more radioactive citrate to CO_2 than did diaphragms incubated in alkaline media, a result identical with that found by Simpson in renal cortical slices. Even the percentage change in oxidation rate of the two tissues caused by variations in bicarbonate and P_{CO_2} were almost identical. A possible explanation of the apparent discrepancy between intracellular

pH or bicarbonate concentrations and the citrate content of the diaphragm may lie in the heterogeneous nature of the cell (3, 26, 27). Changes in local pH in mitochondria or other organelles may occur in a direction opposite to those ocurring in the large mass of cell cytoplasm. The intracellular pH determined by the 5,5 dimethyl-2, 4-oxazolidinedione (DMO) method may therefore not give a true indication of what is happening at the enzymatic site concerned with citrate oxidation.

Regardless of the exact mechanisms involved, the data presented seem to have physiologic significance since the bicarbonate, P_{CO_2}, and pH values employed in this study lie well within the limits found in animal and human subjects. In addition, these in vitro citrate alterations agree well with in vivo experiments that demonstrate significant changes in the citrate content of liver, kidney, intestine, and diaphragm of rats made acidotic or alkalotic (25). Cellular pH appears to be regulated by metabolic means (28), and differences in the accumulation or disposal of organic acids might contribute toward the defense of a cell faced with an external acid-base disorder (3). If this hypothesis is correct, it would be expected that organic acids should decrease in acidosis and increase in alkalosis—exactly what was found for the citrate in these experiments. Further indirect evidence in favor of this hypothesis is given by the time experiments. The length of time required to reach a new steady state in both the metabolic and respiratory experiments closely approximates in vitro diaphragm (3) and in vivo rat skeletal muscle (5) intracellular pH time responses, showing that a temporal relationship between intracellular pH and citrate accumulation exists.

Phosphofructokinase is exquisitely sensitive to pH in vitro (29) and seems to be one of the major rate-limiting steps in glycolysis (30, 31). Decreasing pH causes decreased activity of the enzyme, while alkalosis increases it. It has been shown that citrate can inhibit phosphofructokinase in rat heart muscle (32). Citrate could thus act as a feedback mechanism for the control of glycolysis. This might function in cellular pH control in the following manner. In alkalosis, phosphofructokinase would be activated, but as time progressed intracellular pH would be adjusted towards normal by increased organic acid accumulation. The changes in intracellular pH, plus the increased citrate level in the tissue, would act to inhibit the enzyme and return glycolysis towards normal. Exactly how citrate content changes cannot be stated since in these, as well as in Simpson's experiments, turnover rate was not measured (10).

Finally, there are definite clinical implications which can be drawn. This study has shown that at equal extracellular pH values, even when the pH is at physiologic levels, citrate content can vary markedly, depending on the particular combination of bicarbonate or P_{CO_2} employed.. If these in vitro results can then be extrapolated to circumstances in vivo, a situation could be present in which altered cellular metabolism would coexist with extracellular acid-base compensation. How costly this metabolic derangement would be has not been studied, but these experiments call for a reexamination of the recommendation that in treating low bicarbonate states it is unnecessary to return the bicarbonate concentration to normal levels unless the patient has become symptomatic (33).

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