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Regulation of Renal Citrate Metabolism by Bicarbonate Ion and pH: Observations in Tissue Slices and Mitochondria *

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Summary. The effect of acid-base balance on the oxidation and utilization of citrate and other organic acids has been studied in tissue slices and isolated kidney mitochondria. The results show that: *1*) With bicarbonate-buffered media, citrate oxidation and utilization are inhibited in slices of renal cortex and in kidney mitochondria when [HCO_3^-] and pH are increased within the physiologic range (pH 7.0 to 7.8; 10 to 60 μmoles HCO_3^- per ml). When phosphate or Tris buffers are used, no comparable effect on citrate oxidation occurs when pH is varied. *2*) This effect is not demonstrable in heart or liver slices when a physiologic buffer is used. *3*) α-Ketoglutarate utilization is inhibited in slices of renal cortex under similar conditions. Pyruvate and L-malate utilization are not inhibited in slices or mitochondria. *4*) Citrate content in slices of renal cortex incubated with a high [HCO_3^-] is considerably greater than the concentration found with a low [HCO_3^-] in the medium. This effect is not duplicated by pH change in a nonbicarbonate buffer system. In mitochondria citrate content is also increased markedly at high bicarbonate concentrations. *5*) The kinetic characteristics of the inhibition of citrate oxidation are those of a competitive type of inhibition. *6*) When pH was varied with a constant [HCO_3^-] in the media, citrate oxidation was inhibited by increasing pH in slices of renal cortex but not in mitochondria. On the other hand, when [HCO_3^-] was increased without change in pH, no decrease in citrate oxidation occurred in slices, but a marked inhibitory effect was found when mitochondria were used.

From a comparison of these results with those previously obtained in intact animal experiments, we conclude that the inhibition of citrate oxidation caused by increasing pH and [HCO_3^-] in slices of renal cortex and kidney mitochondria is an *in vitro* representation of the inhibition of citrate reabsorption in the nephron that occurs in metabolic alkalosis. Thus, citrate clearance increases in metabolic alkalosis because of inhibition of oxidation of reabsorbed citrate within cells of the renal tubules. This inhibition is the result of an inhibitory effect of bicarbonate ion on citrate oxidation in mitochondria.

Introduction

The renal clearance of citrate is greatly increased during metabolic alkalosis (1–3). In normal animals nearly all of the citrate filtered through the glomerulus is reabsorbed in the nephron with only small quantities escaping into the urine. In

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the presence of metabolic alkalosis the tubular reabsorption of citrate is inhibited, and the excretion of citrate increases manyfold with little concomitant change in the plasma level. The physiologic characteristics of this phenomenon have been described in numerous reports, but the cellular mechanism responsible for it has not been defined.

Three possibilities exist to explain the effect of metabolic alkalosis on citrate clearance. First, secretion of citrate, derived either from renal blood or from synthesis within the cells of the tubules, could be enhanced by metabolic alkalosis, resulting in increased urinary citrate. However, evidence from physiologic studies suggests that secretion of citrate is not primarily responsible for increasing the clearance of citrate in metabolic alkalosis (4). Second, metabolic alkalosis could inhibit the transport of citrate across cell membranes in the tubules, thus decreasing the reabsorption of citrate. Such an effect would be analogous, for example, to the effect of phlorizin on glucose reabsorption. Third, metabolic alkalosis could increase citrate clearance by inhibiting the intracellular oxidation of citrate. This possibility must be considered because an important characteristic of the renal physiology of citrate is the rapid oxidation of this substance by cells of the cortex. Recently it has been shown that citrate oxidation may account for as much as 10% of the oxygen consumption of the kidney (5). The consumption of citrate by the kidney normally exceeds the amount of filtered citrate by about one-third (4, 6). Therefore, both reabsorbed citrate and significant amounts of unfiltered citrate from the blood are metabolized in the kidney and provide energy for other renal functions. Thus, the normal fate of reabsorbed citrate is oxidation in the cells of the tubules; interference with this process could decrease the fraction of citrate reabsorbed.

The available data from intact animal studies do not allow a distinction to be made between an effect of metabolic alkalosis on citrate transport and an effect on the intracellular metabolism of citrate. In the present investigation an *in vitro* system was sought that would permit investigation of the cellular mechanism underlying this effect of metabolic alkalosis. In tissue slices of renal cortex and in kidney mitochondria, increasing pH and bicarbonate concentrations in the medium were found to inhibit citrate oxidation. The properties of this *in vitro* effect closely resemble those which have previously been described for the effect of metabolic alkalosis on citrate clearance in the intact animal.

Methods

Preparation and incubation of tissue slices. Fresh kidneys obtained from exsanguinated rabbits were placed in ice-cold slicing medium consisting of 0.14 M NaCl and 0.01 M KCl. Slices were cut from pieces of cortex with a Stadie-Riggs-type slicer calibrated to cut slices 0.25 mm thick. After slicing, a group of slices was blotted gently on filter paper and weighed on a torsion balance. Between 190 and 200 mg of slices was used in each flask. The weighed groups of slices were then kept in individual beakers containing ice-cold slicing medium until it was time to place them in the incubation flasks.

The rate of citrate oxidation was determined by measuring the rate of formation of $^{14}CO_2$ from citrate-1,5-^{14}C. Slices were incubated in Dixon-Keilin-type Warburg vessels (7). (These flasks are designed for use with a bicarbonate buffer system in which a constant CO_2 concentration is maintained in the gas phase during incubation. They are constructed with a stopcock on the bottom of each vessel containing a small hollowed-out chamber in which sodium hydroxide solution is placed. During incubation this solution is sealed off from the interior of the flask; at the end of the experiment the stopcock is turned until the stopcock chamber communicates with the center well of the flask and the CO_2 in the flask is absorbed by the sodium hydroxide solution.) A typical incubation medium contained, per milliliter, 10 μmoles $NaHCO_3$, 122 μmoles NaCl, 10 μmoles KCl, 1 μmole KH_2PO_4, 1 μmole Na citrate, 2 μmoles Na pyruvate, and 2 μmoles Na L-malate. We used high medium K^+ concentration in order to ensure adequate potassium to replace intracellular losses occurring while the slices were kept in the cold slicing medium. However, the effects to be described can be demonstrated even in the absence of potassium in the medium. Organic acid solutions were adjusted to pH 7.2 to 7.4 before being added to the medium. Variations in the composition of the medium were compensated for by increasing or decreasing the amount of NaCl as necessary to keep the osmolarity of the medium constant. We placed 1.8 ml of medium in each flask and 0.5 ml 1 N HCl in the sidearm. The stopcock chamber was filled with 6 N NaOH and closed; a strip of filter paper was placed in the center well. We placed 25 μl of radioactive citrate solution in a microvessel attached to the inside wall of the Warburg flask with a small drop of paraffin. Before the slices were placed in the flasks the latter were warmed in a Warburg bath to 30° C and gassed with 5% CO_2–95% O_2. In some experiments we used other concentrations of CO_2 in order to vary the CO_2 tension in the medium. Before passing through the flasks, gas mixtures were saturated with water vapor. After equilibrium with the gas mixture was obtained, the tissue slices were added and the gassing continued for 5 minutes. The

flask vents were then closed and the radioactive citrate released into the medium by gently melting the paraffin holding the microvessel to the flask wall. The slices were incubated with the radioactive substrate for 15 minutes at 30° C and the experiment stopped by tipping acid in from the sidearm. The stopcock on the bottom of the flask was turned until the chamber within it was under the center well of the flask, allowing the NaOH solution to wet the filter paper. Shaking of the flask was continued for 2 hours while the CO_2 was being absorbed. Control observations indicated that all of the CO_2 in the flasks was absorbed during this interval.

When experiments with nonbicarbonate buffers were performed, 10 μmoles phosphate buffer per ml or 10 μmoles (with respect to Cl^-) Tris-HCl per ml was substituted for bicarbonate. The flasks were gassed with 100% O_2. Filter paper saturated with 6 N NaOH was present in the center well throughout the incubation so that evolved CO_2 was collected continuously. When bicarbonate plus phosphate or Tris buffer was used, the appropriate pH of the nonbicarbonate buffer for a given concentration of HCO_3^- was calculated with the Henderson-Hasselbalch equation and a pK of 6.10; an α-value of 0.0315; and a carbon dioxide pressure (P_{CO_2}) of 728 × (per cent CO_2 of the gas mixture), (728 = 760 mm Hg − water vapor pressure at 30° C). The other conditions of incubation were the same as those described for media buffered with bicarbonate alone.

In some experiments substrate utilization was determined by measuring the amount of organic acids remaining in the medium after incubation with the tissue slices. The same slicing and incubation procedure was followed as that described above, except that the incubation time was longer and the experiment was terminated by opening the flask and rapidly adding 2 ml of 20% trichloroacetic acid (TCA). The slices and medium were then transferred to a Potter-Elvehjem homogenizer, and the slices were homogenized. The suspension was centrifuged and the supernatant saved for measurement of the organic acids. The term "citrate utilization" is used throughout the paper to describe results of experiments in which the amount of citrate remaining in the medium after incubation was measured. Citrate oxidation, on the other hand, is used to refer to experiments in which the conversion of radioactive citrate to $^{14}CO_2$ was measured as described in the preceding paragraph.

In further experiments the total citrate content in slices and medium was compared in slices incubated with various combinations of bicarbonate and phosphate or Tris buffers. Citrate was omitted from the media so that substrate was provided entirely by pyruvate and malate. About 200 mg of slices was incubated in 1.8 ml of medium in each flask for 15 minutes. The incubation was stopped with 2 ml of 20% TCA. After homogenizing the slices and centrifuging, we removed the supernatant for citrate determination.

Preparation and incubation of mitochondria. Mitochondria were prepared by the short method of Lehninger (8). Fresh rabbit kidneys were homogenized for 60 seconds in a Waring blendor in ice-cold 8.5% sucrose containing 0.001 M EDTA. pH was maintained during homogenization at about 7.0 by the dropwise addition of 6 N KOH. The homogenate was centrifuged at 0° C at 1,000 × g for 5 minutes and the sediment discarded. A sufficient amount of 1.5 M KCl was added to the supernatant to bring the KCl concentration of the solution to 0.15 mole per L. The solution was allowed to stand for 10 minutes in an ice bucket and then centrifuged for 15 minutes at 0° C at 1,500 × g. The supernatant was discarded, and the mitochondrial pellet was resuspended in 0.15 N KCl and centrifuged at the same speed for 10 minutes. This washing step was repeated once again. After the final wash, the mitochondrial pellet was suspended in about 5 ml of ice-cold 0.15 M KCl, providing a thick slurry of mitochondria.

We added 0.5 ml of the mitochondrial preparation to 1.5 ml of medium that had previously been equilibrated at 30° C with 5% CO_2–95% O_2 or another gas mixture in a Dixon-Keilin flask in a Warburg bath. A typical medium contained in each milliliter, after addition of the mitochondria, 10 μmoles $KHCO_3$, 70 μmoles KCl, 3 μmoles $MgSO_4$, 10 μmoles phosphate buffer, 4 μmoles Na_4ATP, and 1 μmole K citrate. This composition does not include the KCl used to suspend the mitochondria. In calculating the osmolarity of the final incubation solution we assumed that the mitochondrial suspension contained 80% of 0.15 M KCl so that the total osmolarity of the medium after addition of the mitochondria was about 270 mOsm per L. We neutralized citrate and other organic acid solutions to pH 7.2 before preparing the medium. The ATP[1] solution was neutralized immediately before use and added to the rest of the medium just before the mitochondria were added. The proper pH of the phosphate buffer for a given concentration of HCO_3^- was calculated in the same manner as described for the slice experiments. When changes in the composition of the medium were made, the concentration of KCl was adjusted to keep the osmolarity constant. The mitochondria were gassed and incubated in the same manner as the tissue slices except that the gassing time after introduction of the mitochondria was reduced to 3 minutes and the incubation time to 10 minutes.

In two experiments the total citrate content of mitochondria and medium was measured after incubation for 12 minutes with phosphate or bicarbonate buffers. Two μmoles pyruvate and malate per ml was used as substrate. The incubation was stopped with 3% perchloric acid; samples were assayed for citrate.

Analytical methods. In experiments in which radioactive citrate was used the NaOH solution in the center well of the Warburg flask was transferred quantitatively to a centrifuge tube. The filter paper was removed from the center well and washed carefully with water; the washings were added to the centrifuge tube. Carrier Na_2CO_3 was added and the carbonate precipitated with $BaCl_2$. The precipitate was washed twice with water and placed in a planchet. After drying, planchets were counted in a gas flow proportional counter and the counts per minute corrected for self-absorption.

[1] Sigma Chemical Co., St. Louis, Mo.

Citrate was determined by McArdle's modification of the pentabromacetone method (9). Pyruvate was measured by the method of Friedemann and Haugen (10) in the tissue slice experiments with ethyl acetate as the extracting solvent, and by Huckabee's modification (11) of this method in the experiments on mitochondria. Alpha-ketoglutarate was measured by the method of Friedemann and Haugen (10). Malate was determined fluorometrically (12). Twenty-five-μl samples of the tissue extract were used for the assay; under the conditions of these experiments it was not necessary to precipitate the malate with calcium as originally described.

In the Figures and Tables the results of individual experiments are shown to illustrate the typical changes observed. In the text additional data obtained in confirmatory experiments are presented in the form of the mean slope of the least squares lines plus or minus the standard error of the mean. In order to permit comparisons of the mean slopes obtained in different types of experiments, we have calculated the regression coefficients in all instances using the same scale—linear for bicarbonate concentration—for the abscissa.

Results

Tissue slice experiments

Effect of increasing pH on citrate oxidation. Figure 1 shows the result of an experiment in which citrate oxidation was measured in slices of renal cortex with a bicarbonate buffer system. The CO_2 concentration in the gas phase was 5% in all flasks. $[HCO_3^-]$ in the medium was varied between 10 and 60 μmoles per ml. Calculated pH ranged between 7.0 and 7.8. As pH and bicarbonate concentration increased, the rate of citrate oxidation diminished linearly. In four such experiments the average slope was -0.163 ± 0.011. In a control experiment, measurement of the pH of the medium after incubation under conditions similar to those of the above experiments showed that a gradual fall in pH occurred; after 40 minutes this decrease averaged 0.08 pH U and was of similar magnitude at all pH levels.

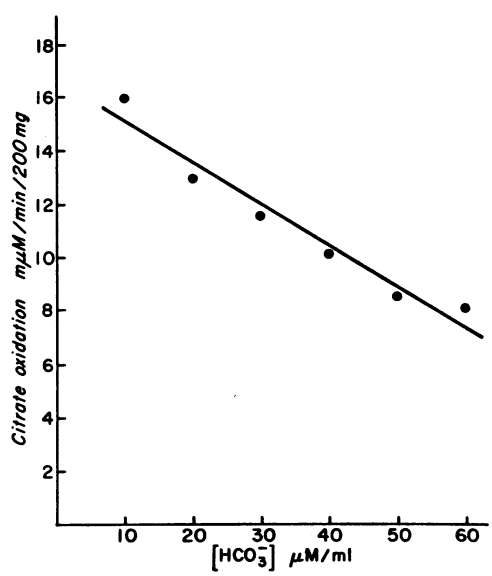

FIG. 1. RATES OF CITRATE OXIDATION IN SLICES OF RENAL CORTEX WITH MEDIA OF DIFFERENT BICARBONATE CONCENTRATIONS. The organic acid substrate in each milliliter consisted of 1.0 μmole Na citrate, 2.0 μmoles Na pyruvate, and 2.0 μmoles Na malate.

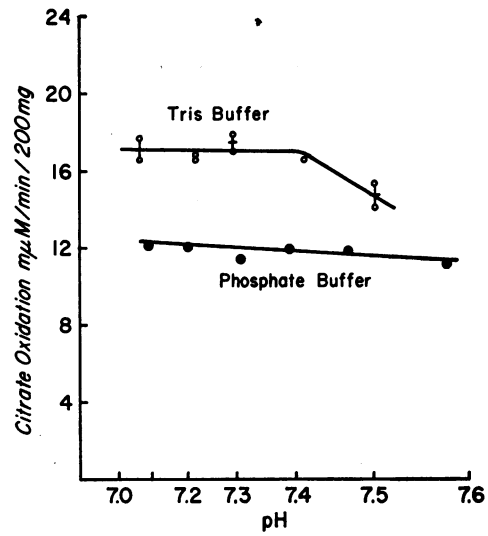

FIG. 2. RATES OF CITRATE OXIDATION AT DIFFERENT pH VALUES IN RENAL CORTICAL SLICES WITH MEDIA BUFFERED WITH PHOSPHATE OR TRIS. Organic acid substrate was the same as in the experiment of Figure 1.

When phosphate buffer was used in the medium in place of bicarbonate buffer, there was little change in the rate of citrate oxidation between pH 7.0 and 7.6 (Figure 2). In six experiments with phosphate buffer the mean slope was -0.035 ± 0.010. The result of a similar experiment with Tris buffer is also shown in Figure 2. The mean slope of three experiments in which Tris buffer was used was -0.037 ± 0.011. With Tris, a rapid decline in citrate oxidation occurred above pH 7.5, as is illustrated in Figure 2, but little inhibitory effect was present below this pH. These experiments suggest that the carbonic acid buffer system is essential for demonstrating the particular linear inhibition of citrate oxidation related to increasing pH and $[HCO_3^-]$ within the physiologic range.

TABLE I

*Comparison of the effects of changes in $[HCO_3^-]$ and changes in $[Cl^-]$ in the medium on the rate (V) of citrate oxidation in slices of renal cortex**

Flask no.	$[HCO_3^-]$	$[Cl^-]$	V
	μmoles/ml	*μmoles/ml*	*mμmoles/min/200 mg*
1†	20	82.5	19.2
2†	20	82.5	18.3
3	35	82.5	14.7
4	50	82.5	11.8
5	20	112.5	16.2
6	20	97.5	17.5

* Total osmolarity of each medium was kept constant by varying the concentration of glucose.
† Identical incubation media.

Comparison of the effect of changing $[Cl^-]$ or $[HCO_3^-]$ on citrate oxidation. In the media used in the preceding experiments, increases in $[HCO_3^-]$ were accompanied by decreases in $[Cl^-]$; the osmolarity of the media was thus kept constant. Therefore, the inhibition of citrate oxidation in these experiments could have been related to the decrease in $[Cl^-]$ rather than to the increase in pH and $[HCO_3^-]$. In order to study this possibility, in one experiment we compared citrate oxidation in media in which the osmolarity was kept constant by addition or subtraction of glucose and either $[Cl^-]$ or $[HCO_3^-]$ was varied while the concentration of the other anion was held constant. The results are shown in Table I. The data in the top two sections of the Table show that when $[Cl^-]$ was held constant at 82.5 μmoles per ml, citrate oxidation was inhibited by increasing $[HCO_3^-]$. The data in the first and last sections indicate that as $[Cl^-]$ decreased with a fixed $[HCO_3^-]$ in the medium, citrate oxidation increased slightly. Thus, when $[Cl^-]$ was varied alone the effect on citrate oxidation was in the opposite direction to that found in experiments of the type shown in Figure 1. Therefore, the inhibitory effect of increasing pH and $[HCO_3^-]$ on citrate oxidation is not related to the accompanying decrease in $[Cl^-]$. Additional evidence that chloride variation is not a significant factor in this inhibitory effect is provided in the experiments described below, in which $[HCO_3^-]$ and $[Cl^-]$ were varied as in the experiment of Figure 1, but the pH was held constant; no change in the rate of citrate oxidation occurred under these conditions.

Effect of bicarbonate buffer on citrate content of slices and media. When slices of renal cortex were incubated with phosphate or Tris buffers of high and low pH, total citrate content of slices and media was significantly greater at the higher pH (Table II). When bicarbonate buffer was present as well as phosphate or Tris, the citrate content was increased at each pH level. In addition, the magnitude of the difference between citrate content at high and low pH levels was considerably enhanced. Thus, the presence of a bicarbonate buffer causes an increase in citrate content with increasing pH that is independent of the relatively small increase in citrate content produced by non-bicarbonate buffers. In one experiment of this type, slices and bicarbonate-buffered media were separated after incubation and citrate measured in each. After incubation with 10 μmoles HCO_3^- per ml of medium, citrate content in three flasks averaged 0.184 ± 0.008 μmole per 500 mg slices, and the citrate concentration in the medium was 0.035 ± 0.0015 μmole per ml. In slices incubated with 50 μmoles HCO_3^- per ml, mean citrate content in three flasks was 0.335 ± 0.016 μmole per 500 mg slices, and medium citrate concentration was 0.109 ± 0.006 μmole per ml.

Effect of varying pH and $[HCO_3^-]$ on citrate oxidation in heart and liver. Figure 3 shows the

TABLE II

*Effect of bicarbonate on citrate content of renal cortical slices incubated with phosphate or Tris buffers**

pH	Without bicarbonate	With bicarbonate	
	Total citrate	$[HCO_3^-]$	Total citrate
	μmoles	*μmoles/ml*	*μmoles*
Phosphate buffer			
	0.118		0.174
6.99	0.123	10	0.204
	0.120		0.192
	0.160		0.298
7.60	0.144	40	0.312
	0.142		0.308
Tris buffer			
	0.126		0.156
6.99	0.120	10	0.143
	0.115		0.145
	0.143		0.253
7.47	0.139	30	0.270
	0.147		0.290

* Substrate in each milliliter of medium was 2.0 μmoles of pyruvate and of malate. Incubation time was 15 minutes. Gas phase was 100% O_2 in flasks containing media without bicarbonate and 5% CO_2–95% O_2 in those with bicarbonate.

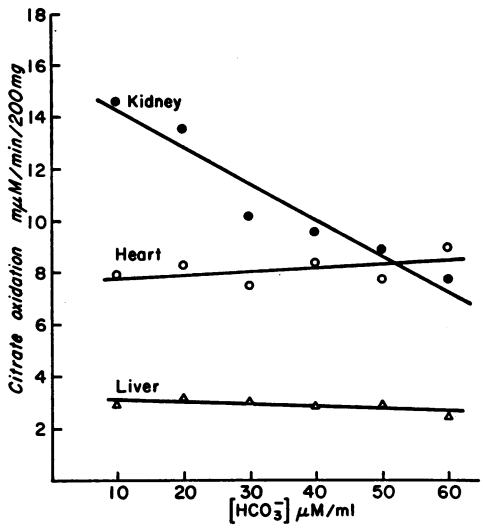

FIG. 3. RATES OF CITRATE OXIDATION IN TISSUE SLICES OF DIFFERENT ORGANS INCUBATED IN MEDIA CONTAINING VARYING CONCENTRATIONS OF BICARBONATE. Media contained 2.5 μmoles $CaCl_2$ per ml.

results of three experiments with tissue slices from kidney cortex, heart, and liver. The conditions for these experiments were identical to those of the experiment shown in Figure 1, except for the presence of 2.5 mM Ca^{++} in the media. In neither heart nor liver was there any significant effect on citrate oxidation when bicarbonate concentration varied between 10 and 60 μmoles per ml. In four experiments with heart slices the mean slope was -0.014 ± 0.011. Similarly, in two experiments in which liver slices were used no significant change in the rate of citrate oxidation occurred. In contrast, when kidney slices were used, citrate oxidation decreased as the $[HCO_3^-]$ and pH rose. In addition, the rate of citrate oxidation in slices of heart and especially in slices of liver was slow compared to that in kidney slices.

Comparison of the effect of varying pH and $[HCO_3^-]$ on the utilization of citrate, pyruvate, malate, and α-ketoglutarate. To determine if the inhibitory effect of increasing pH and $[HCO_3^-]$ on citrate oxidation was a general effect on Krebs cycle and related substrates, we measured the rates of utilization of pyruvate, malate, and α-ketoglutarate at various bicarbonate concentrations. In three experiments of this type, one of which is shown in Table III, the mean slopes of substrate utilization vs. concentration of bicarbonate for citrate, malate (two experiments only), and pyruvate were -0.337 ± 0.046, $+0.072 \pm 0.015$, and $+0.132 \pm 0.050$, respectively. The positive slopes for pyruvate and malate are chiefly the result of deviations at the highest and lowest bicarbonate concentrations, whereas relatively constant rates of pyruvate and malate utilization were present between 20 and 50 μmoles HCO_3^- per ml as illustrated by the data in Table III. Thus, although citrate utilization was markedly inhibited as $[HCO_3^-]$ increased, there was no change or perhaps a slight increase in the rates of malate and pyruvate utilization. In Table IV a similar experiment is shown in which α-ketoglutarate was used as substrate. Both in the presence and absence of citrate in the medium, α-ketoglutarate uptake was inhibited by increasing pH and $[HCO_3^-]$. Similar results were obtained in three other experiments in which α-ketoglutarate utilization was measured. Thus, this inhibition does not affect all Krebs cycle intermediates, but only selected ones.

Kinetics of the inhibitory effect in tissue slices. In order to study the kinetics of this phenomenon,

TABLE III

*Comparison of the effects of different bicarbonate concentrations in the medium on the rates of utilization of citrate, malate, and pyruvate by slices of renal cortex**

$[HCO_3^-]$	Rate of substrate utilization		
	Citrate	Malate	Pyruvate
μmoles/ml	*mμmoles/min/200 mg*		
10	16.3	54	46
20	12.5	56	49
30	8.5	57	50
40	6.3	57	50
50	5.0	57	52
60	2.5	60	57

* Media contained in each milliliter 1.0 μmole Na citrate, 2.0 μmoles Na pyruvate, and 2.0 μmoles Na malate.

TABLE IV

Effect of increasing $[HCO_3^-]$ and pH on the utilization of α-ketoglutarate by renal cortical slices

$[HCO_3^-]$	α-Ketoglutarate utilization	
	Medium A*	Medium B*
μmoles/ml	*mμmoles/min/200 mg*	
10	35.4	50.2
30	28.0	40.2
50	23.2	29.4

* Medium A contained 1.0 μmole α-ketoglutarate per ml and 1.0 μmole citrate per ml as substrate; medium B contained 1.0 μmole α-ketoglutarate only per ml.

in two experiments we incubated renal cortical slices with varying concentrations of citrate and either 10 or 50 μmoles HCO_3^- per ml. In this type of experiment citrate was the only substrate present in the medium. A Lineweaver-Burk plot of the result of one experiment is shown in Figure 4. In each experiment of this type, the lines representing the two different bicarbonate concentrations intersect at a point on the ordinate, indicating a competitive type of inhibition (13). The maximal velocities (point of intersection on ordinate) in the two experiments were 75 (Figure 4) and 50 mμmoles per minute.

Effect of variations in the components of the CO_2-bicarbonate buffer system on citrate oxidation. Since both pH and $[HCO_3^-]$ varied in the preceding experiments, the inhibitory effect on citrate oxidation could be related to the change in one or the other or both of these components of the buffer system. By varying the per cent CO_2 in the gas phase with a constant $[HCO_3^-]$ in the medium, pH was varied independently of $[HCO_3^-]$ and a significant inhibitory effect on citrate oxidation was found (Figure 5, a). In five experiments of this type the mean slope obtained was

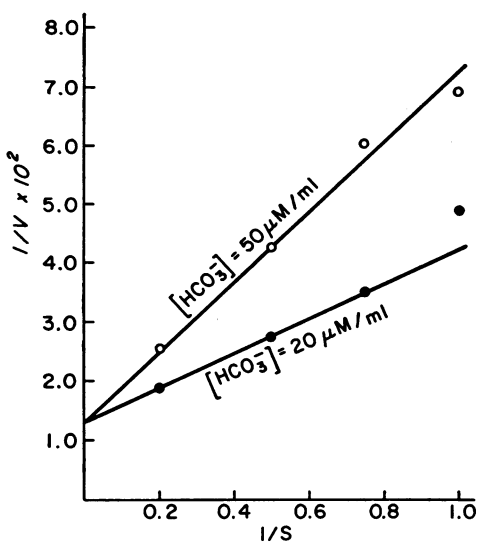

FIG. 4. LINEWEAVER-BURK RECIPROCAL PLOT OF THE RATES OF CITRATE OXIDATION WITH DIFFERENT CONCENTRATIONS OF CITRATE IN THE MEDIA AND WITH TWO DIFFERENT CONCENTRATIONS OF BICARBONATE. Organic acid substrate consisted of Na citrate in the concentrations (S) shown inversely on the abscissa. V = rate of citrate oxidation in millimicromoles per minute per 200 mg slices.

-0.148 ± 0.010. When $[HCO_3^-]$ and per cent CO_2 in the gas phase were varied proportionately, with pH kept constant no inhibitory effect occurred, as shown in Figure 5, b. The mean slope in six experiments in which the $[HCO_3^-]$ was varied while the pH was held constant was $+0.004 \pm 0.016$. The contrast between the independent effects of changes in pH and changes in $[HCO_3^-]$ is well shown in the experiment described in Table V. Here, three pairs of flasks were used, and in each pair one of the three components of the buffer system, the CO_2 tension, the pH, or the $[HCO_3^-]$, was kept constant while the other two components were varied. Media were composed so that a particular component of the buffer had identical pairs of values in the two pairs of flasks in which its concentration was varied. When CO_2 tension was held constant, the rate of citrate oxidation decreased by 4.8 μmoles per ml (pair A). When pH was held constant and $[HCO_3^-]$ varied between 20 and 45 μmoles per ml, citrate oxidation decreased by only 0.5 mμmole per minute per 200 mg slices (pair B). When $[HCO_3^-]$ was held constant, a change in pH comparable to that in the first pair of flasks caused citrate oxidation to diminish by 5.1 mμmoles per minute per 200 mg slices (pair C). Thus, in tissue slices the inhibitory effect of increasing pH and $[HCO_3^-]$ in the medium is due primarily to an effect related to the change in pH, whereas the change in $[HCO_3^-]$ alone has no effect on citrate oxidation.

Experiments with mitochondria

Effect of increasing pH on citrate oxidation. When mitochondria were incubated under conditions similar to those used in the tissue slice experiments, increasing pH and $[HCO_3^-]$ were again accompanied by decreasing rates of citrate oxidation (Figure 6). In four experiments in which citrate oxidation in kidney mitochondria was studied under these conditions, the mean slope was -1.45 ± 0.12. Under these conditions the inhibitory effect was even more pronounced than in tissue slices. When a phosphate buffer was substituted for the bicarbonate buffer, no significant decrease in citrate oxidation by mitochondria occurred within the pH range 7.0 to 7.5.

Effect of increasing pH and $[HCO_3^-]$ on citrate, pyruvate, and malate utilization. When

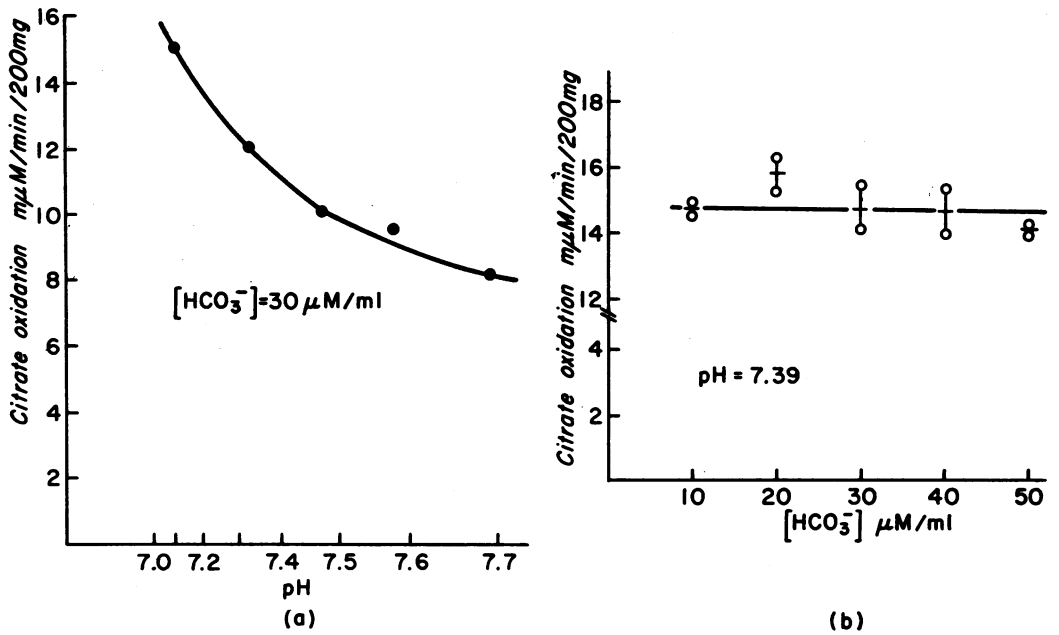

FIG. 5. THE EFFECTS OF INDEPENDENT VARIATIONS IN pH AND IN $[HCO_3^-]$ ON CITRATE OXIDATION IN RENAL CORTICAL SLICES. (a) Rates of citrate oxidation with varying pH and a constant $[HCO_3^-]$ (30 μmoles per ml). The pH of the media was altered by using different concentrations of CO_2 in the gas phase. (b) Rates of citrate oxidation with varying $[HCO_3^-]$ and a constant pH (7.39). pH was held constant by varying $[HCO_3^-]$ and per cent CO_2 proportionately. Two flasks were used at each pH level.

the rates of utilization of pyruvate, malate, and citrate were compared in media with varying concentrations of bicarbonate, results similar to those shown in Table VI were obtained in three experiments. Citrate utilization decreased as pH and bicarbonate concentration increased; this decrease was similar in magnitude to that observed when citrate oxidation was measured by using radioactive citrate. Pyruvate and malate utilization, on the other hand, increased as pH and bicarbonate concentration increased.

TABLE V

Effect of variations in the concentrations of components of the HCO_3^--CO_2 buffer system on the rate (V) of citrate oxidation by renal cortical slices

Pair*	Medium $[HCO_3^-]$	Medium CO_2	Medium pH	V
	μmoles/ml	*per cent*		*mμmoles/min/200 mg*
A	20	5	7.29	15.5
	45	5	7.65	10.7
B	20	4	7.39	14.2
	45	9	7.39	13.7
C	36	9	7.29	15.8
	36	4	7.65	10.7

* In each of the lettered pairs of flasks one of the three components of the buffer system was held constant while the other two were varied.

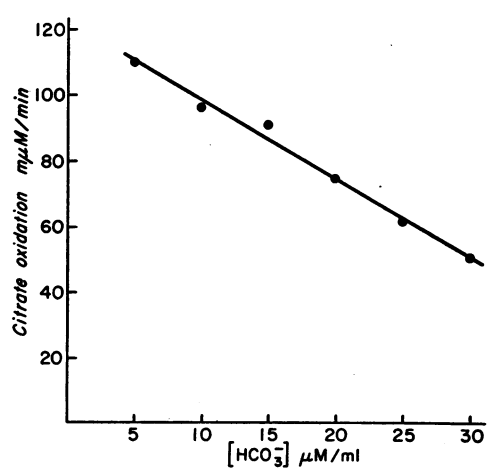

FIG. 6. RATES OF CITRATE OXIDATION IN KIDNEY MITOCHONDRIA WITH BICARBONATE BUFFERS WITH VARYING $[HCO_3^-]$ AND pH. Organic acid substrate in each milliliter consisted of 1.0 μmole K citrate, 2.0 μmoles K pyruvate, and 2.0 μmoles K malate.

TABLE VI

*Comparison of the effects of varying [HCO_3^-] and pH on the rates of utilization of citrate, malate, and pyruvate by kidney mitochondria**

[HCO_3^-]	Rate of substrate utilization		
	Citrate	Malate	Pyruvate
μmoles/ml		*mμmoles/min*	
5	38	60	47
10	34	70	57
15	30	74	53
20	24	83	66
30	12	84	65

* Media contained in each milliliter 1.0 μmole K citrate, 2.0 μmoles K pyruvate, and 2.0 μmoles K malate.

Kinetics of the inhibitory effect in mitochondria. In two experiments incubation of mitochondria in media containing varying amounts of citrate and either 10 or 20 μmoles HCO_3^- per ml resulted in a Lineweaver-Burk plot indicative of competitive inhibition (Figure 7). Since a pure enzyme system was not used to obtain these data, further analysis of the Michaelis-Menten characteristics of this plot does not seem worthwhile. However, demonstration of the competitive nature of this inhibitory effect is of value in characterizing the type of phenomenon being studied and for comparing the results obtained in tissue slices with those obtained in mitochondria.

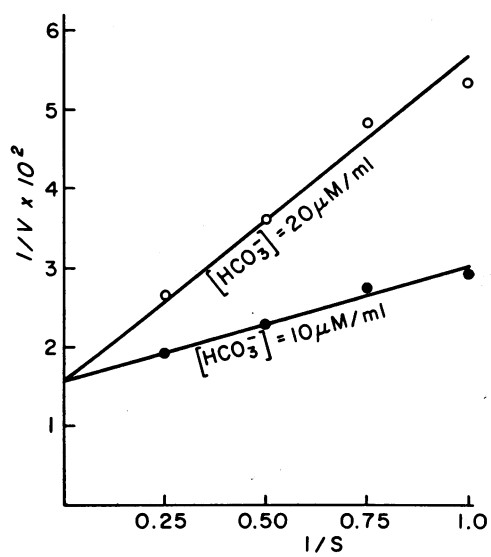

FIG. 7. LINEWEAVER-BURK PLOT OF THE RATES OF CITRATE OXIDATION IN KIDNEY MITOCHONDRIA WITH DIFFERENT CONCENTRATIONS OF CITRATE AND TWO DIFFERENT CONCENTRATIONS OF HCO_3^-. S = concentration of citrate in micromoles per milliliter. Organic acid substrate consisted only of citrate in the concentrations shown.

Citrate content of mitochondria after incubation with bicarbonate and phosphate buffers. The results of one of two experiments in which citrate content was determined after incubating mitochondria with pyruvate and malate are shown in Table VII. The bicarbonate-buffered media contained 10 μmoles phosphate buffer of the appropriate pH in each milliliter; the composition of the phosphate-buffered media was similar, except that KCl replaced the $KHCO_3$. In both experiments, as in similar experiments with slices, citrate content increased with increasing pH when mitochondria were incubated with a phosphate buffer. However, when bicarbonate was also present in the medium, the citrate content at each pH level was considerably increased, and the magnitude of the change in citrate content with increasing pH was enhanced by bicarbonate.

Effect of variations in the CO_2-bicarbonate buffer system on citrate oxidation. In the tissue slice experiments it was found that the inhibitory effect was related to the change in pH of the medium rather than to the change in [HCO_3^-] (Figure 5 and Table V). When similar experiments were repeated with mitochondria, the results were reversed. In this case, when pH was held constant and [HCO_3^-] and per cent CO_2 varied, citrate oxidation decreased with increasing [HCO_3^-] (Figure 8, a). In four experiments in which pH was held constant at 7.17 the mean slope of the curve between 10 and 30 μmoles HCO_3^- per ml was -1.88 ± 0.42. A similar result was obtained when the pH was maintained at 7.39 and [HCO_3^-]

TABLE VII

*Citrate content of kidney mitochondria incubated in phosphate-buffered media with or without bicarbonate**

pH	Without bicarbonate	With bicarbonate	
	Total citrate	[HCO_3^-]	Total citrate
	μmoles	*μmoles/ml*	*μmoles*
	0.190		0.383
6.97	0.193	10	0.363
	0.217		0.393
	0.330		0.707
7.46	0.330	30	0.713
	0.330		0.667

* Substrate in each milliliter consisted of 2.0 μmoles of pyruvate and of malate. Incubation time was 12 minutes. Gas phase was 100% O_2 in flasks containing media buffered with phosphate only and 5% CO_2–95% O_2 in those with bicarbonate.

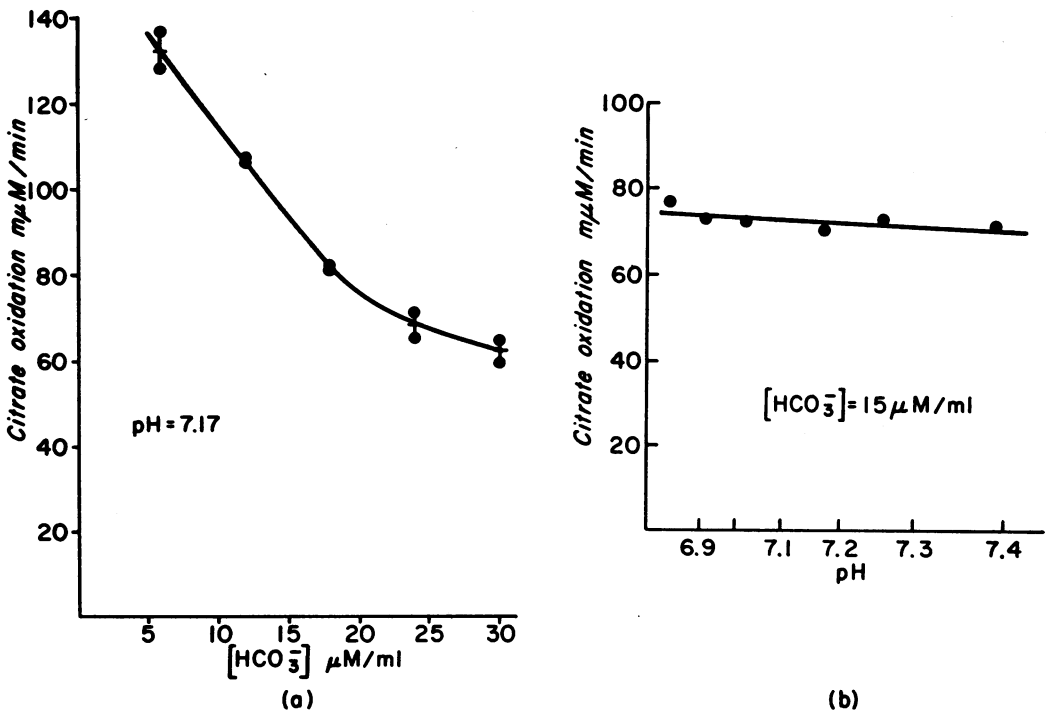

FIG. 8. THE EFFECTS OF INDEPENDENT VARIATIONS IN pH AND $[HCO_3^-]$ ON CITRATE OXIDATION IN KIDNEY MITOCHONDRIA. Substrate consisted of 1.0 μmole K citrate per ml. (a) Rates of citrate oxidation with varying $[HCO_3^-]$ and a constant pH (7.17). Two flasks were used at each pH level. (b) Rates of citrate oxidation with varying pH and fixed $[HCO_3^-]$ (15 μmoles per ml).

varied. When $[HCO_3^-]$ was held constant and pH varied by changing the per cent CO_2 in the gas phase, no significant inhibition of citrate oxidation occurred (Figure 8 b). The mean slope obtained in five experiments, three with 15 μmoles HCO_3^- per ml and two with 30 μmoles HCO_3^- per ml, was $+ 0.051 \pm 0.097$. This relationship was also demonstrated in a single experiment in which three pairs of flasks were used, with one parameter of the CO_2–bicarbonate buffer system held constant in each pair. The results of this experiment, shown in Table VIII, indicate no effect on citrate oxidation by a change in pH without change in $[HCO_3^-]$ (pair C), and considerable change in citrate oxidation with a change in $[HCO_3^-]$, whether pH changed (pair A) or was held constant (pair B). Thus, in mitochondria the inhibitory effect is related to the change in $[HCO_3^-]$ and not to the change in pH.

TABLE VIII

Effect of variations in the concentrations of components of the HCO_3^--CO_2 buffer system on the rate (V) of citrate oxidation by kidney mitochondria

Pair*	Medium			V
	$[HCO_3^-]$	CO_2	pH	
	μmoles/ml	*per cent*		*mμmoles/min*
A	10	5	6.99	60.0
	23.3	5	7.36	32.2
B	10	3	7.21	47.2
	23.3	7	7.21	28.9
C	14	7	6.99	43.7
	14	3	7.36	44.1

* In each of the lettered pairs of flasks one of the three components of the buffer system was held constant while the other two were varied.

Discussion

It was first noted in the early 1930's that the state of acid-base balance in an animal has an important influence on the excretion of citrate (14). The more important properties of this phenomenon, as revealed by many physiologic investigations since that time in man and other mammals, can be summarized as follows.

1) In animals on a normal diet the clearance of citrate is usually less than 3% of the glomerular filtration rate (13, 15). Within a few minutes after induction of metabolic alkalosis, citrate clearance increases to many times the previous nonalkalotic value (1, 3). At the same time little change in plasma citrate occurs. Consequently, the change in citrate excretion in metabolic alkalosis is due entirely to an alteration in the renal handling of citrate.

2) In metabolic alkalosis citrate concentration in kidney tissue increases (1). When other organs are examined, no comparable change in citrate concentration occurs (16). Also, the uptake and oxidation of extracellular citrate are unimpaired in organs other than the kidney (17). Thus, the effect of metabolic alkalosis on the metabolism of extracellular citrate appears to be limited to the kidney.

3) Metabolic alkalosis also increases the clearance of α-ketoglutarate, and the properties of this effect are similar to those described for citrate (1, 18). Comparable changes in pyruvate and malate clearances, however, have not been described (19, 20).

4) Alkalinization of the urine in the absence of systemic alkalosis, as produced by acetazolamide administration, for instance, does not increase citrate clearance (1, 21, 22). Induction of alkalosis in the presence of acetazolamide, however, leads to a prompt increase in citrate clearance (23). Therefore, citrate clearance is not altered by a change in the pH or bicarbonate concentration of the tubular fluid alone. In potassium deficiency, where an intracellular acidosis and an extracellular alkalosis coexist (24, 25), citrate clearance is not increased (19, 25, 26). During recovery from potassium deficiency, citrate excretion increases as the intracellular acidosis is corrected. These facts suggest that the alteration in citrate clearance by metabolic alkalosis is related to the presence of an intracellular alkalosis and not to concomitant changes in the acid-base balance of the extracellular fluid or urine.

5) The effect of metabolic alkalosis on citrate clearance appears to be due principally to inhibition of citrate reabsorption rather than to stimulation of citrate secretion. The evidence for this is based on an experiment by Grollman, Harrison, and Harrison (4). These investigators infused simultaneously bicarbonate and malate, which also increases citrate clearance substantially, in order to obtain a maximal citrate excretion. The two agents together resulted in a large increase in citrate clearance to levels that approximated but did not exceed the filtration rate of citrate. In addition to this evidence Gordon, using ^{14}C-labeled citrate and acetate, has obtained data which suggest that most of the urinary citrate in metabolic alkalosis is derived directly from plasma citrate (27). However, these results do not entirely eliminate the possibility that metabolic alkalosis, in addition to inhibiting the reabsorption of citrate, also stimulates the secretion of some citrate synthesized in the renal tubules. Indeed, Runeberg and Lotspeich have recently reported that infusion of ^{14}C-labeled succinate into one renal artery of an alkalotic dog results in the appearance in the urine of labeled citrate synthesized by the infused kidney (28).

The results summarized above indicate that the presence of metabolic alkalosis within the cells of the renal tubules interferes with the reabsorption of citrate from the tubular fluid in a specific manner. We undertook the present study to investigate this phenomenon in an *in vitro* system. As pointed out in the introduction, this approach is feasible because citrate is removed from both the tubular fluid and the peritubular fluid and is oxidized within the cells of the renal cortex. After demonstrating inhibition of citrate oxidation in slices of renal cortex under conditions comparable to those in metabolic alkalosis, we studied the properties of this *in vitro* effect in order to compare it with the effect of metabolic alkalosis in the intact animal. These experiments show that in tissue slices: *1*) increasing pH and bicarbonate concentration cause inhibition of citrate oxidation in kidney tissue but not in heart and liver when identical media are used for incubation of slices from the different organs; *2*) citrate content is considerably increased in slices of renal cortex after incubation in a medium with a high bicarbonate concentration; *3*) when pH and bicarbonate concentration increase, the utilization of α-ketoglutarate as well as of citrate is inhibited, but pyruvate and malate utilization are not diminished; *4*) the pH range, 7.0 to 7.8, in which this effect was studied is small and lies within the limits that can be produced in physiologic experiments in

healthy animals or human subjects; *5*) a bicarbonate buffer system is essential for demonstrating this effect. Thus, the *in vitro* effect has the same organ and substrate specificities and occurs in the same pH range as the *in vivo* effect. From these results we conclude that the inhibition of citrate oxidation by increasing pH and $[HCO_3^-]$ in slices of renal cortex is a reflection of the same underlying mechanism as that responsible for the inhibition of citrate reabsorption by metabolic alkalosis in the intact animal.

To locate the site of this effect more precisely, we used a mitochondrial system and carried out experiments similar to those performed with tissue slices. Inhibition of citrate oxidation by increasing pH and $[HCO_3^-]$ was shown at the mitochondrial levels. The following properties, similar to those found with tissue slices, were demonstrated. *1*) Inhibition of citrate oxidation occurs in the presence of increasing pH within the physiological range when a bicarbonate buffer is used and $[HCO_3^-]$ is varied, but not when a phosphate buffer is substituted. *2*) Pyruvate and malate utilization are not inhibited under these circumstances. *3*) This effect on citrate oxidation has the properties of a competitive type of inhibition. *4*) Total citrate content is increased when $[HCO_3^-]$ in the medium increases.

The results in tissue slices and mitochondria differ in one important aspect. In tissue slices the inhibitory effect is primarily related to the change in pH of the medium; the change in $[HCO_3^-]$ contributes only slightly. With mitochondria, however, an increasing concentration of bicarbonate ion is responsible for the inhibition of citrate oxidation; change in pH by itself does not alter the rate of mitochondrial citrate metabolism. Because both the tissue slice and mitochondrial systems require a bicarbonate buffer to demonstrate the inhibitory effect and because of the similarity of the other properties of the two systems, the same basic mechanism must be responsible for altering citrate metabolism in both systems. Thus, in slices of renal cortex the fundamental influence on citrate oxidation is the effect of intracellular $[HCO_3^-]$ on mitochondrial metabolism. The differences in the effects of independent changes in pH and $[HCO_3^-]$ on citrate oxidation in slices and mitochondria suggest an interesting relationship between hydrogen ion concentration and intracellular bicarbonate. The results in mitochondria suggest that, when conditions of acid-base balance are varied in an experiment, a decrease or increase in the rate of citrate oxidation implies a corresponding increase or decrease, respectively, in intracellular $[HCO_3^-]$. If this conclusion is correct, then the results obtained in tissue slices when pH and $[HCO_3^-]$ are varied independently can be interpreted in the following manner. When the pH of the medium is increased without change in $[HCO_3^-]$ citrate oxidation in slices is inhibited, implying that a change in intracellular $[HCO_3^-]$ has occurred without any change in extracellular $[HCO_3^-]$. Conversely the results obtained when the $[HCO_3^-]$ in the medium is varied without change in pH suggest that no change in intracellular $[HCO_3^-]$ has occurred under these conditions. Thus, intracellular $[HCO_3^-]$ would appear to change only if the pH of the medium is altered. An understanding of how pH changes could regulate intracellular $[HCO_3^-]$ in this manner must await the development of more sophisticated techniques for measuring the transport and intracellular concentration of hydrogen and bicarbonate ions.

The present study indicates that citrate clearance is increased in metabolic alkalosis because of an intracellular inhibitory effect of bicarbonate ion on citrate metabolism. Thus, under these circumstances, the rate of citrate excretion is regulated by the rate of intracellular oxidation of this substance in the cells of the nephron. Two hypotheses have been advanced previously to explain the inhibition of citrate reabsorption by metabolic alkalosis. Grollman and co-workers (4) speculated that alkalosis interferes with citrate transport across the cell membrane. Crawford, Milne, and Scribner (1), on the other hand, believed the effect was due to inhibition of the intracellular oxidation of citrate. These authors noted that the activity of condensing enzyme was markedly increased by increasing pH (29). They suggested that in metabolic alkalosis more citrate is synthesized in cells of the renal tubules due to the enhanced activity of this enzyme. As a result, the intracellular concentration of citrate increases, and the rate of entry of extracellular citrate into the cell diminishes. The results of the present study suggest that Crawford and associates were correct in relating the effect of metabolic alkalosis on

citrate clearance to intracellular inhibition of citrate oxidation rather than to an effect on the membrane transport of citrate. However, since their hypothesis does not provide a role for bicarbonate ion in the cause of this phenomenon, it is not an adequate explanation of the inhibitory mechanism.

Further investigation is necessary to provide insight into the biochemical mechanism by which bicarbonate influences the oxidation of extramitochondrial citrate. Few effects on metabolism are known to be caused by bicarbonate as an entity distinct from CO_2. Bicarbonate may participate in some types of "CO_2 fixation," but in general the molecular species involved in reactions concerning CO_2 incorporation are undefined. Measurement of the extent of CO_2 fixation in renal cortex, as well as definitive information on the molecular species involved, will be necessary before conclusions can be drawn on its relationship to the phenomenon under investigation. In attempting to find a biochemical explanation for the effect of bicarbonate on citrate oxidation, two points established in the present experiments are worth emphasizing. First, the inhibition of citrate oxidation is demonstrable in the absence of any substrate from the medium other than citrate itself. Second, this effect has the characteristics of competitive inhibition, suggesting that bicarbonate stimulates the entry of some substance into the Krebs cycle that competes with extramitochondrial citrate for entry into the cycle. These points may be helpful in formulating further experiments to define the cause of this effect.

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