# pH and Bicarbonate Effects on Mitochondrial Anion Accumulation

## PROPOSED MECHANISM FOR CHANGES IN RENAL METABOLITE LEVELS IN ACUTE ACID-BASE DISTURBANCES

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ABSTRACT Mitochondria from rabbit and dog renal cortex were incubated with 1 mM $^{14}$C-weak acid anions in media containing low (10 mM) or high (40 mM) concentrations of bicarbonate and the steady-state accumulation of labeled anion in the matrix was measured. In the absence of an energy source, no concentration of $^{14}$C-anion in the mitochondrial matrix space was present, but the anion concentration was significantly higher at low- than at high-bicarbonate concentration. Addition of an energy source, usually ascorbate plus tetramethyl-*p*-phenylenediamine, led to increases in matrix space anion levels and to accentuation of the difference in anion uptake between low- and high-bicarbonate media, so that two to four times as much anion was present at low- than at high-bicarbonate concentrations. The anions affected included substrates for which inner membrane carriers are present in mitochondria, such as citrate, α-ketoglutarate, malate, and glutamate, as well as substances which diffuse passively across the inner membrane such as acetate and formate. When a nonbicarbonate medium buffered with Hepes was used, pH change did not alter anion uptake although anion concentrations exceeding those in the medium still developed when an energy source was present. The difference in mitochondrial anion accumulation between low- and high-bicarbonate levels diminished with decreasing temperature or with increasing anion concentration in the medium. Estimation of intramitochondrial pH with [$^{14}$C]5,5-dimethyloxazolidine-2,4-dione showed that the pH gradient across the inner mitochondrial membrane was significantly greater with 10 than with 40 mM bicarbonate in the medium.

A hypothesis is described that relates this effect of pH and bicarbonate on mitochondrial anion accumulation to the very rapid changes in substrate levels in renal cortex, which develop when acute metabolic acidosis or alkalosis is produced in the intact animal. It is suggested that an abrupt fall in systemic pH and bicarbonate is associated with a shift in substrate in renal cortex out of the cytoplasm and into mitochondria, where some of the added substrate is metabolized. Reduction in the size of the cytoplasmic pool of substrate occurs with relatively little accompanying change in the size of the mitochondrial pool, thus causing a net reduction in the total tissue pool. This mechanism accounts for the reduction in tissue levels of many mitochondrial substrates observed acutely in metabolic acidosis. In metabolic alkalosis, reversal of these effects leads to expansion of the cytoplasmic pool, thereby resulting in the rise in tissue levels of substrates which occurs in this condition.

## INTRODUCTION

Changes in systemic acid-base balance provide one of the major mechanisms responsible for the physiologic regulation of renal metabolism and serve to adjust homeostatic functions of the kidney such as ammonia production. Among the well-recognized responses of the kidney to variations in acid-base balance are fluctuations in the concentrations of metabolic intermediates in renal cortex. In metabolic acidosis of a few hours to several days duration, decreases in the concentrations of citrate, malate, α-ketoglutarate, and glutamate occur (1–5). In contrast, metabolic alkalosis leads to changes in the opposite direction so that increases in substrate levels develop in renal cortex

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(6, 7). These distinct alterations in response to acid-base changes have played an important role in formulating hypotheses to explain regulation of renal ammoniagenesis and its relationship to gluconeogenesis (8).

One feature of this phenomenon that has not received wide recognition is the rapidity with which changes in substrate levels occur in the kidney when a systemic acidosis or alkalosis is induced. Within a few minutes after the administration of hydrochloric acid or sodium bicarbonate, marked decreases or increases, respectively, are detectable in the concentrations of mitochondrial intermediates in the renal cortex (9).[1] The cause of these very rapid alterations in substrate levels is unknown. The experiments described in this paper define changes in anion accumulation by isolated mitochondria from renal cortex, which are produced in response to variations in pH and bicarbonate concentration in the incubation medium. These observations are an extension of earlier work in which high pH and bicarbonate concentrations were shown to inhibit citrate uptake by rabbit kidney mitochondria, an effect we suggested was responsible for the increase in citrate clearance in metabolic alkalosis (10). In this paper we propose the hypothesis that this newly described effect of pH and bicarbonate on accumulation of weak acid anions by mitochondria is the underlying mechanism responsible for the very rapid alterations in substrate levels in renal cortex in response to acid-base manipulations.

## METHODS

The general methods used in these experiments have been previously described in detail in papers from this laboratory (10, 11). Mitochondria were prepared from rabbit or dog renal cortex by differential centrifugation in isotonic sucrose solution buffered with Hepes. 0.1 ml of the final mitochondrial suspension, containing 20–30 mg protein/ml, was added to a sealed flask containing 0.9 ml of incubation medium. The flask had been previously gassed with 95% $O_2$-5% $CO_2$ (or 100% $O_2$ when a nonbicarbonate-containing medium was used) and was warmed to 37°C. After the gassing, potentially volatile substrates such as formate were added to the medium by injection through the serum stopper before adding the mitochondria.

Incubation was carried out for 2 min at 37°C. Unless otherwise noted the incubation medium contained 117 mM KCl plus $KHCO_3$, 5 mM Hepes of appropriate pH for the bicarbonate concentration, 5 mM $MgSO_4$, 30 mM sucrose (added with the mitochondrial suspension), 1 μg/ml rotenone, 3.7 mM ascorbate, 0.3 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD),[2] 1 mM $^{14}C$ substrate, and [$^3H$]sucrose or -water. When substrate concentrations were varied appropriate adjustments in KCl concentration were made to keep the osmolality of the medium constant.

At the end of incubation 200 μl of medium was transferred to a microcentrifuge tube containing a layer of silicone oil over 100 μl of 1 N $HClO_4$. The mitochondria were rapidly separated from the suspending medium by centrifugation in a microcentrifuge. 50 μl of the perchloric acid extract of the mitochondria was added to a vial containing 11 ml of a liquid scintillation cocktail (Instagel, Packard Instrument Co., Inc., Downers Grove, Ill.), and the $^{14}C$ and $^3H$ content was determined in a three-channel liquid scintillation spectrometer. Counting efficiency was measured by the automatic external standards ratios method. A separate sample of the mitochondrial incubation solution from each flask was added directly to perchloric acid for measurement of total $^{14}C$ and $^3H$ disintegrations per minute in the medium.

In each experiment separate flasks were prepared containing incubation medium identical to that of the other flasks, except that unlabeled substrate was used and [$^{14}C$]-sucrose plus [$^3H$]water were present. The results from these flasks, which were incubated and handled in the same way as the other flasks, enabled measurement of the sucrose (outer) and total water spaces of the mitochondrial preparation. The proportion of the matrix to total water space was then calculated. This information together with the volume of the $^3H$ space measured in each flask enabled determination of the individual matrix space of the flask. From the $^{14}C$ content of the mitochondrial extract, the volume of the outer and matrix spaces, and the specific activity of the incubation medium, the concentration of labeled substrate in the matrix space was calculated.

In some experiments intramitochondrial pH was determined from the distribution of [$^{14}C$]5,5-dimethyloxazolidine-2,4-dione ([$^{14}C$]DMO) (12). [$^{14}C$]DMO accumulation by mitochondria was determined in the same manner as described for measurement of uptake of labeled substrates. By using a Beckman Expandomatic pH meter (Beckman Instruments, Inc., Fullerton, Calif.) medium pH was continuously measured and recorded in separate flasks under conditions identical to those in which [$^{14}C$]DMO measurements were made. The medium pH after 2 min of incubation was used to calculate intramitochondrial pH.

To check on the integrity of the mitochondria, measurements of respiratory control ratios were performed on each preparation with succinate and citrate as substrates; all preparations used showed an appropriate abrupt transition between respiratory states III and IV. Protein was determined by the biuret method. Mean results are reported ±SE. The unpaired *t* test was used to determine the significance of mean differences. The term "significant" in this paper indicates a difference with a $P$ value of $<0.01$ unless noted otherwise. "Nonsignificant" refers to a $P$ value of $>0.20$.

All radioisotopes were obtained from New England Nuclear, Boston, Mass. Unlabeled substrates were products of Sigma Chemical Co., St. Louis, Mo. or Calbiochem-Behring Corp., San Diego, Calif.

## RESULTS

*General effect of pH and $HCO_3^-$ on mitochondrial substrate accumulation.* In our previous study (10) in which citrate accumulation by mitochondria was noted to be inhibited by high pH and bicarbonate concentration, a medium was used which contained phosphate, ADP, and succinate. In subsequent experiments we found that the effect on citrate could be shown in a simpler medium containing only an energy source and a counter-ion (malate). An extensive series

[1] Simpson, D. P., and R. Richards. Unpublished observations.

[2] *Abbreviations used in this paper*: DMO, 5,5-dimethyloxazolidine-2,4-dione; PEP, phospho-enol-pyruvate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

TABLE I

*Representative Effects of High- and Low-Bicarbonate Concentrations on Matrix Accumulation of Various Anions by Mitochondria from Renal Cortex*

| | [$^{14}$C Substrate]$_{matrix}$ | | | | | |
|---|---|---|---|---|---|---|
| Species | Rabbit | | | | Dog | |
| Experiment | A | | B | | | |
| Medium [$HCO_3^-$], *mM* | 10 | 40 | 10 | 40 | 10 | 40 |
| | *mM* | | | | | |
| $^{14}$C Substrate: | | | | | | |
| Citrate* | 7.13 | 2.12 | 8.30 | 2.22 | 8.17 | 2.39 |
| L-Malate | 5.82 | 1.92 | 3.28 | 1.09 | 5.20 | 2.85 |
| α-Ketoglutarate* | 5.90 | 3.67 | 6.01 | 3.47 | 5.45 | 2.67 |
| Pyruvate | 4.56 | 2.90 | 6.29 | 2.92 | 5.40 | 3.60 |
| Glutamate | 2.41 | 1.48 | 2.17 | 1.71 | 3.90 | 2.60 |
| PEP | 3.13 | 2.07 | 3.85 | 2.20 | – | – |
| Acetate | 5.05 | 2.56 | 2.19 | 1.43 | 3.04 | 2.21 |
| β-Hydroxybutyrate | 3.28 | 1.84 | 3.69 | 1.86 | – | – |

Values represent mean of results from two flasks with identical medium. Medium also contained 0.3 mM TMPD, 3.7 mM ascorbate, and 1 μg/ml rotenone in each case. $^{14}$C Substrates were present at 1 mM concentration.

* 1 mM malate also present in medium.

of experiments was undertaken to test the effect of pH and bicarbonate concentration on the accumulation of other mitochondrial substrates. For each of a group of labeled substrates separate experiments were carried out in which mitochondria were incubated in media containing low (10 mM) or high (40 mM) concentrations of bicarbonate and labeled substrate only or labeled substrate plus an energy source (TMPD and ascorbate) or labeled substrate, an energy source, and malate; duplicate flasks were used for each experimental condition studied. The general type of result obtained is described in more detail in the next section, *Effects on formate accumulation.*

Table I shows representative results of such experiments for a variety of substrates incubated in media containing TMPD and ascorbate. In each case the concentration of substrate in the matrix space was distinctly higher when the mitochondria were incubated with 10 mM bicarbonate in the medium, compared with 40 mM. The magnitude of this effect was greatest with citrate, where the difference in concentration between low- and high-bicarbonate media was about threefold, but it was evident for each anion tested. Similar differences were noted with dog mitochondria. (The remainder of the experiments reported in this paper were performed on rabbit kidney cortex mitochondria.) The range of compounds affected includes both compounds which have specific carriers in the inner mitochondrial membrane, such as citrate and malate, and compounds such as acetate which are believed to enter mitochondria passively (13, 14). The presence of malate in the medium was not necessary to demonstrate the effect of pH and bicarbonate except in the cases of citrate and α-ketoglutarate (*vide infra*). Even at the high-bicarbonate level, each substrate was concentrated in the matrix space to levels considerably exceeding the 1-mM concentration in the medium.

The intramitochondrial extracts were not analyzed to identify the labeled compounds present. In a few cases a significant amount of the total $^{14}$C in the matrix may have been in a form other than that of the added substrate. For example, some (probably <20%) of the malate may have been converted by fumarase to fumarate. Because of this process the total amount of malate in the matrix may have been somewhat less than that indicated in Table I. However, the measured differences in $^{14}$C accumulation would still provide an accurate reflection of relative effects of low- and high-bicarbonate concentration on malate uptake. The amount of rotenone in the medium was sufficient to inhibit oxidative metabolism almost completely (15), and thus was also sufficient to block conversion of any substrate by NAD-requiring steps. Therefore, for most of the substrates used, the label in the matrix space must have remained almost entirely in the original $^{14}$C substrate present in the medium.

*Effects on formate accumulation.* The preceding type of experiment demonstrated that the effect of pH and bicarbonate on anion accumulation is quite nonspecific. Further evidence on the characteristics of this effect was obtained with formate, the simplest organic weak acid anion and one which has negligible intramitochondrial metabolism. Fig. 1 shows the results of five experiments similar to those summarized

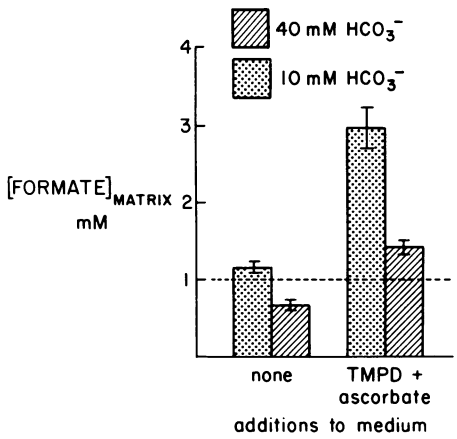


FIGURE 1 Accumulation of 1 mM [$^{14}$C]formate in mitochondrial matrix space with different concentrations of $HCO_3^-$ in the medium ($n$ = 5). When indicated, 0.3 mM TMPD and 3.7 mM ascorbate were present in the medium.

in the preceding section, except that rotenone was omitted from the media. When [$^{14}$C]formate was incubated in the basic medium alone, the matrix concentration of formate did not exceed that in the medium; a significant difference between the formate concentration found with 10 and 40 mM $HCO_3^-$ was still evident however, with matrix space levels below those in the medium when high bicarbonate was used. In the presence of TMPD and ascorbate, the levels of formate in the matrix space were increased to three times the medium concentration in low-bicarbonate medium and the difference between low- and high-bicarbonate media was expanded to over twofold.

When the experiments described in the preceding paragraph were done in the presence of rotenone, similar results were obtained (16). With TMPD and ascorbate in the medium, [$^{14}$C]formate concentration in the matrix was 3.87±0.47 and 1.49±0.07 mM at 10- and 40-mM bicarbonate concentrations, respectively. Without TMPD and ascorbate, the respective concentrations were 0.91±0.03 and 0.60±0.03 mM. These results are not significantly different from those obtained in the absence of rotenone. Addition of 1 mM potassium cyanide abolished the effect of TMPD and ascorbate on [$^{14}$C]formate uptake, indicating that the effect of these substances is related to the energy supplied by their metabolism.

Previously, we demonstrated that development of the marked pH-dependent differences in mitochondrial citrate uptake required a bicarbonate buffer (10). This result was also confirmed for [$^{14}$C]formate accumulation. With a buffer system containing Hepes only, labeled formate concentration in the matrix space at pH 7.1 was 3.5 mM and at pH 7.6 was 3.8 mM in one experiment; in another experiment the values were 4.2 and 4.9 mM at low- and high-medium pH (16).

Further studies on [$^{14}$C]formate accumulation were carried out to evaluate the effect of stepwise variation in bicarbonate concentration. Fig. 2 shows that throughout the range of 5–40 mM $HCO_3^-$, as bicarbonate concentration decreased in increments, each change was accompanied by an increase in formate uptake. Similar results to those shown in Fig. 2 were previously reported with citrate instead of formate (10).

*Role of counter-ion in citrate, α-ketoglutarate, and malate accumulation.* The mitochondrial carriers for citrate and α-ketoglutarate involve the exchange of these substances for malate (13, 14). Therefore, the role of malate in influencing the effect of pH and bicarbonate on the uptake of citrate and α-ketoglutarate was examined. Fig. 3 shows studies on α-ketoglutarate accumulation in media with various combinations of this substrate, malate, and TMPD and ascorbate. Unlike other substrates studied, no significant effect of pH and bicarbonate was present when the medium contained only TMPD and ascorbate without malate. However, the matrix space concentration of α-ketoglutarate was several times that in the medium, demonstrating transport of this compound into the mitochondria in the absence of an added counter-ion; whether or not this transport required endogenous malate was not investigated. Addition of malate to the system containing an energy source resulted in the appearance of a significant difference between high-

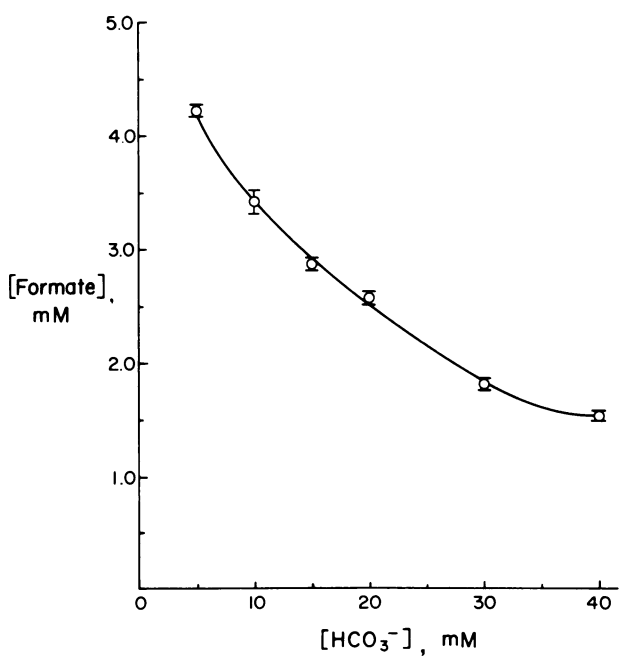


FIGURE 2 Effect of incremental changes in bicarbonate concentration on [$^{14}$C]formate accumulation. 1 mM [$^{14}$C]formate, TMPD, ascorbate, and rotenone were present in each medium. Gas phase was 5% $CO_2$-95% $O_2$ in each flask. Mean values for four flasks are shown.

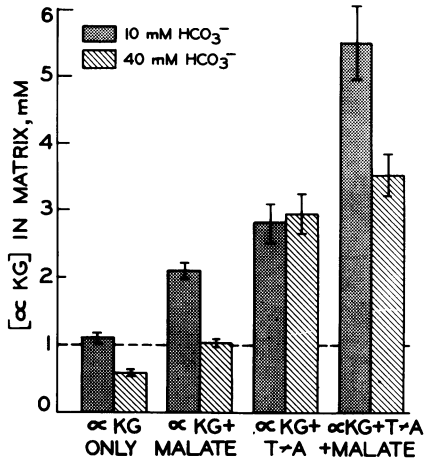


FIGURE 3 Influence of a counter-ion (malate) and energy source on accumulation of 1 mM [$^{14}$C]α-ketoglutarate (α KG) by mitochondria incubated with high- or low-bicarbonate concentrations ($n$ = 4). T + A indicates presence of 0.3 mM TMPD and 3.7 mM ascorbate in medium.

and low-bicarbonate levels and in the development of an almost twofold greater concentration of α-ketoglutarate in the mitochondria incubated with 10 mM bicarbonate.

Similar experiments with citrate (Fig. 4) show an even greater importance of the presence of a counter-ion. In the absence of malate, only a twofold accumulation of citrate occurred at low-bicarbonate concentration and none developed in the matrix space at high-bicarbonate levels. When malate was added, citrate concentration in the matrix with low bicarbonate rose to over six times that present in the medium but less

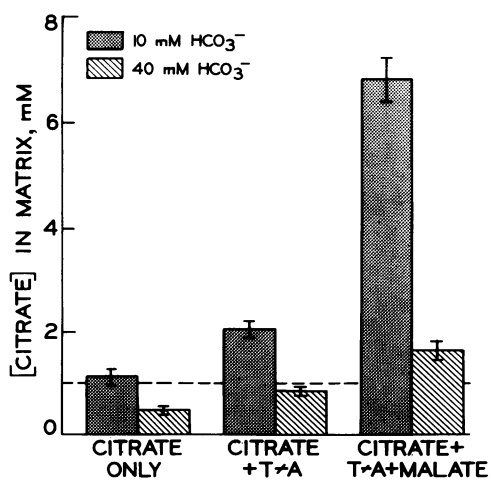


FIGURE 4 Influence of malate and TMPD plus ascorbate (T + A) on the accumulation of 1 mM [$^{14}$C]citrate in mitochondria incubated at high- or low-bicarbonate concentrations in the medium ($n$ = 4).

than a twofold rise occurred in high-bicarbonate medium. Table II demonstrates the interactions of citrate and malate on the accumulation of the other substrate at high-and low-bicarbonate concentrations. When both substrates were present at equal concentrations, the matrix concentration of each was higher at low bicarbonate than at high. Omitting citrate from the medium resulted in an increase in the matrix concentration of malate in the 10-mM bicarbonate medium, enhancing the difference between low- and high-bicarbonate concentration, indicating the importance of this compound as a counter-ion for citrate transport.

*Effect of high- and low-bicarbonate media on phosphate uptake.* Phosphate is an important anion in metabolism and has a specific mitochondrial transporter. It also acts as a counter-ion for the malate transporter (although, as described above, addition of phosphate to the medium was not necessary for demonstration of the effect of pH and bicarbonate on malate uptake). The influence of pH and bicarbonate on phosphate uptake by mitocondria was studied in the same system used to study effects on organic anion accumulation with 1 mM [$^{33}$P]phosphate in the medium. In the absence of TMPD and ascorbate, the matrix phosphate concentration was 7.8±0.6 mM with 10 mM, and 6.9±0.4 mM with 40 mM $HCO_3^-$ medium. When TMPD and ascorbate were added, these results were 19.3±0.67 and 15.3±0.67 mM, respectively, in three experiments. While these differences between high- and low-bicarbonate media are significant, the magnitude of the difference in the presence of an energy supply is less than that observed with the organic anions (Table I).

*Temperature dependence.* Fig. 5 shows the results of an experiment in which citrate uptake was measured at high and low concentrations of bicarbonate in the medium while the temperature was varied. At 0°C no concentration gradient for citrate was present across the mitochondrial membrane either at high- or low-

TABLE II
*Matrix Accumulation of Labeled Citrate or Malate in Presence of Unlabeled Counter-Ion*

| | [$^{14}$C Substrate]$_{matrix}$ | | |
|---|---|---|---|
| $^{14}$C Substrate | Citrate | Malate | Malate |
| Unlabeled substrate | Malate | Citrate | — |
| [$HCO_3^-$]Medium: | | *mM* | |
| 10 mM | 9.28 | 3.56 | 5.82 |
| 40 mM | 2.48 | 2.02 | 1.92 |

Both labeled and unlabeled substrates were present at 1 mM concentration. TMPD and ascorbate were also present in the media. Values represent mean of results from two flasks with identical medium.

bicarbonate concentration. As the temperature rose, a concentration gradient for citrate developed and increased steadily to >8 mM at 37°C in 10 mM bicarbonate medium; however in 40 mM bicarbonate, medium levels of only 2–3 mM occurred.

*Concentration dependence.* The preceding experiments were all carried out with 1 mM $^{14}C$ substrate in the medium. Fig. 6 shows the effect of increasing substrate concentrations on accumulation of citrate and formate. As substrate concentration increased, the magnitude of the difference in matrix concentration between low- and high-bicarbonate levels declined progressively. At 1 mM concentration, a two- to fourfold higher matrix level of citrate or formate was observed with 10 mM than with 40 mM $HCO_3^-$ in the medium; with 20 mM concentrations of these compounds, the matrix concentrations were increased at the low-bicarbonate level by only 10–30% above those found with high bicarbonate.

*Energy dependence.* The preceding studies showed that an energy source is necessary to produce high levels of anion in the matrix space and to augment the difference in anion accumulation between media containing low or high concentrations of bicarbonate. Because energy production from succinate is not blocked by rotenone, this substrate can substitute for TMPD and ascorbate. In our earlier study of effects of pH and bicarbonate on citrate uptake (10), succinate was used because it not only provides an energy source but also serves as a counter-ion for citrate transport. Table III provides more evidence on the energy dependence of this phenomenon. Formate accumulation increased with increasing amounts of succinate or of TMPD and ascorbate in the medium. The latter compounds were more effective than succinate in enhancing formate accumulation and increases in their concentration were associated with a steady rise in formate levels in the matrix.

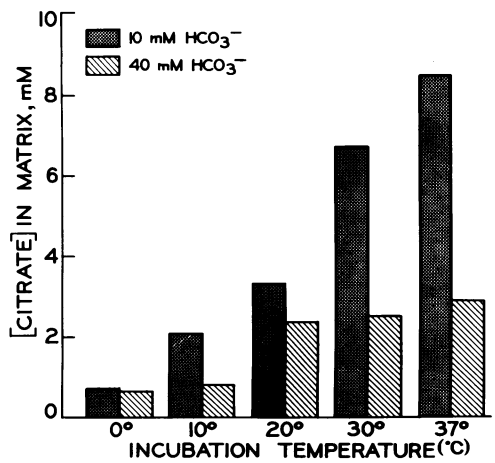


FIGURE 5 Effect of temperature on the accumulation of 1 mM [$^{14}C$]citrate incubated with high or low concentrations of bicarbonate in the medium. Media also contained 0.5 mM succinate and 2 mM potassium phosphate buffer.

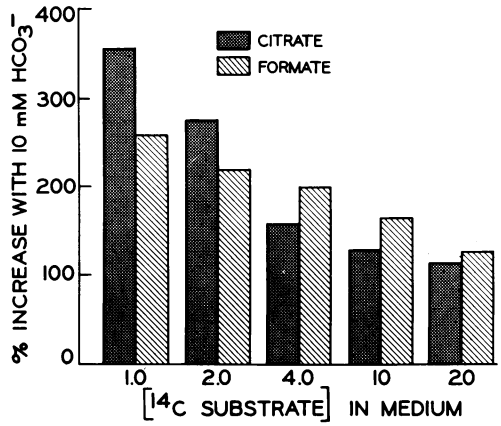


FIGURE 6 Effect of variation in substrate concentration on the increased mitochondrial anion accumulation present at low bicarbonate concentration. The ordinate represents the percentage increase in matrix substrate concentration present with 10 mM, compared with 40 mM $HCO_3^-$ in the medium. All media contained 1 mM $^{14}C$ substrate and TMPD and ascorbate. The medium used for the citrate incubations also contained 1 mM malate.

*Intramitochondrial pH.* Estimation of intramitochondrial pH was made from the distribution of [$^{14}C$]DMO between medium and mitochondria (12). The results shown in Table IV indicate that the pH inside mitochondria was considerably higher than in the medium at both low- and high-bicarbonate concentrations. This intramitochondrial alkalinity is similar to that reported previously for rat liver mitochondria

TABLE III

*Effect of Variation in Energy Supply on Mitochondrial Formate Accumulation at Low- and High-Bicarbonate Concentrations in the Medium*

| | $[Formate]_{matrix}$ | | | |
|---|---|---|---|---|
| | Experiment 1 | | Experiment 2 | |
| Medium $[HCO_3^-]$, *mM* | 10 | 40 | 10 | 40 |
| | *mM* | | | |
| Added energy source: | | | | |
| None | 1.01 | 0.68 | 1.03 | 0.68 |
| Succinate, 0.5 mM | 1.64 | 0.89 | 1.50 | 0.89 |
| Succinate, 1.0 mM | 1.91 | 1.22 | 2.05 | 1.14 |
| Succinate, 5.0 mM | 2.68 | 1.45 | 2.40 | 1.37 |
| Ascorbate, 0.4 mM + TMPD, 0.05 mM | 1.77 | 1.08 | 1.60 | 1.18 |
| Ascorbate, 1.8 mM + TMPD, 0.15 mM | 2.94 | 1.59 | 2.43 | 1.34 |
| Ascorbate, 3.7 mM + TMPD, 0.3 mM | 4.87 | 1.76 | 3.69 | 1.61 |

1 mM [$^{14}C$]formate was present in each medium. Values represent mean of determinations in two duplicate flasks.

TABLE IV
*Intramitochondrial pH Gradients at Low- and High-Medium pH and Bicarbonate Levels*

| $[HCO_3^-]$ | $pH_M$ | $pH_I$ | ΔpH |
|---|---|---|---|
| *mM* | | | |
| 10 | 7.24±0.01 | 7.84±0.02 | 0.60±0.02 |
| 40 | 7.78±0.01 | 8.05±0.02 | 0.27±0.02 |
| | | | $P < 0.001$ |

$n = 5$.
$pH_M$, medium pH; $pH_I$, intramitochondrial pH calculated from distribution of [$^{14}$C]DMO. Medium contained rotenone, TMPD, and ascorbate.

incubated in the presence of an energy source with nonbicarbonate buffers (17). In addition, the pH gradient across the inner membrane was significantly greater, by about 0.3 pH U, at low- than at high- pH and bicarbonate levels.

## DISCUSSION

*Effect of pH and bicarbonate in isolated mitochondria.* These studies have defined a general mitochondrial property by which changes in pH and $HCO_3^-$ concentration alter the concentration of weak acid anions in the matrix space, with low pH and $HCO_3^-$ being associated with high anion levels and the converse being seen with high pH and $HCO_3^-$. The presence of an energy source markedly accentuates the difference in anion accumulation between high- and low-bicarbonate levels as well as increasing matrix space anion uptake to levels considerably in excess of that in the medium. The magnitude of this effect is also affected by the temperature of the medium, by the concentration of the anion, and, in the case of compounds with specific mitochondrial carriers, by the presence of counter-ions for transport.

The matrix space levels obtained at 2 min with the silicone oil technique represent steady-state levels so that the rate of anion exit must equal the rate of anion entry. Thus, the differences in matrix space levels found in these experiments suggest that at low pH and bicarbonate the rate of anion entry into mitochondria is accelerated and(or) the rate of exit is reduced compared to the rates present at high pH and bicarbonate. Direct evidence for stimulation of anion exit by increasing bicarbonate concentration in the medium is available from a recent study by Robinson et al. (18), in which the rate of labeled citrate exit from mitochondria was shown to be inversely related to the bicarbonate concentration of the medium.

Previous studies on mitochondrial anion uptake suggest that the pH gradient across the inner mitochondrial membrane is a major determinant of anion movement across the inner membrane (14, 19, 20). The results obtained with [$^{14}$C]DMO (Table IV) suggest that a difference in pH gradient across the inner mitochondrial membrane may be responsible in large part for the differences in anion accumulation at high- and low-medium pH and bicarbonate levels. Bicarbonate appears to play a critical role in altering the magnitude of this gradient in response to pH changes, because changes in pH in bicarbonate-free media did not influence anion accumulation within the narrow physiologic pH range studied (10, 16). Also, we previously found that with bicarbonate-buffered media, changes in both pH and bicarbonate were necessary to demonstrate maximum difference in citrate uptake, but change in medium bicarbonate alone without change in pH had a partial effect (10).

Demonstration of an effect in isolated mitochondria raises the question of whether or not the effect occurs in an intact organ in vivo. The requirement of energy production for the maximum effect of pH and bicarbonate on anion accumulation would be met in vivo. The use of rotenone in the medium in most of these studies is necessary to distinguish effects on substrate transport from ones on metabolism. However, in the absence of rotenone the effects of pH and bicarbonate are readily demonstrable in the cases both of formate (Fig. 1), which is not metabolized, and of citrate (10). The magnitude of the effect increases with decreasing substrate concentration so that the low tissue levels of most substrates in vivo would be appropriate for the occurrence of the effect in the intact organ. The range of bicarbonate concentrations used in the medium embraces those present in the intact animal in the blood and presumably in cells as well. Studies on isolated kidney tubules suggest that metabolic acid-base disturbances in renal cortex are associated with comparable intracellular changes in acid-base state (21). These considerations indicate that the conditions in the intact animal are suitable for the effect of pH and bicarbonate to be expressed in mitochondria within cells of renal cortex.

*Proposed role of phenomenon in acid-base-induced changes in renal substrate levels.* Nagata and Rasmussen (9) found that in parathyroidectomized rats alteration in acid-base state by infusion of hydrochloric acid or sodium bicarbonate caused changes within 5 min in the levels of citrate, isocitrate, α-ketoglutarate, malate, and pyruvate; levels of phospho-enol-pyruvate (PEP) were unchanged. Studies from our laboratory[3] have confirmed these findings in nonparathyroidectomized animals. Typically the results of these studies show a decrease in α-ketoglutarate levels of 40% in metabolic acidosis and an increase of almost 100% in metabolic

[3] Simpson, D. P., and R. Richards. Unpublished observations.

alkalosis; citrate levels diminish by about 35% in acute acidosis and rise by over 50% in alkalosis. Parallel but smaller effects occur in malate, isocitrate, and pyruvate levels. A small (15%) but significant decrease in glutamate levels is evident in 5 min in acidosis with no increase in alkalosis. The lack of change in the levels of PEP in very acute acid-base disturbances makes it unlikely that the changes in renal substrate levels at this early stage are the result of altered activity of PEP carboxykinase, an explanation that has been invoked to account for reduction in substrate levels later in acidosis when increased activity of this enzyme develops (22, 23).

We propose that the effect of pH and bicarbonate on anion accumulation in isolated mitochondria acts at the cellular level to produce the observed changes in acute metabolic acidosis and alkalosis. In metabolic acidosis, for example, acute decrease in intracellular pH and bicarbonate alters the steady-state balance between mitochondrial substrate influx and efflux by increasing the rate of entry (Fig. 7). This shift in balance reduces the size of the cytoplasmic pool of substrate and increases that inside mitochondria. The increase in the concentration of substrate inside mitochondria leads to a transient increase in the rate of metabolism of that substrate, reducing the size of the mitochondrial pool towards the initial level. As the concentration of substrate in the cytoplasmic pool diminishes, the net rate of influx into the mitochondria will decrease. At some point a new steady-state will be established. Compared to the initial condition, the new steady-state will be characterized by a smaller cytoplasmic pool of substrate, a normal (or slightly increased) mitochondrial pool, and thus a reduced total substrate pool in the cell. Net rate of transport into and out of mitochondria will be reduced; the rate of efflux from mitochondria will be slowed by the effect of decrease in pH and bicarbonate on this rate; the intrinsic rate of influx into mitochondria will be increased because of this same effect, but the net rate of influx will fall as the cytoplasmic pool shrinks until the influx rate equals that of the efflux. At this point also the rate of mitochondrial metabolism of substrate will have returned toward that which was initially present. In metabolic alkalosis the changes will be reversed leading to expansion of the cytoplasmic pool and to an increase in total tissue concentration of substrate.

For the sake of simplicity the above description ignores many modulating effects which would alter the magnitude of the observed effects of acid-base change on the concentration of individual substrates. These modulating influences include the size of the substrate pool, the relative rate of the mitochondrial carrier system for the substrate, the rate of substrate uptake from or release into the peritubular blood, and the rates of cytoplasmic and mitochondrial synthesis and metabolism of the substrate. For example the pool size of glutamate in renal cortex is over 10 times as large as that of $\alpha$-ketoglutarate. Therefore, it might be anticipated that in acute acidosis a longer time would be required to reduce the size of the glutamate pool than to alter that of $\alpha$-ketoglutarate. This would account for the observation that, while the $\alpha$-ketoglutarate concentration falls sharply in acute acidosis, the concentration of glutamate is only slightly decreased in 5 min but after a few hours a much greater reduction develops (2, 4, 5).

A major requirement of this hypothesis is that the cytoplasmic rather than mitochondrial pool is the major contributor to the total amount of most mitochondrial substrates present in renal cortex. Estimates of the distribution of substrates between cytoplasm and mitochondria in kidney and liver have been made,

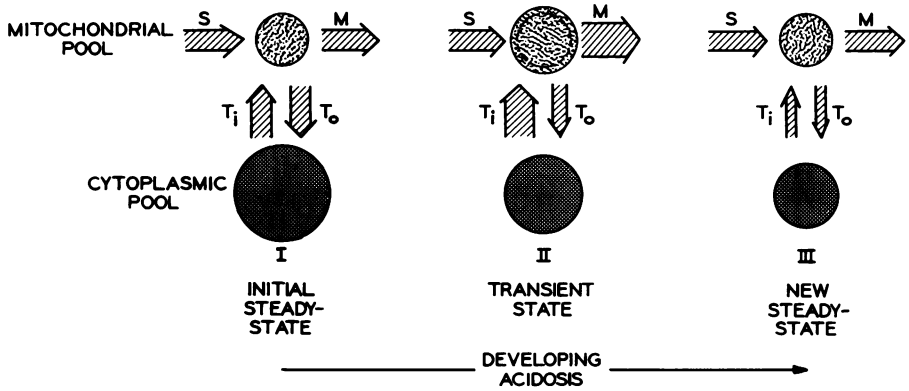


FIGURE 7 Diagramatic illustration of the proposed role of changes in anion transport in causing changes in substrate levels in renal cortex in response to acute acid-base variations. The response to an acute metabolic acidosis is shown. The area of a circle depicts the size of the cytoplasmic or mitochondrial pool of a substrate. The width of an arrow represents the relative rate of transport or metabolism. S, mitochondrial synthesis; M, mitochondrial metabolism; $T_i$, net rate of transport into mitochondria $T_o$, net rate of transport out of mitochondria.

usually with indirect methods, with widely varying results depending upon the techniques used and the assumptions made (14). Recently, a direct method, in which digitonin is used to release cytoplasmic but not mitochondrial constituents from isolated hepatocytes, has shown that the amount of most mitochondrial substrates is substantially greater in the cytoplasm than in the mitochondria (24). If the hypothesis proposed here is correct, a similar distribution of metabolites should exist in renal cortex; however, evidence on this point is not yet available.

Because acute metabolic acidosis leads to a reduction in the amount of citric acid cycle and related compounds in renal cortex without any accompanying increase in any one of these compounds, net removal of the carbon skeletons of compounds entering mitochondria from the cytoplasm must occur in this condition. The hypothesis does not attempt to specify the routes by which this removal occurs but presumably these would be the same as the routes by which metabolic products of citric acid cycle substrates are normally transferred out of mitochondria, eventually forming either glucose, through the reactions of gluconeogenesis, or pyruvate, which can be recycled back into mitochondria and oxidized.

Reductions in the level of most substrates of intermediary metabolism in renal cortex, as well as occurring immediately after acidosis is initiated, are found in states of chronic acidosis. While other influences are probably also involved in causing these chronic changes, as long as intracellular pH and bicarbonate remain low the altered substrate distribution produced acutely between cytoplasm and mitochondria should persist. Thus, the acute effect of pH and bicarbonate on mitochondrial anion accumulation may contribute to the reduced substrate levels present in renal cortex in chronic as well as acute acidosis.

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