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REGULATION BY BICARBONATE ION OF INTRAMITOCHONDRIAL CITRATE CONCENTRATION IN KIDNEY MITOCHONDRIA

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SUMMARY

1. Steady-state concentrations of citrate in the matrix space ($[Citrate]_M$) in mitochondria from rabbit renal cortex were measured with low (10 mM) or high (40 mM) concentrations of bicarbonate in the medium. $[Citrate]_M$ was much less at high than at low bicarbonate concentration either in the presence or absence of rotenone. With rotenone and 1 mM citrate in the medium $[Citrate]_M$ was over twice as great at 10 as at 40 mM bicarbonate.

2. The greatest effects on $[Citrate]_M$ occurred between 5 and 20 mM bicarbonate.

3. The largest differences in $[Citrate]_M$ were observed when both pH and bicarbonate in the medium varied concomitantly. However significant differences in $[Citrate]_M$ occurred when bicarbonate concentration changed without change in pH; pH change with constant bicarbonate concentration caused little or no change in $[Citrate]_M$.

4. These results suggest that the concentration of bicarbonate ion regulates citrate transport across the mitochondrial membrane. This mechanism accounts for the inhibitory effect of bicarbonate on citrate oxidation by slices and mitochondria from renal cortex and provides an explanation for the physiologic increase in citrate clearance in metabolic alkalosis.

INTRODUCTION

In recent years active transport systems located in the inner mitochondrial membrane have been described for di- and tricarboxylic acid intermediates of the citric acid cycle as well as for phosphate and adenine nucleotides[1,2]. In general these transport systems are capable of increasing the concentration of a substrate in the matrix space several fold over that existing in the medium and can be inhibited by specific analog compounds. Different translocators have been identified for several substrate groups including separate ones for malate and succinate, for citrate and isocitrate and for α-ketoglutarate. The existence of these various carriers raises the possibility that physiological variations in the rate of substrate metabolism may in

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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some circumstances result from alterations in the rate of substrate transport into or out of mitochondria. The present study was undertaken to see if changes in bicarbonate concentration, which are known to control the rate of citrate oxidation in renal cortex[3], might regulate the rate of citrate transport by renal mitochondria. The results indicate that bicarbonate ion does have a strong influence on the steady state concentration of citrate in the matrix space and presumably alters the rate of citrate transport across the inner mitochondrial membrane.

METHODS

Preparation of mitochondria

Rabbit kidneys were placed in an ice-cold solution of 0.14 M NaCl, 0.01 M KCl, cut into sections and the cortex separated from medulla. 3 g of cortex were minced with scissors in 15 ml of 0.3 M sucrose, 1 mM EDTA, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4) and homogenized with six strokes of a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged for 5 min at $700 \times g$ at 0 °C and the supernatant centrifuged for 10 min at $8000 \times g$ (maximum) at 0 °C in an 870 head of an International B-20 centrifuge. After decanting the supernatant the mitochondrial pellet was suspended in 0.3 M sucrose, 5 mM HEPES (pH 7.4) using the 'cold finger' technique[4]. The suspension was centrifuged for 10 min at $8000 \times g$ and the pellet suspended in a small volume of 0.3 M sucrose.

Measurement of mitochondrial [^{14}C]citrate content

0.2 ml of the mitochondrial fraction was added to 1.8 ml of an incubation medium in a 25-ml erlenmeyer flask closed with a serum stopper which had previously been gassed at 37 °C with CO_2–O_2 (5:95, v/v) or other gas mixture. The medium contained 108 mM KCl *plus* $KHCO_3$, 5 mM $MgSO_4$, 2.5 mM phosphate, 5 mM HEPES, 3 mM ADP, 0.5 mM succinate, 1 mM citrate and 1.0 μCi [6-^{14}C]citric acid (New England Nuclear Corp.). In most of the experiments rotenone was present in a concentration of 1 μg/ml. Before bicarbonate was added the pH of the medium was adjusted with KOH to approximate that expected from the final bicarbonate concentration after equilibration with CO_2. In some experiments the pH of the medium was measured anerobically after gassing at 37 °C with a Radiometer Microelectrode Unit and a Beckman Expandomatic pH meter. Preliminary experiments indicated that the citrate concentration in the matrix space ($[\text{Citrate}]_M$) reached a steady state level in less than 2 min of incubation. Hence incubation times of 2 or 4 min were used after which two 0.2-ml portions of medium were transferred to microcentrifuge tubes containing 100 μl of 1M $HClO_4$ beneath a layer of silicone oil (Versilube F-50, General Electric Co.), and the mitochondria rapidly separated from medium[5]. A 50-μl portion of medium was also added directly to 100 μl $HClO_4$ for measurement of dpm in medium.

The $HClO_4$ extract of mitochondria was transferred from beneath the silicone oil to another microcentrifuge tube and allowed to stand, with several mixings, for 1 h to permit diffusion of labeled CO_2 out of the solution. Control studies showed that this procedure was adequate to eliminate $^{14}CO_2$ formed during incubation. An aliquot of mitochondrial extract was transferred to a glass vial, 15 ml of a scintillation

solution (25% Triton X-100 in a solution containing 6 g 2,5-diphenyloxazole and 0.5 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene per l of toluene) were added and the radioactivity measured by liquid scintillation counting using internal standards for measurement of efficiencies.

Measurement of water spaces and calculation of $[Citrate]_M$

In most of the experiments measurements of the total water and sucrose-permeable spaces of mitochondria were made in separate incubation flasks containing tritiated water and ^{14}C-labeled sucrose. The mean total water space in these experiments was 3.04 ± 0.15 (S.E.) μl per mg protein and mean sucrose permeable space was 2.14 ± 0.09 μl per mg protein. In a few experiments the water spaces were not measured but the average values from other experiments were used in calculation of $[Citrate]_M$.

Calculation of the dpm of citrate in the sucrose-permeable space was made from the volume of this space times the measured dpm in the medium. This value was subtracted from the total dpm per mg protein in the mitochondrial extract to give the dpm in the matrix space. $[Citrate]_M$ was calculated from the latter value by dividing by the specific activity of citrate in the medium. This calculation ignores the fact that about 10% of the measured dpm were in aconitate and isocitrate as required by the equilibrium positions of aconitate hydratase[6]. Because the C-6 of citrate is removed as CO_2 by the isocitrate dehydrogenase reaction, metabolites of the citric acid cycle beyond isocitrate will not be labeled when [6-^{14}C]citrate is used as substrate.

Other measurements

In each experiment the production of $^{14}CO_2$ from [6-^{14}C]citrate was measured. Incubation was carried out in the same manner as in the flasks in which citrate accumulation was measured except that one-tenth as much labeled citrate was used per flask. At the end of the incubation period 0.5 ml of 1M $HClO_4$ was added to the medium through the serum stopper and 0.25 ml of ethanolamine was placed in a plastic cup suspended from the stopper. CO_2 was absorbed for 2 h and the cup transferred to a counting vial. 10 ml of a solution of 20% methylcellosolve in toluene containing 6 g 2,5-diphenyloxazole and 0.5 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene per l were added and the radioactivity determined.

The respiratory control ratio of each mitochondrial preparation was determined using a Clark type oxygen electrode (Yellow Springs Instrument Co.). With 10 mM malate *plus* pyruvate as substrates the respiratory control ratios ranged from 2.8 to 6.0.

RESULTS

Effect of high and low bicarbonate concentrations on $[Citrate]_M$

The $[Citrate]_M$ at two different bicarbonate concentrations, 10 and 40 mM (pH 7.07 and 7.65, respectively) are shown in Fig. 1 together with the $^{14}CO_2$ produced during 4 min of incubation. In the presence of 10 mM bicarbonate, $[Citrate]_M$ was 3–4-fold greater than that in the medium itself (1 mM Citrate). When 40 mM bicarbonate was present the calculated dpm in the sucrose permeable space slightly exceeded the measured dpm in the mitochondrial extract; this result can be explained by continued citrate metabolism during the several seconds required for the mito-

chondria to pass through the silicone oil layer; during this time whatever citrate was initially present in the matrix space was metabolized together with a small amount of citrate transferred from the sucrose permeable space. About 50% more labeled CO_2 was produced during incubation of mitochondria in 10 mM bicarbonate medium than in 40 mM, a result consistent with the previously demonstrated effect of bicarbonate on mitochondrial citrate oxidation[3]. This difference is representative of that found in the other experiments in this series in control flasks incubated without rotenone.

The $[Citrate]_M$ in the above experiment represents the resultant of the rate of citrate entry into mitochondria *minus* the sum of the rate of citrate exit *plus* the rate of citrate metabolism to other products. By adding rotenone the contribution of citrate metabolism to this steady state level was greatly decreased. Fig. 2 shows that in the presence of rotenone oxidation of labeled citrate to $^{14}CO_2$ was markedly reduced and the difference in $^{14}CO_2$ production at high and low bicarbonate concentrations was eliminated. $[Citrate]_M$ at 10 mM bicarbonate in the presence of rotenone was about 3-fold greater than at 40 mM bicarbonate. In contrast to the results without rotenone, at 40 mM bicarbonate $[Citrate]_M$ which exceeded the concentration of citrate in the medium was demonstrable.

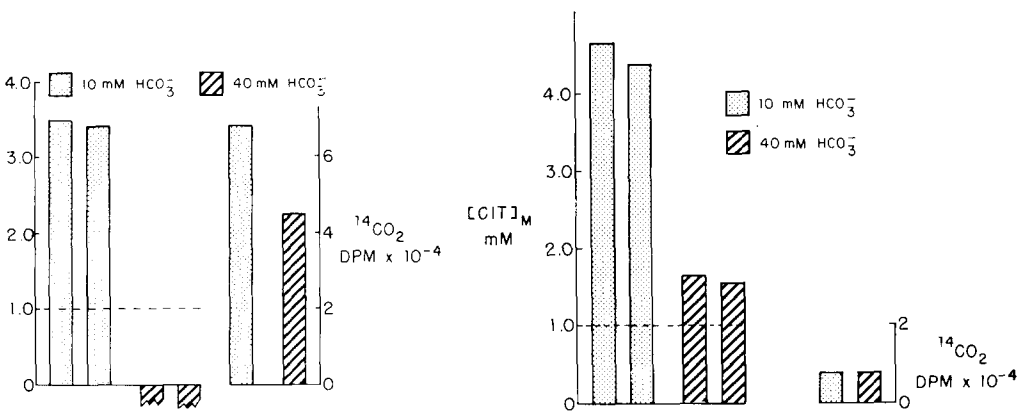

Fig. 1. $[Citrate]_M$ and $^{14}CO_2$ produced at 10 and 40 mM bicarbonate in the medium. Incubation time was 4 min. Each vertical bar represents results from one flask. The bars below the horizontal axis indicate that fewer dpm were present in the mitochondrial extract than were calculated to be present in the sucrose-permeable space (see text for explanation).

Fig. 2. $[Citrate]_M$ and $^{14}CO_2$ produced at 10 and 40 mM bicarbonate in the presence of rotenone. Incubation time was 4 min.

A direct comparison of $[Citrate]_M$ in uninhibited and rotenone-inhibited mitochondria is shown in Fig. 3. In this experiment mitochondria were incubated with or without rotenone at high and low concentrations of bicarbonate. Inhibition of respiration increased $[Citrate]_M$ at both bicarbonate concentrations by about 1.5 mM. With 40 mM bicarbonate again no citrate was detectable in the matrix space in the absence of rotenone. $[Citrate]_M$ at 10 mM bicarbonate greatly exceeded that present at 40 mM bicarbonate both in the presence and absence of inhibitor.

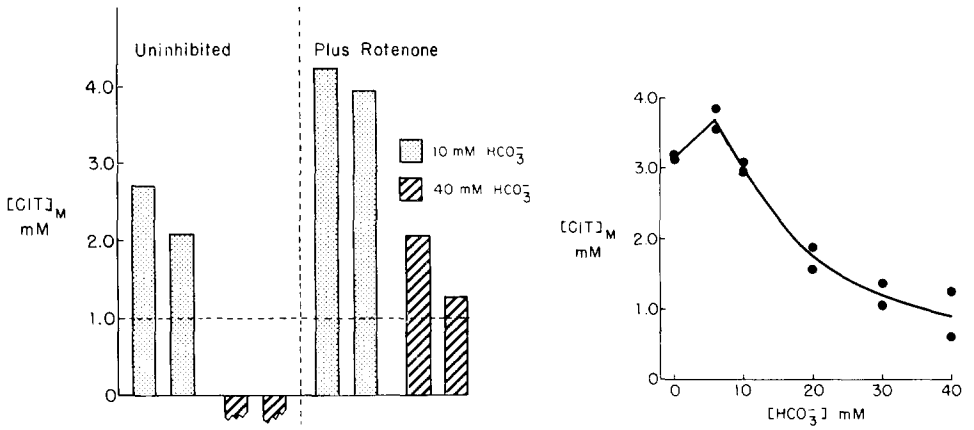

Fig. 3. Comparison of effects of high and low bicarbonate concentration in the presence and absence of rotenone. Incubation time was 4 min.

Fig. 4. $[Citrate]_M$ with varying concentrations of bicarbonate. Incubation time was 4 min. The flasks without bicarbonate were gassed with 100% O_2; the remainder were gassed with 5% CO_2–O_2 (5:95, v/v). Rotenone was present in each flask.

$[Citrate]_M$ measured over a range of bicarbonate concentrations

In the experiment shown in Fig. 4 the concentration of bicarbonate in the medium was varied from 0 to 40 mM. The flasks with no bicarbonate contained phosphate and HEPES at pH 7.07 and were gassed with 100% O_2; in the absence of bicarbonate $[Citrate]_M$ was slightly less than the maximum observed in the presence of bicarbonate. The effect of a change in bicarbonate concentration on $[Citrate]_M$ was greatest between 5 and 20 mM bicarbonate; above 20 mM bicarbonate the slope of the curve decreased considerably.

TABLE I

EFFECTS ON $[CITRATE]_M$ OF VARYING BICARBONATE CONCENTRATIONS WITH OR WITHOUT ACCOMPANYING pH CHANGES

Incubation time was 2 min. 10 mM HEPES and 1 μg/ml rotenone were present in each medium. Separate results from two flasks are shown for each set of conditions.

Expt	*pH*	*% CO_2*	*$[HCO_3^-]$ (mM)*	*$[Citrate]_M$ (mM)*
1	7.17	5	13	3.9, 4,0
	7.65	5	40	1.2, 1.4
	7.65	1.8	13	2.9, 3.0
	7.52	8	40	1.7, 2.0
2	6.96	5	8	4.8, 4.6
	7.54	5	30	1.7, 1.9
	7.42	1.8	8	2.8, 3.1
	7.37	8	30	1.6, 1.7

Relative contributions of H^+ and bicarbonate to changes in $[Citrate]_M$

In the above experiments the atmosphere in the flasks contained 5% CO_2 in oxygen and therefore as concentration of bicarbonate varied so did pH. In order to separate the effect of bicarbonate change from that of H^+ change experiments were carried out in which pH and bicarbonate were varied independently by changing the composition of the gas phase. Table I describes the results of two experiments in which the effects of bicarbonate change were studied with or without accompanying change in pH. When both pH and bicarbonate varied (5% CO_2) $[Citrate]_M$ was about two and a half times greater at low than at high bicarbonate concentration. When P_{CO_2} and bicarbonate were varied proportionately so that medium pH was similar at both bicarbonate concentrations, the flasks with 10 mM bicarbonate again showed distinctly greater concentrations of citrate in the matrix space than did those with 40 mM bicarbonate; the ratios of $[Citrate]_M$ with low and high bicarbonate were 1.5 and 1.8 in the two experiments shown, somewhat less than the 2.5 to 1 ratio present when both pH and bicarbonate changed.

Table II shows results of similar experiments in which pH was varied with or without change in bicarbonate concentration. In Expt 1, when only non-bicarbonate buffers were used in the medium, no distinct difference in $[Citrate]_M$ was discernible between flasks containing media with different pH values. In Expt 2, Table II, pH and P_{CO_2} were varied while bicarbonate concentration was kept fixed at 15 mM. Again little difference in $[Citrate]_M$ was present between the two pH levels. In contrast when pH and bicarbonate were varied together $[Citrate]_M$ was about 2.5 times greater at the lower pH and bicarbonate concentration. Tables I and II indicate that over the pH range studied change in pH has only a small influence on $[Citrate]_M$. The large changes in $[Citrate]_M$ produced by changes in pH and bicarbonate are due primarily to an effect of bicarbonate ion although maximum effect is obtained when both parameters are varied together.

TABLE II

EFFECTS ON $[CITRATE]_M$ OF VARYING MEDIUM pH WITH OR WITHOUT ACCOMPANYING CHANGE IN BICARBONATE CONCENTRATION

Incubation time was 2 min. Rotenone and 10 mM HEPES were present in each medium. Separate results from two flasks are shown for each set of conditions.

Expt	*pH*	*% CO_2*	*$[HCO_3^-]$ (mM)*	*$[Citrate]_M$ (mM)*
1	7.07	0	0	4.9, 5.2
	7.64	0	0	4.3, 4.3
	7.07	5	13	3.0, 2.7
	7.64	5	40	1.5, 1.0
2	6.91	0	0	3.4, 4.4
	7.49	0	0	4.2, 3.7
	7.04	5	10	3.5, 3.0
	7.67	5	40	1.2, 1.2
	7.04	8	15	2.9, 3.1
	7.65	1.8	15	2.3, 2.8

DISCUSSION

Active transport of ions into and out of cells provides an important means of regulating physiological processes. The existence of transport systems in the inner mitochondrial membrane for mitochondrial substrates provides an additional locus at which metabolic control can be exerted. In the present study the steady-state level of citrate inside mitochondria is shown to be regulated by the bicarbonate concentration in the medium. This regulation occurs both in the presence and absence of inhibition of citric acid cycle activity, implying that net citrate transport is controlled by the concentration of bicarbonate inside the cells of the renal cortex. Previous investigations of the ability of mitochondria to maintain a concentration gradient for citrate across the mitochondrial membrane have revealed the presence of a translocator in the inner membrane specific for tricarboxylic acids[1,2]. Active transport of citrate both in and out of mitochondria has been demonstrated by means of inhibitor stop techniques[7,8]. Presumably the ability of bicarbonate ion to regulate intramitochondrial citrate concentration in the absence of significant metabolic activity reflects an effect of bicarbonate on citrate transport by the tricarboxylate translocator.

The effects demonstrated in this study could be accounted for by several types of influences on citrate fluxes across the inner membrane. Bicarbonate could influence citrate transport in one direction only, high bicarbonate either inhibiting citrate entry or stimulating citrate exit without altering the opposite flux. A more powerful regulatory effect could be obtained if bicarbonate affected the inward and outward flux of citrate in opposite ways so that high bicarbonate concentration decreased the rate of citrate entry and simultaneously increased its rate of exit. Effects of bicarbonate of equal magnitude on citrate transport in both directions could not account for the results; however if high bicarbonate stimulated both citrate entry and exit but had a disproportionately large effect on the latter, the observed changes in $[\text{Citrate}]_M$ might be explained. No direct evidence bearing on these possibilities is yet available. However it may be pertinent that earlier studies[3] showed that the amount of citrate contained in the medium when mitochondria were incubated with pyruvate *plus* malate was greater at high than at low bicarbonate concentrations. This observation suggests that citrate synthesized inside mitochondria is transported out more rapidly in the presence of a high bicarbonate level and it seems likely therefore that bicarbonate at least enhances the rate of citrate exit from mitochondria. Whether or not it also inhibits citrate entry cannot be determined from these results. Use of the inhibitor stop technique should provide more definitive evidence on effects of bicarbonate on citrate fluxes.

Another explanation which might be entertained to account for the results of these experiments is that of a mechanism by which bicarbonate displaces citrate from the matrix space without any influence on citrate transport into or out of mitochondria. Although such a non-specific effect of bicarbonate cannot be entirely dismissed on the basis of present information, it is unlikely by virtue of the following reasoning. In the absence of rotenone citrate oxidation is reduced at higher bicarbonate concentrations. If the rate of citrate transport into the mitochondria is the same at high and at low concentration of bicarbonate, then the rate of citrate transport out of mitochondria must be increased at high bicarbonate levels to the same

extent as the rate of oxidation is decreased. Thus some effect of bicarbonate on citrate transport out of mitochondria must be postulated to account for the results. Because the steady state level of citrate inside the mitochondria is considerably reduced at higher bicarbonate levels, an increase in passive diffusion of citrate out of mitochondria could only occur in this circumstance if there were a marked change either in the electrical gradient across the membrane or in the average pore size of the membrane. Since movement of citrate out of mitochondria can be effectively blocked with inhibitors of the tricarboxylate carrier, passive diffusion probably plays little role in the mitochondrial transport of citrate. Thus an effect of bicarbonate on facilitated diffusion or active transport of citrate is a more likely explanation for the observed effects.

Tables I and II indicate that the observed effects are primarily related to bicarbonate rather than H^+. Although pH effects on citrate accumulation have been described[9,10], pH appears to have little effect separate from that of bicarbonate within the relatively small pH range used in these studies. As shown in Fig. 4 the maximum effect occurs between 5 and 20 mM bicarbonate. Intracellular bicarbonate concentration probably lies within this range in cells of renal cortex which have an estimated intracellular pH of 7.3 when extracellular pH is 7.4 and P_{CO_2} 40 mM[11]. Renal cortical cells, unlike those of muscle, show a linear variation in intracellular pH in response to changes in extracellular pH and bicarbonate concentration. Thus changes in intracellular bicarbonate will occur in the 5 to 20 mM range in response to variations in extracellular pH and bicarbonate and will cause maximum changes in citrate transport.

Metabolic alkalosis causes a marked increase in renal citrate clearance[12,13] and is associated with an increase in citrate content in renal cortex[12,14]. Normally more citrate is removed from blood entering the kidney than is filtered by the glomeruli[15,16]. In metabolic alkalosis little change in plasma citrate concentration occurs but renal utilization of citrate decreases considerably. Earlier investigation of these effects demonstrated a marked inhibitory effect of high pH and bicarbonate levels on citrate oxidation by tissue slices and mitochondria[3]. Other properties of this *in vitro* phenomenon closely paralleled the effects of metabolic alkalosis on renal citrate metabolism in the intact animal and we concluded that the increased citrate clearance seen in metabolic alkalosis resulted from an inhibitory effect of increased intracellular bicarbonate ion on mitochondrial citrate oxidation.

The present study provides an explanation for this inhibitory effect provided that the rate of citrate metabolism by mitochondria is dependent on $[\text{Citrate}]_M$. This latter requirement seems likely in view of the currently known facts regarding the equilibrium of aconitate hydratase (EC 4.2.1.3) and the kinetics of isocitrate dehydrogenase (EC 1.1.1.41). Aconitate hydratase maintains an equilibrium between citrate and isocitrate of about 20 to 1 (ref. 6) so that with $[\text{Citrate}]_M$ in the range of 1 to 4 mM, as were found in the present study, isocitrate concentrations of 0.05 to 2 mM would be expected. These levels are below or close to those of the K_m for NAD-dependent isocitrate dehydrogenase (EC 1.1.1.41), which in beef heart enzyme varies from 0.36 to 1.5 mM depending on the conditions of measurement[17]. The kinetic properties of this enzyme have been noted to be particularly suitable for metabolic regulation and it is believed to play a more prominent role in intramitochondrial metabolic control than NADP-dependent isocitrate dehydrogenase

(EC 1.1.1.42) which has a much lower K_m of about 10^{-3} mM[18]. The present experiments indicate that high bicarbonate concentrations inside the cell decrease $[Citrate]_M$ which in turn decrease the concentration of isocitrate in the matrix space and decrease the rate of intramitochondrial citrate oxidation. Thus these results suggest a regulatory role for bicarbonate ion on the tricarboxylate transport system in the mitochondrial membrane which accounts for the inhibitory effect of bicarbonate ion on citrate oxidation *in vitro* and for the physiological effect of metabolic alkalosis on renal citrate clearance.

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