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TRANSPORT OF POTASSIUM AND SODIUM WITH CITRATE ACROSS THE MITOCHONDRIAL MEMBRANE

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SUMMARY

Citrate is accumulated by rat liver mitochondria by an energy-dependent reaction in which the trivalent anion enters with an equal equivalence of Na^+ or K^+. This uptake is in addition to the incorporation by exchange that takes place in the respiratory-inhibited state. The energy-dependent reaction has the following characteristics: Accumulation of citrate and the cations occurs in the absence of ionophores. The rate is slower than that of the exchange reaction, but in 6–10 min (at 20 °C) the quantity taken up (15–30 μmoles/g mitochondrial protein) may exceed that taken in by exchange (10–15 μmoles/g protein). The energy-dependent reaction is relatively specific for citrate. 50% as much K^+ or Na^+ is gained with *cis*-aconitate but only 10–25% as much with isocitrate and other Krebs-cycle intermediates. In contrast to results obtained with acetate, the energy-dependent accumulations of citrate occur (1) with Na^+ as the cation, (2) from low concentrations of external organic anion (2–4 mM), (3) with relative independence of changes in the pH of the media.

INTRODUCTION

Recent studies of citrate transport in mitochondria have placed emphasis chiefly upon exchange reactions. Citrate is accumulated from suspending media in exchange for endogenous inorganic and organic phosphate components[1]. Citrate is also accepted in exchange for other intermediates of the Krebs-cycle (see recent reviews, refs 2 and 3). Additional demonstration of incorporation by exchange is included in the present study, but the main emphasis is given to description of a second mechanism, namely, a respiratory-dependent incorporation of citrate as a salt with either Na^+ or K^+ as accompanying cations.

Mitochondrial transport of Na^+ and K^+ is not well understood and has generally been considered in terms of separate and highly specific mechanisms. In the case of potassium, reference has been made to a pumping mechanism[4–6] or to electrogenic forces secondary to movements of anions[7–10]. Na^+ movement, on the other hand, is usually attributed to a Na^+–H^+ antiport[7,8]. In the experiments of the present study the two cations are, to a degree, interchangeable. This suggests the existence of a common mechanism which may involve a role for citrate as a cation carrier.

METHODS

Mitochondria were isolated from livers of rats and rabbits using conventional methods[11]. Rat liver was used as the source in all but the first of the two experiments shown in Fig. 1. The experimental manipulations, methods of analysis and procedures for calculating the data were those described in a previous report[12]. Citrate accumulation was measured as gain in ^{14}C-labeled citrate. As reported earlier[1], 80–90% of citrate accumulated by mitochondria remains intact during 10-min incubations, the remainder being converted chiefly to malate. Correction was made for quantities of citrate in the sucrose-permeable space in order to estimate the amounts retained within the inner membrane[12–14]. The permeable space was measured in parallel experiments using ^{14}C-labeled sucrose. The experiments describe changes with reference to control samples: mitochondria added to cold (0 °C) aliquots of the media and centrifuged immediately. Incubated media were adjusted to pH 7.0 with additions of 0.5–0.1 μmoles of NaOH. Buffering reagents were avoided so that additional anions were not introduced into the system. ATP was measured by following the oxidation of NADH in the presence of phosphoglyceric phosphokinase and glyceraldehyde phosphate dehydrogenase. The enzymes were obtained from Sigma. The ^{14}C-labeled compounds ([1,5-^{14}C]citric acid and uniformly labeled sucrose and acetate) were obtained from Amersham, Searle.

RESULTS

An objective in this study is to account for the stoichiometry of the accumulation of citrate—to balance gain in anionic equivalence of the trivalent citrate with both loss of endogenous anion and gain in exogenous cation. With this purpose in mind, the data are presented in terms of equivalence (μequivalents per g protein) rather than in terms of molarity. For citrate, the equivalence is calculated as 2.95

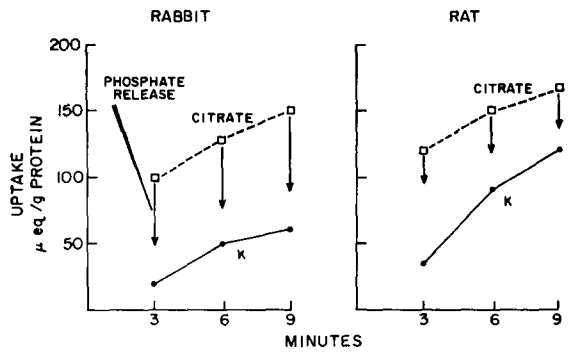

Fig. 1. Accumulation of citrate and K^+ and release of phosphate (downward pointing arrows). Mitochondria from rabbit liver, 2.8 mg protein per ml (left panel) and from rat liver, 2.2 mg protein per ml (right panel) were incubated at 20 °C in 5 ml of sucrose, 0.18 M at pH 7.0. The incubation media also contained 40 mM KCl; 4 mM labeled citrate; 1 mM succinate. Samples were removed and centrifuged at the times indicated. Phosphate release was measured as increase in total phosphate (organic and inorganic) in the supernatants after centrifugation. The equivalence of the released phosphate was estimated as 1.5 times the molarity.

times the molarity. This assumes that the internal pH of the mitochondria does not fall significantly below 7.0. The estimate of the average equivalence of the released inorganic and organic phosphate components (Fig. 1 only) were obtained by multiplying the molarity by a factor of 1.5.

Evidence of two types of reaction for the accumulation of citrate

Described in the first figure are accumulations of citrate and K^+ as measured after 3, 6, and 9 min of incubation. Mitochondria obtained from livers of both rabbit and rat were studied. The temporal relationships give preliminary evidence for two types of reaction. During the first 3 min there is a very rapid influx of citrate with only a small increase in K^+. On the other hand, between 3 and 9 min, there is a progressive increase in K^+, and this is associated with a continued gain in citrate. The first phase, the rapid accumulation of citrate can be ascribed to the mitochondrial anion-exchange reaction[2,3]; but in the second, slower phase the gain also of cationic equivalence suggests that the citrate is entering as a salt rather than in exchange for endogenous anions. The total accumulation of citrate is large. A value of 150 μequiv/g protein is comparable to the equivalence of K^+ in freshly prepared mitochondria[15].

Loss of endogenous inorganic and organic phosphate (downward pointing arrows, Fig. 1) appears to be an important component of the exchange reaction. This loss occurs during the first 3 min and appears not to increase after this time. The larger release from the rabbit mitochondria can be attributed to the larger quantities of phosphoenolpyruvate in the mitochondria from this species[1,16,17]. The uptakes of K^+ are somewhat larger in the mitochondria isolated from rat liver, and this preparation was used for the remainder of the studies.

Effects of respiratory inhibition provide additional evidence for two separate reactions. The mitochondrial anion-exchange reaction does not require respiratory activity and is, in fact, usually studied in the presence of an inhibitor such as rotenone[2,3]. Thus, as shown on the first line in Table I, the accumulation in the presence of cyanide of 45 μequiv/g protein, with only 6 of K^+, can be attributed to the exchange reaction. In the absence of cyanide, the accumulations of citrate and K^+ are increased, respectively, to 120 and 75 μequiv/g protein. Thus with discontinuance of the respiratory inhibition there is a matching gain of approx. 70 μequiv/g protein of both components.

It is of particular interest that qualitatively similar results are obtained if K^+ is replaced by Na^+. In this case the reaction is complicated by a release of endogenous K^+; but if the reference is to net changes in cation content, matching gains of cationic and anionic equivalence can again be demonstrated. Results obtained with sodium citrate are given on the third and fourth lines in Table I. The uptake of citrate in the presence of cyanide is 55 μequiv/g protein when the net change in cation content (Na^+ gain *minus* K^+ loss) is 16 μequiv/g protein. When cyanide is omitted, and the inhibition released, these two values rise to 85 and 50 – a gain of 30 and 34 μequiv/g protein, respectively, for citrate and for the net change in the cation content.

Addition of permeating cations to the medium in the presence of respiratory activity (Fig. 2) is a third means to distinguish the two reactions. The cations would not be required for an exchange reaction but would for uptake of citrate as a salt. In the absence of permeant cations 50–70 μequiv/g protein of citrate are accumulated in routine 6-min incubations (see Table III). This quantity is comparable to the

TABLE I

ACCUMULATION OF CITRATE WITH K^+ AND WITH Na^+ IN THE PRESENCE AND IN THE ABSENCE OF CYANIDE

Mitochondria from rat liver, 1.9 mg protein per ml, were incubated for 6 min at 20 °C at pH 7.0. Reagent concentrations are those described in the legend of Fig. 1. The concentration of cyanide was 2 mM.

Medium	*Accumulation in mitochondria (μequiv/g protein)*		
	Citrate	*K^+*	*Na^+*
Potassium citrate with cyanide	+ 45	+ 6	
Potassium citrate omit cyanide	+120	+75	
Sodium citrate with cyanide	+ 55	− 6	+20
Sodium citrate omit cyanide	+ 85	−22	+72

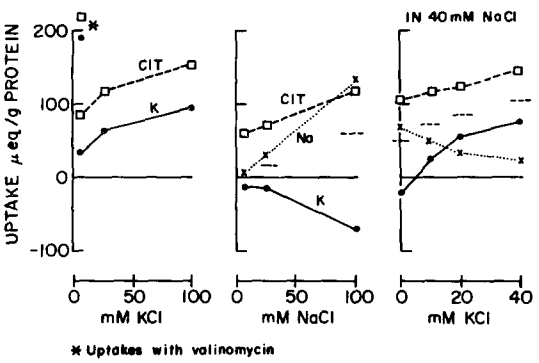

Fig. 2. Effect of change in the external concentrations of KCl or NaCl. Mitochondria, 2.5 mg protein per ml, were incubated at 20 °C, pH 7.0, in 5-ml solutions containing labeled Tris citrate, 4 mM, and Tris succinate, 1 mM. Isotonic concentrations of KCl or NaCl, 0.125 M, replaced sucrose, 0.25 M, in order to increase the concentrations of external Na^+ or K^+ isosmotically. The horizontal dashed lines in the second and third panels refer to net cation uptake, Na^+ uptake *minus* K^+ loss.

rapidly accumulation fraction shown in Fig. 1 or to the amounts accumulated in the presence of cyanide as shown in Table I. This baseline uptake can then be attributed to the anion-exchange reaction. When Na^+ and K^+ are provided in increasing concentrations (Fig. 2), additional accumulations obtain. The additional accumulations of citrate are balanced by equivalent increase in K^+ when in the K^+-containing medium or by an equivalent net increase in Na^+ *plus* K^+ (Na^+ gain *minus* K^+ loss) when in the Na^+-containing medium. The net uptake is given by the horizontal dashed lines. At low external concentrations (5–15 mM), K^+ is considerably more effective than is Na^+. Large accumulations of Na^+ occur only from higher concentrations (50–100 mM), and these are associated with large losses of endogenous K^+. It is evident that increased uptakes of the citrate salts obtain when the concentration

of the cations in the medium is raised, and still larger uptakes occur after adding an ionophore such as valinomycin (refs 18 and 19, see also Fig. 2). It would appear that it is the transfer of the cation that is the rate-limiting step.

Results obtained when there is competition between Na^+ and K^+ are shown in the last panel. K^+ competes effectively when present at one third the Na^+ concentration. When both are present at 40 mM, the accumulation of K^+ is three times that of the Na^+.

The data in Table II are included to demonstrate that the, frequently observed, rapid citrate for citrate exchange[2,3,20] does occur under the experimental conditions used in this study. Labeled citrate is lost rapidly from mitochondria when it can exchange with unlabeled citrate in surrounding fluids. As is the case when the exchange is with phosphate[1], the reaction is insensitive to respiratory inhibition.

TABLE II

LOSS OF CITRATE FROM PRELABELED MITOCHONDRIA

Mitochondria were loaded with labeled citrate using the usual experimental procedure as outlined in Fig. 1. They were recovered by centrifugation and incubated a second time in the presence of the same reagents (sucrose, 0.18 M; KCl, 40 mM; succinate, 1.0 mM) but with labeled and unlabeled citrate, 4 mM. The specific activity of the labeled citrate in the second incubation was identical to that in the first.

Media	*Mitochondria, labeled citrate (μequiv/g protein)*
No citrate, 0 °C	55
No citrate, 20 °C	45
Labeled citrate	65
Labeled citrate *plus* cyanide	60
Unlabeled citrate	3
Unlabeled citrate *plus* cyanide	5

Additional properties of the cation-linked citrate transport mechanism

A survey of effects of various reagents is given in Table III. For these studies mitochondria (2–3 mg protein per ml) were incubated for 6 min at 20 °C at pH 7.0 in a medium containing 0.2 M sucrose; 0.025 M K^+; 0.004 M ^{14}C-labeled citrate; and 0.001 M succinate. The accumulation of citrate and K^+ after incubation in this "complete system" is given in the first line.

As shown in the second line (Table III), omission of citrate reduces the uptake of K^+ to a low level. Succinate is present in these experiments, and incubation with this substrate results in accumulation of small amounts of K^+ even in the absence of ionophores[18,21–24]. When K^+ is omitted (third line), and succinate and citrate are added as the Tris salts, the uptake of citrate is sharply curtailed. Presumably, incorporation of citrate as a salt is inhibited due to the low permeability of the Tris. Here, most of the incorporation can be attributed to the exchange reaction.

TABLE III

REQUIREMENTS FOR CITRATE ACCUMULATION

Experimental conditions and procedure are outlined in the text. The concentration of phosphate (P_i) was 2 mM when added to the usual system and 10 mM when added to the State III and State IV media. Other concentrations were ADP and ATP, 2 mM; dinitrophenol, $5 \cdot 10^{-5}$ M; rotenone, 2 μg per ml.

Media	*Uptake*	
	Citrate (*μequiv/g protein*)	*K^+* (*μmoles/g protein*)
Complete system	135	80
Omit citrate		22
Omit K^+	58	
Omit succinate	60	11
Add rotenone without succinate	44	7
Add rotenone with succinate	110	60
Add dinitrophenol	20	15
Add inorganic phosphate (P_i)	110	98
Add P_i, omit succinate	25	33
Add P_i and ADP	45	10
Add ATP, omit succinate	80	25
State IV	82	53
State III	41	3

Omission of succinate reduces the accumulation of citrate towards the respiratory-inhibited state (see Table I). A partial effect can be attributed to a low rate of oxidation of citrate or other endogenous substrates. Rotenone is as effective as cyanide. Addition of succinate in the presence of rotenone restores the ability to accumulate both the citrate and the K^+. These effects on citrate metabolism have been described previously by Max and Purvis[25]. Additions of succinate to "depleted" mitochondria were found by Harris[19] to produce large increases in citrate incorporation. However, in these experiments, increase in cation content was not observed without valinomycin. Dinitrophenol inhibits citrate accumulation somewhat more efficiently than cyanide or rotenone[1,25].

Inorganic phosphate is known to compete with citrate for access to the exchange mechanism[26,27]. It is known also to be incorporated as a permeable anion with respiratory-dependent accumulations of K^+[28,29]. Thus when phosphate is added to the complete system, there is a moderate reduction in the uptake of citrate together with an increase in the incorporation of K^+. Reduction of both of these values obtains when succinate is omitted, and there is inhibition of the respiratory-dependent components. Reduction of accumulation of citrate and K^+ occurs also when ADP and inorganic phosphate are added. Presumably, in this system, energy is diverted to oxidative phosphorylation[30]. ATP induces a positive response, but with the conditions used the effect is smaller than that obtained by adding succinate to stimulate respiration.

The last two lines (Table III) give results obtained when the conventional State III and State IV media are used. These media contain Mg^{2+} (5 mM). The

concentration of sucrose is half that used in the other experiments (*i.e.* 0.1 M rather than 0.2 M); and succinate and phosphate are present at 5-fold higher concentrations (*i.e.* 10 rather than 1–2 mM). With these media the accumulations of citrate and K^+ are not as great. Once again, completion of the system for oxidative phosphorylation, that is transition from State IV to State III, inhibits accumulation of both K^+ and citrate.

Anion specificity

Accumulation of the cations, Na^+ and K^+, reflects activity of the respiratory-dependent, second reaction as referred to above. The specificity of the reaction for citrate is considered in Fig. 3. These are measurements of Na^+ or of K^+ contents after incubation with the various intermediates of the Krebs-cycle. Uptakes with citrate are about twice those with *cis*-aconitate, while the other intermediates are much less effective. The gains of Na^+ and K^+ are similar. Supplementation with malate has been shown to stimulate the uptake of potassium citrate[1]; however, addition of malate has no effect on the uptake of K^+ with isocitrate (experiment not shown).

Measurement of changes in endogenous ATP are shown in Fig. 4. The initial level is indicated to be 7 μmoles per g protein (left panel) and this is reduced by approximately half when K^+ is accumulated with citrate. The findings shown on the right hand panel (Fig. 4) provide further examples of the specificity illustrated in Fig. 3. It can be seen that the largest accumulation of K^+ and decrease in ATP occurs with citrate. Intermediate values are obtained with *cis*-aconitate, whereas isocitrate is essentially inactive. Significant changes were recorded with succinate in this experiment.

Although Krebs-cycle intermediates other than citric and *cis*-aconitic appear to be inactive, it is well established that Na^+ and K^+ are accumulated with acetate[8,10,28,29,31–35]. A comparison enunciating three differences in the characteristics of the accumulations of acetate and of citrate is given in Table IV. As shown first, the maximal accumulation of citrate is obtained from the lower concentration of 6 mequiv/l or 2 mM. On the other hand, with acetate, the accumulation reached 45 μmoles per g protein only at the higher concentration of 40 mM. Reported obser-

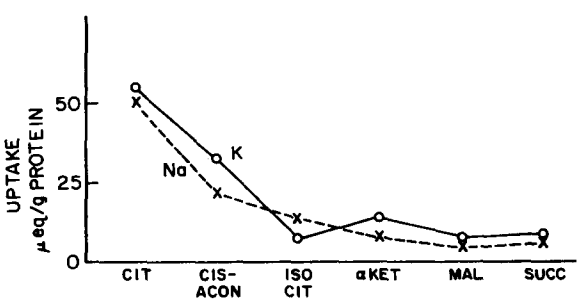

Fig. 3. Accumulation of Na^+ and K^+ after incubation with various di- and tricarboxylic acids. Mitochondria, 2.5 mg protein per ml were incubated for 6 min at 20 °C in sucrose, 0.18 M, at pH 7.0; NaCl or KCl, 40 mM; Tris succinate, 1 mM; and Tris salts of the different carboxylic acids, 4 mM. CIT, citrate; CIS-ACON, *cis*-aconitate; Iso-CIT, isocitrate; αKET, α-ketoglutarate; MAL, malate; SUCC, succinate.

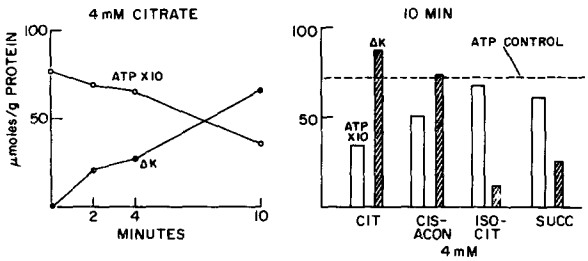

Fig. 4. Changes in the endogenous levels of ATP when K^+ is accumulated with citrate (left panel) and when K^+ is accumulated with other Krebs-cycle intermediates (right panel). The mitochondria, 2.4 mg per ml, were incubated at 20 °C, pH 7.0, in 5-ml solutions containing the citrate or other anions, 4 mM; succinate, 1 mM; KCl, 40 mM; sucrose, 150 mM.

TABLE IV

COMPARISON OF EFFECTS OF ANION CONCENTRATION, MEDIUM pH AND RESPIRATORY INHIBITION UPON THE ACCUMULATION OF ACETATE AND CITRATE

In three separate experiments mitochondria, 2.4–2.7 mg per ml, were incubated for 6 min at 20 °C, pH 7.0, in sucrose, 0.18 M; KCl or NaCl, 28 mM; sodium or potassium acetate, 6 mM; sodium or potassium citrate, 2 mM; succinate, 1 mM. In the acetate experiments, baseline contents of Na^+ or K^+ in the mitochondria were determined in samples added to chilled aliquots of the media which contained KCl or NaCl in place of potassium acetate or sodium acetate. Individual adjustments: (1) The higher concentrations of acetate and citrate displaced Cl^-. (2) The pH was controlled by adjusting the acidity of the acetate or citrate solutions. (3) The concentration of cyanide was 2 mM.

Medium	*External cation*	*Mitochondrial accumulation (μequiv/g protein)*	
		Acetate	*Citrate*
Anion concentration			
6 mequiv/l	K^+	10	98
40 mequiv/l	K^+	45	97
Medium pH			
pH 6.0	K^+	72	110
Hp 8.0	K^+	28	127
Respiratory inhibition			
None	Na^+	29	90
Add cyanide	Na^+	26	53
None	K^+	45	140
Add cyanide	K^+	15	47

vations of acetate accumulation, as measured in the absence of ionophores, refer usually to experiments in which the external concentration is in a still higher range (50–150 mM/l)[28,29,31–35]. The second difference listed for these two organic anions (Table IV) is the response to change in pH. With acetate there is a greater accumulation at the lower pH as would be expected as a consequence of the distribution of

a weak acid across a membrane separating two phases of different pH (alkaline inside). The importance of this relationship has been demonstrated particularly by the experiments of Palmieri and Quagliariello[36]. The lack of an effect on pH change with citrate may be due to a saturation of the mitochondrial capacity for accumulation of anions[20,24,37]. The third dissimilarity, as listed in Table IV, is the absence, with sodium acetate, of a significant response to respiratory activity. Uptakes of sodium citrate (as well as of potassium acetate and potassium citrate) are stimulated by respiratory activity and inhibited by cyanide. However, cyanide has very little effect on the accumulation of sodium acetate.

DISCUSSION

It is clear that two reactions must be recognized. Citrate is incorporated by a rapid exchange without coupling to the movement of Na^+ or K^+, and it is accumulated also by a slower, respiratory-dependent mechanism in which it enters with the cations. Parallelism between the accumulation of cations and of the trivalent anionic equivalence of the citrate is demonstrated here with reference to the progression of time (Fig. 1), with change from the inhibited to the uninhibited state (Table I) and in response to addition of permeating cations to the external media (Fig. 2). The purpose in the present investigation is to characterize the second, energy-dependent reaction. Although the rate is much reduced, the quantities taken up (15–30 μmoles per g protein) may exceed the amounts incorporated by exchange. As shown in Fig. 3, the effect is much larger with citrate than with other Krebs-cycle intermediates. The accumulation of potassium with succinate has been considered in several reports[18,21–24], but the quantity involved is relatively small, 5–10 μmoles per g protein. Valinomycin induces large uptakes of all the Krebs-cycle intermediates with potassium (refs 18, 19 and 38, see also Fig. 2). The results in the present study emphasize that very sizeable incorporations of either Na^+ or K^+ occur with citrate and without added ionophore.

Active accumulation of organic anions has frequently been interpreted as a response secondary to the generation of proton gradients across the mitochondrial membrane[7–10]. The first step is considered to be the splitting of water with extrusion of a proton and retention of the hydroxyl ion. The organic anion then enters as the undissociated acid, and once inside it yields the proton to combine with the inner hydroxyl group. The cation is then drawn in by electrophoretic attraction by the negatively charged anion[7,10]. This hypothesis presents a general explanation designed to account for accumulation of all permeant anions. If it is to explain the accumulation of citrate, the hypothesis must be modified to account for the special effectiveness with reference to other Krebs-cycle intermediates (Fig. 3) and to account also for the differences with respect to acetate (Table IV).

Whereas the results of the present studies emphasize rather specific characteristics for accumulation of citrate as the anion, they present examples of diminished selectivity with respect to the cations, Na^+ and K^+.

Mitochondria are usually regarded as highly cation-specific. They survive preparative procedures as potassium-containing even when isolated from solutions of NaCl (ref. 39), and they have been considered to have a potassium-specific pump[4–6]. In earlier studies, we have been impressed by experiments which demon-

strate that mitochondria swollen in sodium acetate will release this salt when resuspended in sucrose solutions but will continue to retain the endogenous K^{+40}. Such a bi-directional movement of the one cation without mobilization of the other is indicative of a large difference in permeability. In contrast, the results of the present study emphasize that both cations enter with citrate. The effects are somewhat larger with K^+ (Tables I and IV and Fig. 2), but the discrimination almost disappears when citrate rather than acetate is the cation (Table IV). Respiratory stimulation of uptake of sodium acetate from low external concentrations has not been reported.

The close matching between the equivalence of the uptakes of citrate and of the cations suggests ion-pair transport or the possibility of role for citrate as a carrier. In this regard the special effectiveness of citrate in comparison to other Krebs-cycle intermediates is noted (Fig. 3) as is also the rapid turnover demonstrated by the results of Table II.

Reactions in which citrate is accumulated in exchange for dicarboxylic acids have been described[27,41]. These can account for entrance of one cation with one molecule of citrate. However, such a mechanism does not appear to be involved since the respiratory-dependent accumulation of citrate is associated with gain of an equal equivalence of the cations, with three rather than one K^+ or one Na^+ (Figs 1 and 2 and Table I).

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