Factors Affecting the Kinetics and Equilibrium of Exchange Reactions of the Citrate-transporting System of Rat Liver Mitochondria*

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SUMMARY

Benzene 1,2,3-tricarboxylic acid was found to be a sufficiently potent inhibitor of citrate transport by rat liver mitochondria that it may be used in "inhibitor stop" experiments. It was thus possible to measure the initial rates of exchange reactions catalyzed by the citrate-transporting system and to study the dependence of the rate on temperature and the concentrations of citrate, malate, and Mg^{2+}. Both the rate and extent of exchange of L-malate with intramitochondrial [^{14}C]citrate were less than that of citrate with intramitochondrial [^{14}C]citrate. This difference in the rate and extent between the two exchanges was thought to be due to the fact that L-malate-[^{14}C]citrate exchange resulted in a disequilibrium of either charge or pH across the mitochondrial membrane. It is proposed that malate^{2-} exchanges for citrate^{2-} rather than citrate^{3-}, thereby setting up a pH differential which restricts further exchange.

The operation of a tricarboxylate-malate antiport mechanism across the inner mitochondrial membrane has been shown both directly and indirectly in a number of laboratories (1–6). The kinetics of the oxidation of citrate, isocitrate, and cis-aconitate as shown by mitochondrial NAD(P) reduction and the dependence of these oxidations on the presence of L-malate have recently been investigated (7). However, such measurements suffer from the disadvantage that the rate-limiting factor may not be the transport system; *i.e.* rates of entry are monitored by following the oxidation of the transported compound by substrate dehydrogenases. Studies of citrate transport and exchange in isolated mitochondria have suffered from the absence of time resolution (6, 7), this problem being due to the lack of a specific inhibitor for the citrate transporting system. Benzene 1,2,3-tricarboxylic acid is an inhibitor of tricarboxylate transport (8) and we report here its use in "inhibitor stop" experiments (9) designed to elucidate factors affecting the kinetics and equilibrium of this transporting system.

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EXPERIMENTAL PROCEDURE

Materials and Methods—Rat liver mitochondria were prepared as described previously (2). Inhibitor stop experiments were carried out as described by Robinson and Williams (10). Rat liver mitochondria were loaded with [^{14}C]citrate as described by Robinson *et al.* (6).

Citrate was measured by the method of Moellering and Gruber (11), *threo*-D$_s$-isocitrate by the method of Siebert (12), and L-malate by the method of Hohorst (13). Fluorimetric recording of pyridine nucleotide fluorescence changes were made with an Eppendorf fluorimeter.

threo-D$_s$-Isocitrate was obtained from Sigma and benzene 1,2,3-tricarboxylic acid from either K and K Rare Chemicals, Plainsview, New York, or Aldrich.

When "mitochondria loaded with [^{14}C]citrate" are referred to in this paper, it must be borne in mind that small amounts of [^{14}C]isocitrate and [^{14}C]*cis*-aconitate are also present in these mitochondria as dictated by the aconitate hydratase equilibrium (6). Thus all experiments involving exchange of labeled citrate suffer from the disadvantage of having small amounts of the other labeled tricarboxylic acids exchanging at the same time. Since *cis*-aconitate and isocitrate appear to behave as molecules similar to citrate in respect to the tricarboxylate-transporting system (7), we have assumed that their presence and probable participation in the observed ^{14}C label exchanges do not invalidate the results presented here.

RESULTS

Rat liver mitochondria were loaded with [^{14}C]citrate as described in Reference 6 and the exchange of extramitochondrial [^{12}C]citrate (1 mM) or [^{12}C]malate (1 mM) with the labeled material was investigated at 10°. Increasing concentrations of benzene 1,2,3-tricarboxylate reduced the observed exchanges until almost complete inhibition occurred at 25 mM inhibitor (Fig. 1). 2-Ethylcitrate used at 10° rather than 0° (6) was not nearly as

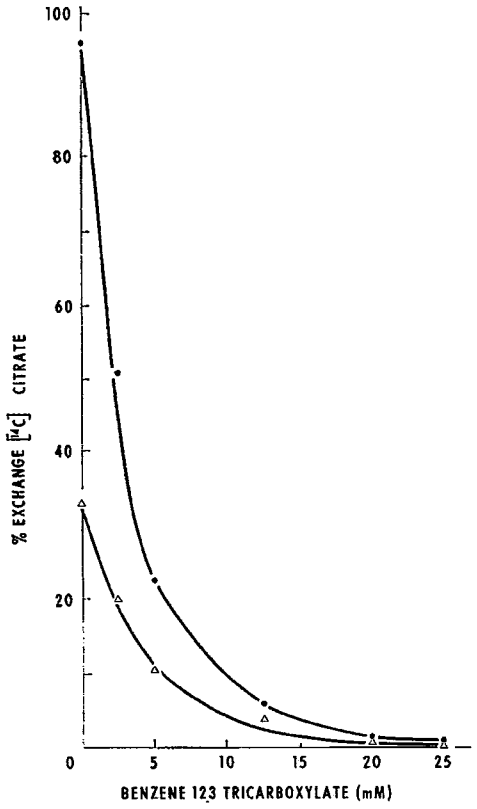

FIG. 1. Benzene 1,2,3-tricarboxylate inhibition of citrate-[^{14}C]citrate and malate-[^{14}C]citrate exchange. Rat liver mitochondria (samples containing 8 mg of protein) loaded with [^{14}C]-citrate were added to 1-ml incubations of a medium containing 125 mM KCl, 20 mM Tris Cl (pH 7.4) at 10°. Increasing concentrations of benzene 1,2,3-tricarboxylate were included in the incubations with either 1 mM citrate or 1 mM malate. After standing for 2 min the mitochondria were separated by centrifugation and the supernatants were deproteinized and evacuated as described in Reference 6 and counted for [^{14}C]citrate. An unseparated sample was also treated and counted to allow calculations of the total [^{14}C]citrate in the system. Control incubations with no citrate or malate added extramitochondrially were also included. The extent of exchange over the time period used was expressed as percentage exchange (6). This is defined as:

$$\frac{\text{Supernatant disintegrations per min in incubation} - \text{supernatant disintegrations per min in control incubation}}{\text{mitochondrial disintegrations per min in control incubation}} \times \frac{100}{1}$$

●——●, 1 mM citrate; △——△, 1 mM L-malate.

effective an inhibitor as benzene 1,2,3-tricarboxylate. Benzene 1,2,3-tricarboxylate (50 mM) gave complete inhibition of both citrate-[^{14}C]citrate and L-malate-[^{14}C]citrate exchange. It is interesting to note that 50% inhibition of exchange occurred at about 3 mM inhibitor for both these exchanges.

Rate of Exchange—Having established the fact that 50 mM inhibitor was capable of fully stopping the exchange, it was decided to follow the time course of exchanges mediated by the citrate-transporting system using the inhibitor stop technique. Incubations were performed at 10°, the exchanges being started by the addition of [^{14}C]citrate-loaded mitochondria to 1-ml incubations of KCl-Tris-Cl buffer containing either 1 mM un-

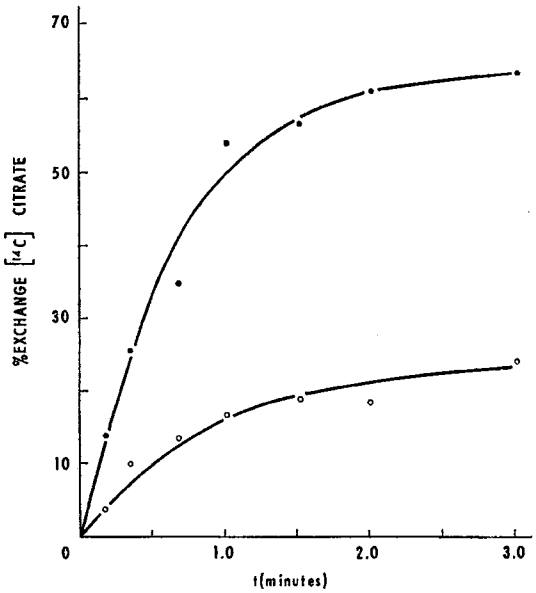

FIG. 2. Time course of citrate-[^{14}C]citrate and malate-[^{14}C]-citrate exchanges as measured by inhibitor stop technique. See text for description of experiment. ●——●, 1 mM citrate; O——O, 1 mM L-malate.

labeled tricarboxylate anion or 1 mM L-malate. After time intervals of 0 to 2 min the exchange was terminated by the rapid addition of benzene 1,2,3-tricarboxylate to 50 mM and the supernatant was separated by centrifugation. After treatment with perchloric acid, neutralization with potassium carbonate, and evacuation over KOH to remove $^{14}CO_2$ (6), samples of the supernatant were counted as described previously (6). An uncentrifuged sample of mitochondria was treated in a similar manner to allow estimation of the total [^{14}C]citrate in the system. Fig. 2 shows the time course of citrate-[^{14}C]citrate and malate-[^{14}C]citrate exchanges with 1 mM [^{12}C]citrate or L-malate present extramitochondrially. Both the rate and extent of citrate-[^{14}C]citrate exchange exceeded that of L-malate-[^{14}C]citrate exchange, although the rate varied from 1.9 to 6.8 nmoles per min per mg of protein for malate-[^{14}C]citrate exchange and 3.5 to 8.0 nmoles per min per mg of protein for citrate-[^{14}C]citrate exchange at 10°.

When the variation of the rate of L-malate-[^{14}C]citrate exchange with temperature was investigated by following the time courses of the exchange with the inhibitor stop technique, it was found that the rate of exchange approximately doubled for every 5° rise in temperature over a range from 0–20° (Fig. 3). The initial rate of exchange at 20° for 1 mM L-malate with [^{14}C]citrate in this experiment was 27 nmoles per min per mg of protein. A doubling in rate for every 5° increase was also found for citrate-[^{14}C]citrate exchange.

The concentration dependence of the initial rate of citrate-[^{14}C]citrate exchange was determined by performing inhibitor stops after exchange had been permitted to take place for 15 sec at varying substrate concentrations. Similarly, for L-malate-[^{14}C]citrate exchange, inhibitor stops with 50 mM benzene 1,2,3-tricarboxylate were performed after 10 sec at varying L-malate concentrations. This method of determining rates assumes linearity of reaction up to the time of addition of the inhibitor (see Fig. 2). This assumption, although probably correct for these exchanges at most substrate concentrations, may be invalid

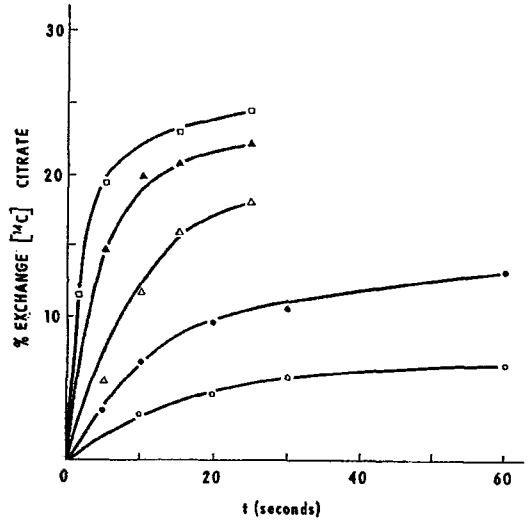

FIG. 3. Temperature dependence of L-malate-[^{14}C]citrate exchange. The experiment was performed as described for Fig. 2 with 1 mM L-malate as the exchanging anion at 0° (○——○), 5° (●——●), 10° (△——△), 15° (▲——▲), and 20° (□——□). Mitochondrial protein (9.1 mg) was added to each incubation and the initial total intramitochondrial citrate was 8.9 nmoles per mg of mitochondrial protein.

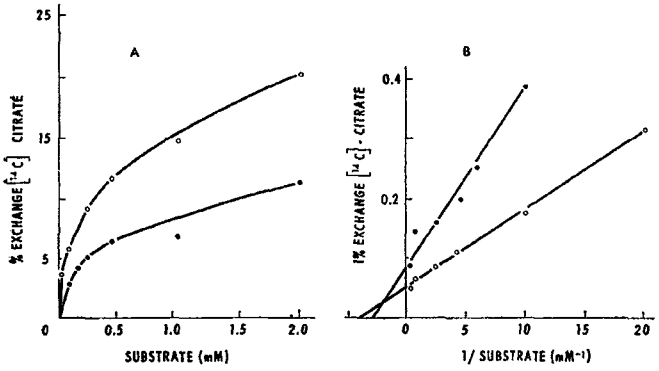

FIG. 4. The concentration dependence of the exchange of L-malate and citrate for [^{14}C]citrate. Rat liver mitochondria (8 mg of protein) loaded with [^{14}C]citrate were rapidly pipetted into 1-ml incubations of KCl-Tris Cl buffer at 10° containing various concentrations of citrate (○——○) or L-malate (●——●). After 15 sec for citrate and 10 sec for L-malate the exchange was stopped by the addition of 50 mM benzene 1,2,3-tricarboxylate and the mitochondria were separated by centrifugation (10). The supernatants were deproteinized, evacuated, and counted as described previously (10). *A* is a plot of percentage exchange against concentration while *B* is a plot of the reciprocals of these values.

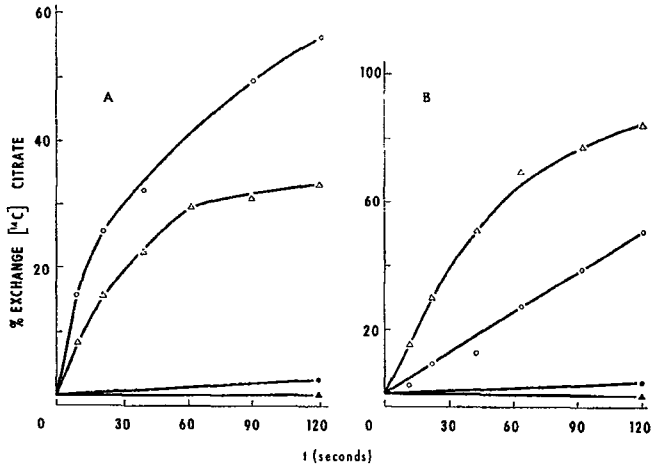

FIG. 5. The effect of Mg^{2+} on the time course of citrate-[^{14}C]-citrate and L-malate-[^{14}C]citrate exchange. The experiments were carried out as described for Fig. 2 with 1 mM L-malate (△——△) and 1 mM L-malate plus 5 mM Mg^{2+} (○——○) in *A* and 1 mM citrate (△——△) and 1 mM citrate plus 5 mM Mg^{2+} (○——○) in *B*. Controls were carried out in each experiment with 50 mM benzene 1,2,3-tricarboxylate added at zero time with 1 mM L-malate (▲——▲) and 1 mM L-malate plus 5 mM Mg^{2+} (●——●) in *A* and 1 mM citrate (▲——▲) and 1 mM citrate plus 5 mM Mg^{2+} (●——●) in *B*.

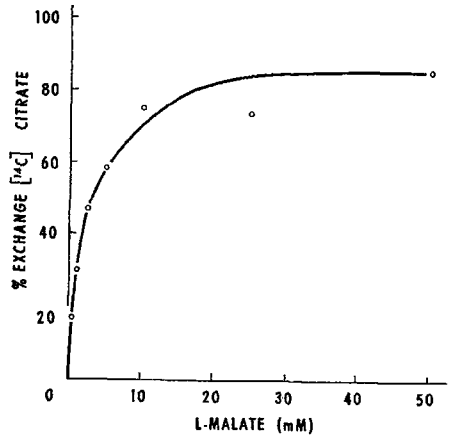

FIG. 6. The dependence on L-malate concentration of the equilibrium position of malate-[^{14}C]citrate exchange. The experiment was carried out as described in Table I.

at low concentrations of L-malate at which the extent of the exchange at equilibrium may be small. Control incubations performed with 50 mM benzene 1,2,3-tricarboxylate present at zero time showed only slight exchange (>1.5%) at the highest substrate concentration used. Again the maximum rate obtained with L-malate (Fig. 4) was lower than that obtained with citrate. Lineweaver-Burk plots of these data gave K_m values of 0.25 mM for citrate and 0.35 mM for L-malate. The rates of exchange of *cis*-aconitate and *threo*-D_s-isocitrate with [^{14}C]citrate slightly exceeded that of citrate, and the K_m values were 0.22 mM for both these species. Similar K_m values were obtained following the time courses at 5°. The presence of 5 mM Mg^{2+} in the incubation medium caused both the rate and extent of L-malate-[^{14}C]citrate exchange to be increased (Fig. 5) while decreasing the rate of citrate-[^{14}C]citrate exchange.

Extent of Exchange—The exchange of L-malate with [^{14}C]citrate was investigated by allowing the added L-malate to exchange for a period of 3 min before separating the mitochondria by centrifugation. This was done so that the exchange would proceed to equilibrium in a fashion similar to that seen when time courses were followed. Allowing exchanges at 10° to proceed for periods longer than 3 min (plus centrifugation time) did not give any further extent of exchange beyond that seen after 3 min of incubation.

It was found that the extent of exchange increased with L-malate concentration (Fig. 6). The supernatant and pellets obtained after centrifugation in this experiment were analyzed

TABLE I

Variation of citrate distribution at equilibrium with calculated malate gradient

Protein aliquots (8.5 mg) of rat liver mitochondria loaded with [^{14}C]citrate were added to 1-ml incubations of a medium containing 125 mM Tris HCl, pH 7.4, at 10° with various concentrations of L-malate included. After 3 min the mitochondria were separated by centrifugation and samples of supernatant and pellet were prepared as described in Reference 8 for assay and counting of radioactive citrate and assay of L-malate. For calculations of intramitochondrial malate concentrations see the text.

L-Malate		Citrate		$[Citrate]_{in}/[citrate]_{out}$	$[Malate]_{out}/[malate]_{out}$
Extramitochondrial	Intramitochondrial	Extramitochondrial	Intramitochondrial		
mM		μM			
0.05	2.4[a]	9.1	7200	793	48
0.52	3.7	20.4	5870	286	7.1
1.05	4.7	28.7	4920	173	4.5
2.55	5.9	39.1	3710	96	2.3
5.0	6.7	45.6	2940	64	1.3
10.0	7.8	55.6	1770	32	0.78
25.0	7.7	54.6	1860	33	0.39
50.0	8.5	62.0	1060	17	0.17

[a] Measured.

for citrate and malate as described in Reference 6 and the resulting equilibrium concentrations of intramitochondrial citrate and L-malate were calculated assuming an intramitochondrial water space of 1 μl per mg of protein (Table I). Calculations of the equilibrium concentration ratios of citrate for each applied malate concentration were made and the log of the citrate concentration ratio was plotted against the log of the corresponding ratios of malate concentration (Fig. 7). In a number of experiments this plot was approximately linear, with a slope of 1.4 to 1.8.

Effect of Uncoupling Agents, Valinomycin, and Phosphate—Experiments designed to test the effect of uncoupling agents such as FCCP[1] upon malate-citrate exchange are complicated by the fact that FCCP alone causes efflux of labeled citrate from the mitochondria. However, this efflux is inhibited by 25 mM pentylmalonate. It has been shown previously that this inhibitor is without action on malate-citrate or citrate-citrate exchange (8). It is thus possible to study the effect of FCCP on these exchanges by performing experiments in the presence of 25 mM pentylmalonate. Comparisons of Columns 3 and 5 of Table II shows that the presence of uncoupling agent has increased the extent of malate-citrate exchange on a carrier system which must be insensitive to pentylmalonate and under conditions in which the uncoupler promoted efflux is completely inhibited.

A similar situation was observed with inorganic phosphate. Increasing concentrations of P_i gave extensive exchange with [^{14}C]citrate after a 3-min incubation but this was inhibited by 25 mM pentylmalonate. However, when L-malate was present with the P_i under these inhibited conditions, the extent of exchange of L-malate with [^{14}C]citrate was much greater than in the absence of P_i (Table III). The data of Table III could of course be equally well described as an overcoming by malate of the pentylmalonate inhibition of P_i-induced citrate efflux, but

[1] The abbreviation used is: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

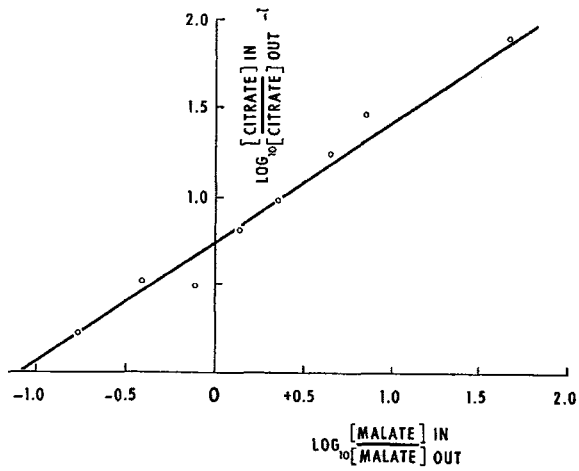

FIG. 7. A graph to show the relationship between the log of the citrate equilibrium gradient and the log of the applied L-malate gradient. Values for the malate and citrate gradients were taken from Table I.

TABLE II

Effect of FCCP on equilibrium of malate-[^{14}C]citrate exchange

Incubations were carried out as described for Fig. 1 with FCCP, pentylmalonate, and L-malate at the concentrations shown.

L-Malate	Exchange			
	No additions	+25 mM pentylmalonate	+1 μM FCCP	+1 μM FCCP + 25 mM pentylmalonate
mM	%			
0	0	0	18.3	0
0.2	13.6	13.8	28.0	27.8
0.5	19.3	19.6	38.8	34.2
5.0	59.0	64.0	70.5	74.2

TABLE III

Effect of phosphate on equilibrium of malate-[^{14}C]citrate exchange

Incubations were carried out as described for Fig. 1.

Anion	Concentration	Exchange			
		No other additions	+25 mM pentylmalonate		
			+0	+0.2 mM L-malate	+0.5 mM L-malate
	mM	%			
None		0	0	11.9	23.0
Phosphate	1.0	25.9	2.3	16.8	30.2
	5.0	37.2	1.1	25.7	32.4
	10.0	36.9	1.1	26.5	37.7

we have chosen to interpret our findings in terms of a set of carriers none of which can bring about a direct P_i-citrate exchange (1, 3, 8, 14, 15) and therefore speak of an effect of inorganic phosphate upon malate-citrate exchange.

Valinomycin added to mitochondria containing [^{14}C]citrate caused little loss of ^{14}C, but in the presence of L-malate the rate of L-malate-[^{14}C]citrate exchange was decreased. When included with increasing concentrations of either citrate or L-malate in a

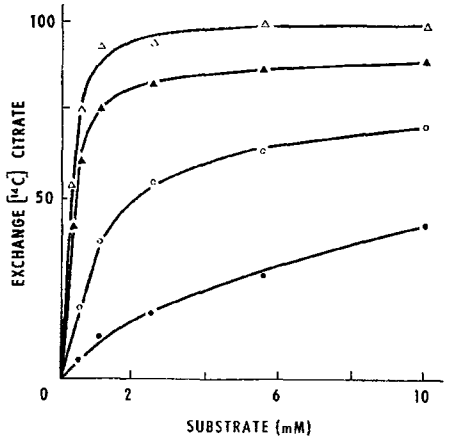

FIG. 8. The effect of valinomycin on the equilibrium of citrate-[^{14}C]citrate and L-malate-[^{14}C]citrate exchanges as a function of citrate and L-malate concentration. The experiment was carried out as described for Table I with increasing concentrations of citrate (△——△), citrate plus 1 μg per ml valinomycin (▲——▲), L-malate (○——○), and L-malate plus 1 μg per ml valinomycin (●——●).

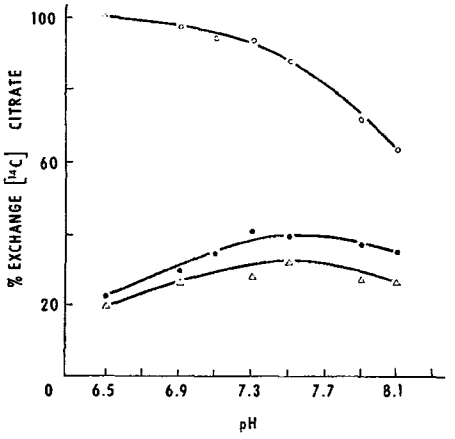

FIG. 9. The effect of pH on the equilibrium of citrate-[^{14}C]-citrate and L-malate-[^{14}C]citrate exchanges. The experiment was carried out as described for Table I with 125 mM KCl, 20 mM Tris chloride medium at the pH values shown at 10°; 1 mM citrate (○——○), 1 mM L-malate (●——●), 1 mM L-malate + 25 mM pentylmalonate (△——△).

3-min incubation experiment with [^{14}C]citrate-loaded mitochondria (Fig. 8), valinomycin reduced the extent of L-malate-[^{14}C]-citrate exchange drastically while having a less marked effect on the extent of citrate-[^{14}C]citrate exchange.

Effect of pH—When the extent of exchange was monitored at varying pH it was found that the L-malate-[^{14}C]citrate exchange was optimal at about pH 7.3, while that of citrate-[^{14}C]citrate exchange showed a marked decline at alkaline pH (Fig. 9). Inclusion of 25 mM 2-pentylmalonate in the exchange incubations did not change the effect of pH on L-malate-[^{14}C]citrate exchange.

DISCUSSION

Rates of Exchange—The rates of exchange observed by the inhibitor stop technique are important since rates of transport by the citrate-transporting system have not been measured independently of the dehydrogenases. Some variability in the rates of exchange were found at 10°, this probably being due to the presence of variable amounts of endogenous intramitochondrial L-malate. Since a doubling of rate of exchange appears to take place every 5°, we may calculate that at 30° the rate of L-malate-[^{14}C]citrate exchange would be 30 to 110 nmoles per min per mg of protein. Since transport rates of 50 nmoles per min per mg of protein have been observed by following pyridine nucleotide reduction by *threo*-D$_s$-isocitrate in intact rat liver mitochondria (7), there seems to be little discrepancy between the rate measured by exchange and that measured by oxidation. However, the K_m values obtained for the tricarboxylic acids (0.22 to 0.25 mM) by exchange were different from those obtained by following oxidation (0.061 mM). However, it may not be valid to compare either the K_m values or the rates of transport obtained by the two methods. The presence of 1 to 3 mM endogenous intramitochondrial L-malate (Table I) in the [^{14}C]-citrate exchange system may alter the kinetics of exchange, making the situation different from that in a system such as is used for oxidation, in which the endogenous L-malate is removed by preliminary incubation with uncoupling agent. The presence of the uncoupling agent itself when the kinetics is followed by oxidation (7) is yet another complicating factor. The situation in which endogenous substrate is present may be much closer to the *in vivo* mitochondrial situation and as such is a valuable model to work with. However, experiments are now being designed so that exchange kinetics may be measured in the absence of this endogenous malate pool.

Equilibrium Positions of Transporting System—The inability of low concentrations of L-malate to promote a complete exchange with [^{14}C]citrate was puzzling. If only a 1:1 stoichiometry of exchange of malate for citrate was involved, it might be expected that citrate would be released from the mitochondria, down the citrate concentration gradient. However, as mentioned above, the situation is complicated by a malate concentration gradient, operating in the same direction as the citrate concentration gradient, when low concentrations of L-malate are used to promote exchange.

Harris and Pressman (16) suggested that the distribution of anion across the mitochondrial membrane would be influenced by a pH differential, the distribution being a function of both charge and concentration so that for any anion A with charge n the following equation applies:

$$\frac{[OH^-]_{in}}{[OH^-]_{out}} = \frac{[A^-]^n_{in}}{[A^-]^n_{out}} \quad (1)$$

The subscripts in and out apply to the intra- and extramitochondrial compartments.

In these experiments the extramitochondrial volume is so far in excess of the intramitochondrial space that the final intramitochondrial concentration could be measured only at the lowest concentration of extramitochondrial malate used (Table I, Line 1).

We have therefore calculated the intramitochondrial concentrations of malate on the assumption that the decrease in intramitochondrial citrate concentration is accompanied by an equivalent increase in malate. There is direct experimental justification for this assumption (*e.g.* Table VB of Reference 15). One may then construct a log-log plot of measured citrate concentrations at equilibrium against calculated malate concentra-

tions. The observed slope of Fig. 7 corresponds to the equation

$$1.5 \log_{10} \frac{[\text{Citrate}]_{in}}{[\text{Citrate}]_{out}} = \log_{10} \frac{[\text{malate}]_{in}}{[\text{malate}]_{out}} + \text{constant}$$

It should be observed that the slope of this log-log plot is relatively insensitive to errors introduced by the assumptions involved in calculating the intramitochondrial malate. Indeed, even if one assumes no exchange to have taken place (*i.e.* one uses the value of 2.4 mM throughout), the slope changes only to a value of 1.6. It is probably therefore justifiable to write

$$\left(\frac{[\text{Citrate}]_{in}}{[\text{Citrate}]_{out}}\right)^3 \propto \left(\frac{[\text{malate}]_{in}}{[\text{malate}]_{out}}\right)^2$$

so that the proposal that mitochondrial anion distribution is a function of charge appears to be correct.

The effects of Mg^{2+} on the equilibrium position of the L-malate-[^{14}C]citrate exchange can be interpreted in light of the above observations. Since the presence of Mg^{2+} causes a decrease in free citrate concentration by formation of magnesium citrate, its presence outside the mitochondria dictates that more citrate has to exit when a malate gradient is applied, to give the same value of $[\text{citrate}]_{in}/[\text{citrate}]_{out}$ at equilibrium as would be obtained in the absence of Mg^{2+}.

Since citrate-[^{14}C]citrate exchange does not appear to be restricted by unequal charge phenomena, Mg^{2+} decreases only the rate and not the extent of exchange, again by decreasing the effective extramitochondrial citrate concentration.

Effect of Valinomycin, FCCP, and Phosphate on Equilibrium—The efflux of citrate from mitochondria may occur either as an exchange of citrate^{3-} for malate^{2-} (*A*) or citrate^{2-} for malate^{2-} (*B*) (Fig. 10). In the former case an excess of positive charges on the inside would build up and tend to restrict the exchange since charge equilibrium is not achieved. In the latter case a build-up of OH^- would result, as, in effect, protons are being exported, and again the exchange would be restricted, this time by a pH gradient. The fact that valinomycin inhibits both the rate and extent of exchange would favor Mechanism *B* rather than *A* since if Case *A* were true then valinomycin would stimulate the exchange by allowing charge equilibrium as K^+ moves outward. Valinomycin, since it can transport K^+ but not protons (17), cannot alleviate the alkalinization which occurs in Mechanism *B*. Uncoupling agents, such as FCCP, as proton conductors (18) may alleviate Situation *B* but not Situation *A* and, in fact, since uncouplers consistently stimulate both the rate and extent of exchange, Mechanism *B* is favored. Phosphate as a potential proton-transporting agent can also remove intramitochondrial OH^- either by $H_2PO_4^-$-OH^- antiport on the phosphate-transporting system or by dissociation of intramitochondrial $H_2PO_4^-$ and H^+ at physiological values of pH. The fact that either uncoupling agent or phosphate added alone results in a considerable efflux of [^{14}C]citrate from the mitochondria is especially interesting since the efflux is blocked by 2-pentylmalonate, an inhibitor of the dicarboxylate-transporting system. This sensitivity indicates that some function of the dicarboxylate carrier is involved. It is proposed that the phosphate or FCCP by virtue of their pH equilibrium properties allows the small amount of exogenous L-malate present (15 to 50 μM) to promote exchange with [^{14}C]citrate. For this process to continue the malate entering on the tricarboxylate carrier must re-exit via the dicarboxylate carrier. Inhibition of this carrier results in the arrest of the exchange at an early state. When FCCP or phosphate is absent little exchange takes place with endogenous L-malate because the citrate^{2-}-malate^{2-} exchange sets up a pH differential, thus restricting further exchange. The pH profiles of citrate-[^{14}C]citrate and L-malate-[^{14}C]citrate exchange are also of help in determining the number of charges carried in the exchange. Mechanism *A*, since it requires citrate^{3-} to be transported, would be expected to show a reduction of citrate-citrate exchange at lower pH values, the concentration of citrate^{3-} decreasing with pH. Conversely Mechanism *B* would show a reduction at higher pH values, as in the case shown in Fig. 9. Also Mechanism *B* would favor a reduction of malate-citrate exchange at lower pH values, which is the case, since the malate^{2-}-citrate^{2-} exchange system is producing extramitochondrial protons. However, this effect is not so marked, this perhaps being due to charged groups on the transport system being affected by pH.[2]

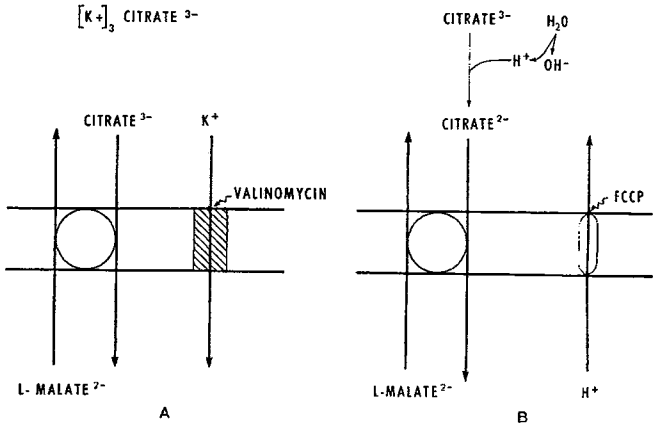

FIG. 10. Diagram to show two possible mechanisms of operation of the citrate-L-malate exchange and the effects of valinomycin and FCCP on these mechanisms. For explanation see text.

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REFERENCES

1. CHAPPELL, J. B., AND HAARHOFF, K. N., in E. C. SLATER, Z. KANIUGA, AND L. WOJTCZAK (Editors), *Biochemistry of mitochondria*, Academic Press, London, 1966, p. 75.
2. ROBINSON, B. H., AND CHAPPELL, J. B., *Biochem. Biophys. Res. Commun.*, **28**, 249 (1967).
3. CHAPPELL, J. B., AND ROBINSON, B. H., *Biochem. Soc. Symp.*, **27**, 123 (1968).
4. HARRIS, E. J., in S. PAPA, J. M. TAGER, E. QUAGLIARIELLO, AND E. C. SLATER (Editors), *The energy level and metabolic control of mitochondria*, Adriatica Editrice, Bari, 1969, p. 31.
5. MEIJER, A. J., TAGER, J. M., AND VAN DAM, K., in S. PAPA, J. M. TAGER, E. QUAGLIARIELLO, AND E. C. SLATER (Editors), *The energy level and metabolic control of mitochondria*, Adriatica Editrice, Bari, 1969, p. 147.
6. ROBINSON, B. H., WILLIAMS, G. R., HALPERIN, M. L., AND LEZNOFF, C. C., *Eur. J. Biochem.*, **15**, 263 (1970).
7. ROBINSON, B. H., AND CHAPPELL, J. B., *Biochim. Biophys. Acta*, **205**, 300 (1970).

[2] Since the original submission of this paper the recent publication of Papa *et al.* (15) has become available to us. These authors have also reached the conclusion that the malate-citrate transporter catalyzes the exchange of doubly charged anions.