ENERGY-LINKED INCORPORATION OF CITRATE INTO RAT LIVER MITOCHONDRIA

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Until recently it has been thought that isolated mitochondria were relatively impermeable to externally added citrate (Schneider, Striebich and Hogeboom, 1956; Bartley and Davies, 1953; Amoore, 1958). Chappell (1964) has shown that added tricarboxylic acids are not oxidized at significant rates and intra-mitochondrial pyridine nucleotides are not reduced if mitochondria are pre-incubated with ADP + P_i or with uncoupling agents. The addition of L-malate is now required before citrate can be oxidized at normal rates. Williams (1965) has come to similar conclusions by continuously monitoring the decarboxylation of citrate. More recently, Gamble (1965) has shown that isolated rabbit-liver mitochondria can accumulate and retain citrate against large concentration gradients. Although 2,4-dinitrophenol blocked the incorporation of citrate, respiration was not required. In this paper evidence will be presented that citrate is incorporated into the matrix of isolated rat-liver mitochondria and that this incorporation requires non-phosphorylated, high-energy intermediates of oxidative phosphorylation derived either from the oxidation of substrate or from ATP.

The incorporation of citrate into mitochondria (cf. Table 1) was stopped by the addition of antimycin a, diluted

and immediately centrifuged at 0^{o}. The mitochondria were washed twice to remove extraparticulate citrate and citrate trapped between the outer and inner membranes in a medium containing 250 mM sucrose, 5 mM Na succinate and 2 mM $MgCl_2$. Klingenberg (1965) has shown that small molecules under the molecular weight of inulin rapidly penetrate the outer membrane but are rapidly washed out of the space between the inner and outer membranes. Since the citrate incorporated in these experiments is not washed out by the washing procedure and is retained against concentration-gradients greater than 1000:1 during the washing procedure, it is concluded that citrate is incorporated across the inner membrane into the matrix of the mitochondria. Experiments with sucrose - ul-C^{14}, which is completely washed out of the mitochondria, show that it is the sucrose-impenetrable space which citrate enters. Zero time controls were done to monitor contamination carried by the washing procedure and are subtracted from all reported values. This contamination is less than 0.25% of the exogenous citrate concentration.

The aerobic incorporation of citrate-1,5-C^{14} as a function of time and the effect of succinate on that incorporation is shown in Fig. 1. If the concentration of citrate in the mitochondria at the plateau is C_{∞}, and the amount in the mitochondria at various times is C_{in}, a plot of $\frac{C^{\infty} - C^{in}}{C^{\infty}}$ vs. time according to the method of Horecker and Monod (1960) yields a straight line characteristic of a first-order reaction, the slope of which equals the exit constant. As can be seen from B., succinate has very little effect on the exit of citrate. An exit constant of 0.20 $min.^{-1}$ indicates that 20% of the citrate entering exits per minute. The entry:exit ratio in the presence and absence of succinate is 5:1. The

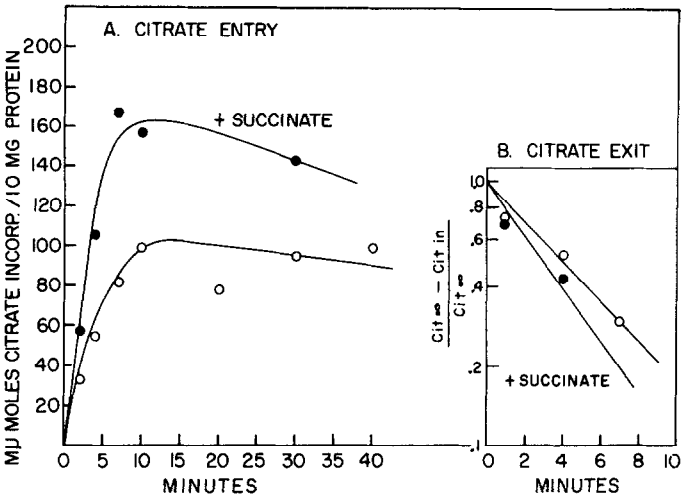

Figure 1: The Effect of Succinate on the Entry and Exit of Citrate-1, 5-C^{14} as a Function of Time

All incubations contained, in addition to 20 mM Na citrate-1, 5-C^{14}, a basic salts medium composed of NaCl, 80 mM; KCl, 4 mM; $MgCl_2$, 10 mM; TRIS-HCl, pH 7.4, 10 mM; sucrose, 62 mM; and nicotinamide, 40mM. Curves: o--o, no additions; •--•, Na succinate, 10 mM. Volume 1.2 ml., 24°, mitochondrial protein 8 mg. Incorporation stopped by addition of antimycin a (2.5 µg/mg. protein), and dilution in wash medium at 0°.

effect of succinate in A. is to increase the entry of citrate into the mitochondria. Direct assays for citrate in the mitochondria by Lowenstein's (1965) modification of the method of Saffran and Denstedt (1948) show that 80% of the counts in the mitochondria can be accounted for as citrate. Thin-layer chromatography on Silica Gel G confirms the accumulation of citrate.

The incorporation of citrate obeys Michaelis-Menton Saturation Kinetics. A Lineweaver-Burke plot of 1/V vs. 1/S in the absence of P_i shows the Km for citrate to be 5.2 mM.

The incorporation of citrate into mitochondria in the resting state can be increased by the addition of Krebs Cycle

intermediates and inhibited by various respiratory inhibitors. Table I shows two experiments with 20 mM citrate. Incorporation of citrate alone is supported for the most part by the State 4 respiration of endogenous substrate. Addition of malate only stimulates slightly but succinate stimulated 2-fold. This stimulation by succinate is probably due to the higher State 4 oxidation rate and higher level of high-energy intermediates. Addition of oligomycin, not shown in the table, doubles the incorporation of citrate and increases the incorporation of citrate 40% in the presence of succinate. This suggests that (1) high-energy intermediates are being hydrolyzed in the absence of oligomycin and that (2) ATP and its phosphorylated precursors are not involved in the incorporation but the energy is supplied directly by non-phosphorylated, high-energy intermediates generated by the respiratory chain. Both rotenone, which blocks the action of DPN-linked dehydrogenases, and malonate, which blocks succinate oxidation, inhibit the resting-state incorporation. If malate is added to the malonate-inhibited mitochondria, the incorporation is stimulated 2-fold; if succinate is added to the rotenone-inhibited system, the incorporation is stimulated 3-fold. In experiment 2, the succinate-supported incorporation is uncoupled by 2,4-DNP. If antimycin a is added to block the respiratory chain, the incorporation is severely inhibited. Now ATP in the presence of succinate can drive the incorporation of citrate into the mitochondria, and this ATP-driven incorporation is completely oligomycin-sensitive. Oligomycin completely blocks the generation of high-energy intermediates from ATP. This ATP driven, oligomycin-sensitive incorporation is greater than the aerobi-

TABLE I

EFFECT OF KREBS CYCLE INTERMEDIATES AND VARIOUS INHIBITORS ON CITRATE INCORPORATION

All incubations contained: 20 mM Na citrate-1,5-C^{14} in basic salts medium. Where indicated: Na malate, 5 mM; Na succinate, 5 mM; Na glutamate, 5 mM; Na malonate, 20 mM; rotenone, 4 μM; ATP, 3 mM; 2,4-DNP, 0.05 mM; antimycin a 1 μg/mg., oligomycin 2.5 μg/mg. Incubation time 10 min. 25°C. Volume 1.20 ml. Mitochondrial Protein Exp. 1, 13.8 mg., Exp. 2, 10.5 mg. Incorporation stopped as in Fig. 1.

	Additions	mμMoles Citrate Incorporated/10 mg.
1.	none	144
	rotenone	88
	malonate	77
	malate	181
	succinate	331
	malonate, malate	160
	rotenone, succinate	238
2.	none	119
	malate, glutamate	159
	succinate	290
	succinate, 2,4-DNP	92
	succinate, antimycin a	42
	succinate, antimycin, ATP	333
	succinate, antimycin, ATP, oligomycin	34

cally supported incorporation. Fig. 2 shows the ATP-driven incorporation of citrate in an antimycin a-blocked system as a function of time. The oligomycin inhibition of this ATP-driven incorporation is shown in the lower tracing. The exit constant is calculated in the insert. This constant, k_{ex}, is almost identical with the exit constant calculated under aerobic conditions with succinate oxidation providing the energy.

The incorporation of citrate in an antimycin a blocked system depends on the ATP concentration in the incubation medium. The incorporation under these conditions shows a typical Michaelis-Menton saturation curve and is linear from

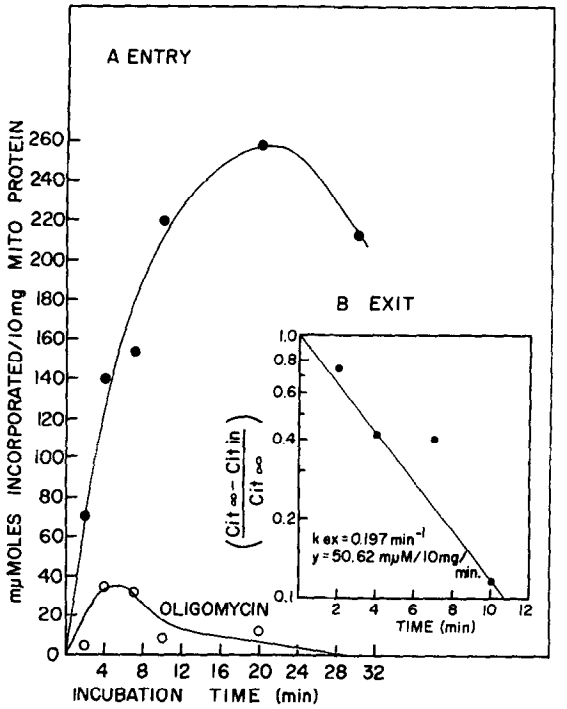

Figure 2: ATP as a Source on Energy for the Incorporation of Citrate-1,5-C^{14} Into Mitochondria in an Antimycin a-Blocked System

All incubations contain 20 mM Na citrate-1,5-C^{14} in addition to basic salts medium. All tubes also contained: ATP, 3 mM, and antimycin a (1 ug/mg. protein). Curves: ●—● no oligomycin, o—o oligomycin (2.5 ug/mg. protein). 25°C. Volume 1.2 ml. Mitochondrial Protein 10.5 mg. Incorporation stopped by dilution in wash medium at 0°.

0.1 mM - 1.0 mM ATP. The Km for ATP was found to be 6 x 10^{-4} M. Approximately 8 mμMoles of ATP were required to incorporate 1 mμMole of citrate. However, this value does not take into account the amount of ATP split in the absence of citrate or in the presence of competing reactions. A better indication of the stoichiometry of the ATP-driven incorporation of citrate was obtained by measuring the increase in P_i occurring during the incorporation of citrate

in the presence and absence of oligomycin (2.5 μg/mg protein) corrected for P_i appearing in the presence and absence of oligomycin when citrate is omitted from the medium. This calculation gave 2.11 and 2.34 mμMoles of ATP split at 5 and 10 minutes respectively per mμMole of citrate incorporated. If 50 μM atractyloside is added with 2 mM ATP, the incorporation of citrate decreases from 206 to 48 mμMoles. Since oligomycin decreases the incorporation to 19 mμMoles, the inhibition by atractyloside is $\frac{206 - 48}{206 - 19} = 85\%$. This indicates that ATP is translocated into the matrix by the adenine translocase system (Klingenberg, 1965) which is sensitive to atractyloside, and is converted to a non-phosphorylated, high-energy intermediate and P_i by the oligomycin-sensitive ATPase. The high-energy intermediate drives the incorporation and P_i exits as citrate enters. Despite the conversion of ATP to P_i in the matrix, the P_i concentration found inside the mitochondria after the incorporation is considerably lower than that before citrate is added. Since no metal ion dependency on the incorporation of 20 mM TRIS-citrate was found, the incorporation of citrate cannot be viewed as a passive accumulation following an active energy-linked incorporation of K^+ or Mg^{2+} ions.

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