CONTROL OF CITRATE AND ACETOACETATE SYNTHESIS IN RAT LIVER *

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Recent studies on the kinetic properties of a number of enzymes have suggested that the supply of reducing equivalents from the citric acid cycle to the respiratory chain is controlled by negative feedback, with the adenine nucleotides acting as allosteric modifiers (see Atkinson, 1966; 1968a,b). Thus, citrate synthase is inhibited by ATP while NAD-linked isocitrate dehydrogenase is activated by ADP. However, there is very little evidence that in more integrated systems the citric acid cycle is, in fact, controlled in this manner.

Experimental support for the concept of adenine nucleotide control has been advanced by Shepherd et al. (1965), Nicholls et al. (1967), Garland et al. (1967), Garland (1968a,b), on the basis of studies with rat liver mitochondria. These authors found that palmitylcarnitine in the presence of malate and malonate was metabolized largely to acetoacetate in the coupled state (high intramitochondrial ATP) and to citrate in the presence of uncoupler (low intramitochondrial ATP). Furthermore, isocitrate oxidation by rat liver mitochondria was inhibited by palmitylcarnitine in the coupled, but not in the uncoupled state.

In our own experiments with rat liver mitochondria (Williamson et al., 1967a,b), measurements of endogenous adenine nucleotides showed that there was no correlation between the rate of citrate formation and the ATP content. In an attempt to resolve the discrepancy between the two sets of experiments, we have reinvestigated this problem using the same experimental conditions as those reported by Shepherd et al. (1965). We have found that the lack of citrate formation observed by Garland and coworkers at high adenine nucleotide phosphorylation ratios is not necessarily caused by an ATP inhibition of citrate synthase, since omission of malonate from the incubation medium

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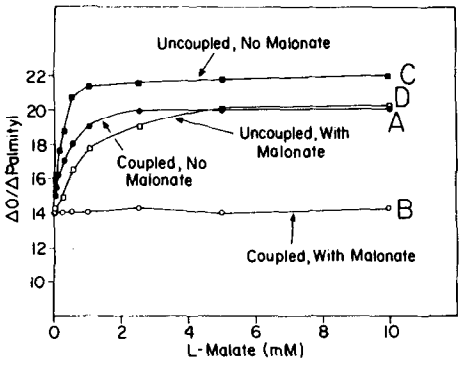

Figure 1. Effect of malate on the ΔO/Δ palmityl ratio. Rat liver mitochondria (1.7 mg/ml) were incubated in 80 mM KCl, 20 mM Tris-Cl, 2 mM Tris-phosphate, 2 mM $MgCl_2$, 1 mM EDTA, and 10 μM fluorocitrate, pH 7.2. Other additions were AMP (1 mM), FCCP (0.5 μM) malonate (12 mM). The respiration induced (ΔO) by addition of 10 μM L(-)palmitylcarnitine (Δpalmityl) was measured. FCCP (p-trifluoromethoxyphenylhydrazone of carbonyl cyanide) was used as an uncoupling agent.

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suffices to enhance citrate production with coupled mitochondria. Presumably malonate competes effectively with malate for entry into the mitochondria.

Methods. Rat liver mitochondria were prepared according to Johnson and Lardy (1967) as modified by Chance and Mela (1966). Incubation conditions are described in the figure legends. Metabolic intermediates were measured in neutralized perchloric acid extracts of mitochondria by fluorometric enzyme methods (Williamson and Herczeg, 1968).

Results. When fluorocitrate is added to rat liver mitochondria incubated in the presence of palmitylcarnitine and malate, the major end products of metabolism are citrate, acetoacetate and β-hydroxybutyrate. With limiting amounts of palmitylcarnitine, the major form of the end product may be assessed quantitatively from the stoichiometry of the oxygen consumed (Shepherd et al., 1965; Garland et al., 1967). Thus, when β-hydroxybutyrate, acetoacetate or citrate is the only end product, the ratio of oxygen atoms consumed to L(-)palmitylcarnitine added, (ΔO/Δ palmityl), is equal to 10, 14, and 22, respectively. When the end products are mixed, intermediate ratios are obtained.

Fig. 1 shows the effect of increasing malate concentration on the ΔO/Δ palmityl ratio. Results obtained in the presence of 12 mM malonate are similar to those reported by Garland et al. (1967); namely a ratio of approx-

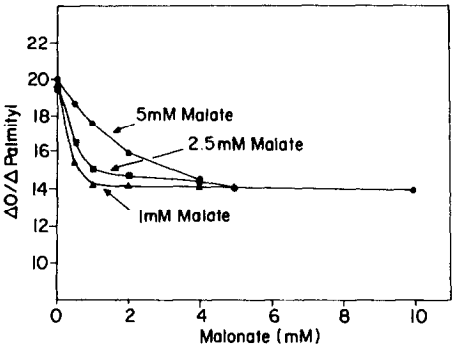

Figure 2. Effect of malonate on the ΔO/Δ palmityl ratio at different malate concentrations. The experimental conditions were the same as those of Fig. 1.

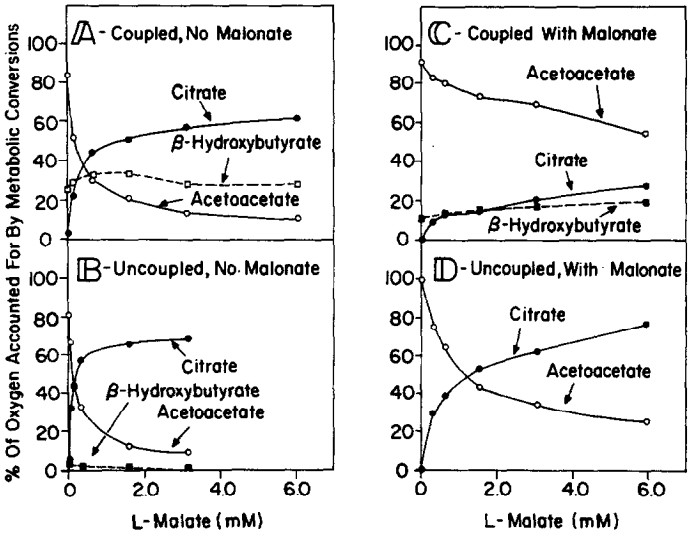

Figure 3. Effect of malonate on the conversion of palmitylcarnitine to citrate and acetoacetate. The buffer contained 80 mM KCl, 20 mM Tris-Cl, 2 mM Tris-phosphate, 2 mM MgCl, 1 mM EDTA, pH 7.2. Other additions were fluorocitrate (15 μM), malonate (10 mM), AMP (1 mM), FCCP (0.3 μM), L(-)palmitylcarnitine (31 μM) and mitochondria (2.2 mg/ml). Samples were taken for analyses immediately before and 1.5 to 3 minutes after the addition of palmitylcarnitine to obtain rates of production of metabolites. The contributions of citrate acetoacetate and β-hydroxybutyrate to the respiration were calculated on the basis of 2.75, 3.5 and 2.5 μg. atoms of oxygen consumed for every μmole of citrate, acetoacetate and β-hydroxybutyrate formed, respectively.

imately 14 with coupled mitochondria which was independent of malate concentration. With uncoupled mitochondria, the ratio increased from 14 to about 20 at high malate concentrations. However, in the absence of malonate, the $\Delta O/\Delta$ palmityl ratio showed a strong dependence on malate concentration with either coupled or uncoupled mitochondria.

These results suggest that malonate may be competing with malate for entry into the mitochondria. Further support for this possibility is obtained from the data in Fig. 2, which shows the effect of malonate on the $\Delta O/\Delta$ palmityl ratio obtained with coupled mitochondria at different malate concentrations. It is seen that a malonate/malate ratio of unity is effective in decreasing the $\Delta O/\Delta$ palmityl ratio from 20 to 14. In other words, the presence of malonate promotes the formation of acetoacetate.

In another set of experiments, the rates of formation of citrate and ketones were measured during constant rates of palmitylcarnitine oxidation with different concentrations of malate. These results are shown in Fig. 3 and are expressed in the form of the percentage of the oxygen consumed accounted for by the measured rates of citrate, acetoacetate and β-hydroxybutyrate appearance. The switch in metabolism from acetoacetate to citrate formation is clearly seen by the rise of citrate and fall of acetoacetate as the malate concentration is increased. The results obtained in the absence of malonate were similar with coupled and uncoupled mitochondria, except that in the uncoupled state there was a decrease in the apparent Km for malate and no production of β-hydroxybutyrate, reflecting a more oxidized state of the mitochondrial NAD-system.

In the presence of 10 mM malonate (Figs. 3C and 3D), acetoacetate formation predominated at all malate concentrations with coupled mitochondria, although the contribution of citrate formation to the respiration increased gradually with the malate concentration. In the uncoupled state (Fig. 3D), however, acetoacetate formation was greater at low than at high malate concentrations, whereas citrate formation predominated at malate concentrations above 1.5 mM. β-Hydroxybutyrate formation was negligible at all malate concentrations with uncoupled mitochondria. A comparison of Fig. 3B and Fig. 3D shows that with uncoupled mitochondria, the maximum relative rates of citrate formation were similar in the absence and presence of malonate. However, malonate greatly increased the concentration of malate required to achieve maximum citrate rates. Since pyridine nucleotides are highly oxidized in uncoupled mitochondria, a low extramitochondrial malate concentration (approximately 250 μm) provided sufficient intramitochondrial oxalacetate to sustain high rates of citrate formation only in the absence of malonate.

Discussion. These results support our previous proposal (Williamson et al., 1967a) that the intramitochondrial availability of malate (and hence oxalacetate) is more important than the ATP level for the control of citrate synthase activity in liver. Total tissue measurements of oxalacetate provide no measure of the mitochondrial content since 99% of the total is extramitochondrial. The calculated mitochondrial oxalacetate concentration in the intact liver is 0.1-0.4 μM and is too low to measure directly in isolated mitochondria (Williamson et al., 1968a). These ratios are well below the reported Km values of 2-5 μM for isolated citrate synthase (Jangaard et al., 1968; Garland, 1968b). The major determinants of the intramitochondrial oxalacetate level are; 1) the intramitochondrial malate concentration and 2) the pyridine nucleotide redox potential. When the malate concentration is held constant, the oxalacetate concentration is controlled by the NAD/NADH ratio through equilibrium with malate dehydrogenase, and the rate of citrate production correlates with the oxidation-reduction state of the NAD-system as determined by fluorescence techniques (Williamson et al., 1967a) as well as by direct analyses (unpublished observations). Similar conclusions have been reached by Wojtczak (1968) in experiments with isolated rat liver mitochondria.

In addition to exerting control at the citrate synthase step, the increased pyridine nucleotide redox potential and acetyl CoA levels observed during conditions of enhanced fatty acid oxidation control the activities of several other enzymes. Namely, (1) pyruvate carboxylase (activation by increased acetyl CoA), (2) pyruvate dehydrogenase (inhibition by increased NADH and/or acetyl CoA), (3) glyceraldehyde-3-P dehydrogenase (stimulation by the mass action effect of increased NADH/NAD), (4) NAD-specific isocitrate dehydrogenase (inhibition by increased mitochondrial NADH and NADPH), (5) NADP-specific isocitrate dehydrogenase (inhibition by decreased mitochondrial NADP), (6) acetoacetate synthesis (enhancement by increased acetyl CoA/CoA ratio), (7) acetyl CoA carboxylase (inhibition by increased fatty acyl CoA). For reference citations see Garland (1968a) and Williamson et al. (1968c). Detailed studies with perfused rat liver and rat liver in vivo (see Williamson et al., 1966, 1967a, 1968a,b,c), have shown that the locations of control sites and the directional changes of postulated enzyme modifiers were all consistent with an interplay of interactions caused by elevated ratios of acetyl CoA/CoA, NADH/NAD and NADPH/NADP.

The physiological significance of the observed inhibition of isolated citrate synthase by ATP is difficult to assess at present. Jangaard et al., (1968) report that with the beef liver enzyme, ATP is competitive with acetyl CoA, but does not change the Km for oxalacetate. On the other hand, the rat

liver enzyme appears to be competitive with both substrates (Shepherd and Garland, 1966). Furthermore, the ATP-Mg complex is noninhibitory (Kosicki and Lee, 1966; Jangaard et al., 1968). Possible control of citrate synthase in vivo, therefore, is intimately related to competition between ATP and other chelating agents (eg. citrate) for Mg^{2+}. In any case, the relevance of ATP as a control factor in the increased ketogenesis associated with enhanced fatty acid oxidation by the liver is minimized by the observed fall in the ATP/ADP ratio under these conditions (Williamson et al., 1966; 1968a,b).

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