CONTROL OF ACETOACETATE AND β-HYDROXYBUTYRATE PRODUCTION IN RAT LIVER MITOCHONDRIA

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Oxidation of fatty acids in liver can result either in their complete oxidation to CO_2 and water in the tricarboxylic acid cycle, or in the formation of acetoacetate which is not further metabolized in liver, except of being eventually reduced to β-hydroxybutyrate. The question therefore arises as to the regulatory mechanism controlling the two pathways. At present, two hypotheses exist concerning this control mechanism. Hathaway and Atkinson (1965) and Shepherd and Garland (1966) have shown an inhibitory effect of ATP on isolated citrate synthetase. Shepherd, Yates and Garland (1965) suggest therefore that the level of mitochondrial ATP is the controlling factor in citrate synthesis and that an increased formation of this nucleotide can switch the metabolism of acetyl-CoA from citrate formation towards the synthesis of acetoacetate. However, Williamson et al. (1967) found that variations in intramitochondrial concentration of ATP are not sufficient to explain on that basis the variations in citrate synthesis under various experimental conditions. On the other hand, they observed a positive correlation between the redox state of mitochondrial nicotinamide nucleotides and the rate of citrate formation. Compatible with this are earlier suggestions of Wieland, Weiss and Eger-Neufeldt (1964) and Exton (1964) that availability of oxaloacetate controls the production of citrate.

The present investigation provides a further experimental support for the assumption that the availability of oxaloacetate rather than the concentration of ATP is the controlling factor in acetoacetate production.

Methods. Rat liver mitochondria were isolated according to Hogeboom (1955). Samples were incubated as described in the legend to Table I. Acetoacetate was determined according to Walker (1954) and the sum of ketone bodies (acetoacetate + β-hydroxybutyrate) by the method of Lester and Greenberg (1944) as described by Bremer (1966). ATP was determined enzymatically according to Lamprecht and Trautschold (1963). In isotopic experiments with uniformly labelled sodium [^{14}C]palmitate acetoacetate was estimated by decarboxylation with aniline citrate (Edson, 1935) and trapping $^{14}CO_2$ by NaOH solution.

ATP was the product of Boehringer, Mannheim, Germany. Uniformly labelled [^{14}C]palmitic acid was purchased by the Radiochemical Centre, Amersham, England. Fluorocitrate was a generous gift of Dr. P.B. Garland and DL-palmitoyl carnitine of Dr. J. Bremer.

Results and Discussion. Table I shows the effect of various concentrations of malate on the production of acetoacetate. In state 3 (Chance and Williams, 1955) with AMP, 0.5 mM malate has little or no effect on the total production of ketone bodies, but it changes the acetoacetate/β-hydroxybutyrate ratio. This ratio is lower with palmitoyl carnitine than with palmitate as substrates, presumably reflecting the higher $NADH_2$/NAD ratio in the former than in the latter case. With increasing concentrations of malate the production of both acetoacetate and β-hydroxybutyrate decreases. However, even in the presence of 5 mM malate there is a substantial production of ketone bodies.

In mitochondria uncoupled by 2,4-dinitrophenol, both malate (Table I) and succinate (not shown) strongly decrease the formation of ketone bodies. Under these conditions synthesis of citrate is greatly enhanced (Williamson et al., 1967). 5 mM arsenate added in state 3 has no effect

Table I. Formation of acetoacetate and β-hydroxybutyrate from palmitoyl carnitine and palmitate in various metabolic states of mitochondria.

The incubation was carried out at 18°- 20° in open tubes under constant shaking. Each tube contained: 120 mM KCl, 10 mM Tris-Cl (pH 7.4), 2 mM P_i, 3 mM $MgCl_2$, 2.5 mM AMP or 0.3 mM 2,4-dinitrophenol (DNP) or 10 mM glucose + hexokinase 0.6 mg/ml (Type III, Sigma), 0.2 ml mitochondria suspension in 1.2% KCl corresponding to 10 - 13 mg protein, and either 1.0 μmole DL-palmitoyl carnitine or 1.0 μmole sodium palmitate + 2 mM ATP + 1 mM DL-carnitine. Fluorocitrate (Fc) and arsenate (As_i) concentrations were 40 μM and 5 mM respectively where indicated. Total volume was 2.0 ml.

Palmitoyl − carnitine + Additions	Aceto-acetate (μmoles)	Aceto-acetate + β-hydroxy-butyrate (μmoles)	ATP (μmoles)
AMP - - - -	0.25	0.25	0.44
+ 0.5 mM malate	0.07	0.25	0.64
+ 1.0 mM malate	0.05	0.15	
+ 5.0 mM malate	0.05	0.15	
+ As_i	0.27	0.27	
+ 0.5 mM malate + As_i	0.22	0.22	0.16
Glucose + hexokinase			
+ 0.5 mM malate	0.11		
+ 0.5 mM malate + As_i	0.24		
DNP - - - -	0.22	0.22	
+ 0.5 mM malate	0.05		
+ 5.0 mM malate	0.00	0.00	

Palmitate + ATP + carnitine + Additions	Aceto-acetate (μmoles)	Aceto-acetate + β-hydroxy-butyrate (μmoles)
AMP - - - -	1.81	2.01
+ 0.5 mM malate	1.00	1.70
+ 5.0 mM malate	0.70	1.13
+ 5.0 mM malate + As_i	0.88	1.00
+ Fc	2.53	2.50
+ 0.5 mM malate + Fc	1.72	1.70
+ 5.0 mM malate + Fc	1.13	1.20
DNP + 5.0 mM malate	0.20	0.20

on the sum of ketone bodies, but less β-hydroxybutyrate and more acetoacetate are formed. At low P_i concentration as that used in experiments described here arsenate is a moderate uncoupler of the respiratory chain oxidative phosphorylation. As shown in Table I, arsenate decreases the formation of ATP by 75%. In spite of that the production of ketone bodies remains unchanged. There is only a shift of the equilibrium between β-hydroxybutyrate and acetoacetate towards the more oxidized compound, presumably reflecting the change in the $NADH_2/NAD$ ratio, as caused by a partial uncoupling. The production of acetoacetate in state 3 with glucose and hexokinase is higher than with AMP. This is also against the assumption that ATP favours the synthesis of acetoacetate. It is, however, likely that also in this case the total amount of ketone bodies remains unchanged and only the ratio acetoacetate/β-hydroxybutyrate varies.

In the presence of fluorocitrate + malate no β-hydroxybutyrate is formed (Table I), indicating that the reduction of acetoacetate to β-hydroxybutyrate occurs during the operation of the tricarboxylic acid cycle. This is in agreement with observations of Exton (1964) on suspensions of liver cells oxidizing palmitate.

Garland, Shepherd and Nicolls (1967) used the ratio $\Delta O/\Delta$palmitoyl as an index of the end products of fatty acid oxidation. On that basis they found that in coupled mitochondria addition of ATP results in the formation of β-hydroxybutyrate, while in an uncoupled system it produces a switch from the citrate pathway towards the synthesis of acetoacetate. Our results (Table II) also show that in coupled mitochondria ATP substantially decreases the acetoacetate/β-hydroxybutyrate ratio without changing the sum of ketone bodies. However, we were unable to show any stimulatory effect of ATP on the production of acetoacetate in the uncoupled state. In fact, in this case the production of acetoacetate in the presence of malate was negligible both in the absence and in the presence of 10 mM ATP. Fluorocitrate had no effect.

Table II. The effect of ATP on the formation of ketone bodies from palmitoyl carnitine.

Experimental conditions as in Table I. The amount of mitochondrial protein was 8.8 mg in Expt. 1 and 13.2 mg in Expt. 2. 6 μg oligomycin was added where indicated.

Expt. No.	Additions	Acetoacetate (μmoles)	Acetoacetate + β-hydroxybutyrate (μmoles)
1.	AMP	0.31	0.42
	AMP + 10 mM ATP	0.00	0.42
	AMP + 5 mM As_i	0.49	0.49
	DNP + 1 mM malate	0.03	0.00
	DNP + 1 mM malate + 10 mM ATP + oligomycin	0.07	
2.	AMP + Fc	1.06	
	DNP + Fc	0.87	
	DNP + Fc + 10 mM ATP + oligomycin	1.19	
	DNP + Fc + 5 mM malate + 2 mM ATP + oligomycin	0.03	
	DNP + Fc + 5 mM malate + 10 mM ATP + oligomycin	0.04	

In the present study only fluorocitrate but not malonate was used to block the tricarboxylic acid cycle, as it has been observed that malonate increased the production of acetoacetate, especially in the presence of ATP. This is compatible with observations of Björntorp (1966).

In the uncoupled state and in the absence of malate, addition of ATP slightly increased the production of acetoacetate from palmitoyl carnitine (Table II, Expt. 2), but this was presumably due to the fact that the sample of palmitoyl carnitine contained some amount of free palmitate which was activated in this case.

The effect of oxaloacetate on the production of acetoacetate from [^{14}C]palmitate is shown in Table III. Oxaloacetate is more effective in decreasing this production than is malate, but this difference is sub-

Table III. Production of acetoacetate from [^{14}C]palmitate in the presence of various concentrations of malate, succinate and oxaloacetate.

Experimental conditions as in Table I. The medium contained 1.0 µmole uniformly labelled palmitate corresponding to 200 000 counts/min. and 11.2 mg mitochondrial protein. Incubation was stopped by perchloric acid and the protein-free supernatant was neutralized with patassium bicarbonate. Total counts in this supernatant corresponded to intermediates of the tricarboxylic acid cycle plus ketone bodies. Acetoacetate was determined as the amount of counts in CO_2 obtained by decarboxylation and multiplied by four.

Additions	Acid soluble fraction (counts/min.)	Acetoacetate (% of acid soluble fraction)
ADP	23 800	100
ADP + 1 mM malate	34 600	60
ADP + 5 mM malate	37 600	31
ADP + 10 mM malate	41 400	26
ADP + 5 mM succinate	18 900	16
ADP + 1 mM oxaloacetate	41 400	77
ADP + 5 mM oxaloacetate	42 400	46
ADP + 10 mM oxaloacetate	37 800	9
ADP + 20 mM oxaloacetate	30 600	8
DNP + 5 mM malate	26 400	6
DNP + 5 mM oxaloacetate	26 000	7

stantial only at concentrations of 10 mM or more. This can be explained by the fact that oxaloacetate can be reduced intramitochondrially to malate when the $NADH_2$/NAD ratio is high enough (and this is the case during the oxidation of palmitoyl carnitine). Therefore, only high concentrations of oxaloacetate are effective. On the other hand, very little acetoacetate is formed in the uncoupled state (with 2,4-dinitrophenol) in the presence of either malate or oxaloacetate. This is in agreement with experiments carried out with unlabelled palmitate or palmitoyl carnitine (Tables I and II).

Conclusions. The end products of fatty acid oxidation in liver mitochondria depend primarily on the redox state of mitochondrial nicotinamide nucleotides which controls the availability of oxaloaceta-

te for citrate synthesis. This redox state is, in turn, controlled by the degree of coupling between electron transport and ATP synthesis, by the phosphorylation potential (Klingenberg, 1963), and by the supply of reducing equivalents. Thus, in the uncoupled state when the respiratory chain is maximally active, the production of oxaloacetate is high enough to ascertain high rate of citrate formation. In the coupled system with an efficient phosphate trap (glucose + hexokinase, low phosphorylation potential) production of oxaloacetate is lower and therefore acetoacetate is formed. In the coupled system with ATP present (high phosphorylation potential) or with a high supply of reducing equivalents by the tricarboxylic acid cycle β-hydroxybutyrate predominates.

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