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KETOGENESIS IN ISOLATED RAT LIVER MITOCHONDRIA

I. RELATIONSHIPS WITH THE CITRIC ACID CYCLE AND WITH THE MITOCHONDRIAL ENERGY STATE

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SUMMARY

1. A method is described to calculate the distribution of acetyl-CoA over the citric acid cycle and ketogenesis during the oxidation of fatty acids in the presence of added malate.

2. Increasing concentrations of added Krebs cycle intermediates lower the rate of ketogenesis both in the low-energy state (State 3; in the presence of glucose and hexokinase (EC 2.7.1.1)) and in the high-energy state (State 4).

3. In State 3 acetyl-CoA is initially used almost exclusively for the synthesis of citrate. Citrate accumulates in the medium, the concentration of malate decreases and a parallel increase in the rate of ketogenesis is observed.

4. The rapid accumulation of citrate in State 3 is also found during oxidation of pyruvate *plus* malate.

5. Uncouplers have no effect on the distribution of acetyl-CoA and on the accumulation of citrate in State 3.

6. Transition from State 3 to State 4 is accompanied by an inhibition of the Krebs cycle and an increased ketogenesis.

7. Under all conditions tested the relative rate of ketogenesis and the 3-hydroxybutyrate/acetoacetate ratio were positively correlated.

8. Addition of ATP and oligomycin to uncoupled mitochondria did not affect ketogenesis.

9. Our results indicate that the concentration of added Krebs cycle intermediates and the NADH/NAD^+ ratio are the only factors controlling the entry of acetyl groups into the cycle.

INTRODUCTION

The enzymes catalyzing β-oxidation, the formation of ketone bodies and the citric acid cycle are localized predominantly in the mitochondrial compartment of the liver cell. Therefore, isolated rat liver mitochondria represent the lowest level of complexity for the study of control of ketone body formation.

An increase in the acetyl-CoA level has been reported in the ketotic liver[1,2],

in the liver perfused with oleate[3] and in isolated mitochondria oxidizing palmitate[4]. These findings support the view[5–7] that ketogenesis is mainly controlled by the rates of acetyl-CoA production and of acetyl-CoA utilization in non-ketogenic pathways.

An important role in the control of the flow of acetyl-CoA into the Krebs cycle has been attributed to the availability of oxaloacetate already more than 25 years ago[8,9] and this hypothesis has since been substantiated by the work of various laboratories[3,10–16]. Depletion of intramitochondrial oxaloacetate has been postulated to be brought about by an increase in the rate of gluconeogenesis[17,18] or by the reduction of oxaloacetate to malate as a result of the increased NADH/NAD^+ ratio during fatty acid oxidation[10–14].

More recently, adenine nucleotides have been implicated in the control of the Krebs cycle[19]. Purified citrate synthase (citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7) from yeast is inhibited by ATP[20]. This inhibition has been confirmed for the isolated enzyme of rat liver[21–24]. However, the importance of this inhibition as a physiological control mechanism has been questioned[25].

Experimental support for the concept of adenine nucleotide control has been advanced by Garland and co-workers[19,26], but these studies with rat liver mitochondria have been challenged by the group of Williamson[27–29].

An unambiguous method to measure the rates of metabolic pathways is a prerequisite for the study of the control of these pathways. The rate of ketone body formation as compared with that of β-oxidation can be expressed as the "acetyl ratio", defined as that part of the total acetyl-CoA, produced during β-oxidation which is converted into ketone bodies. Garland and co-workers[19,26] calculate this ratio from the oxygen uptake during the complete oxidation of a known amount of palmitylcarnitine in the presence of fluorocitrate, malonate and malate. This way of calculation has some serious drawbacks. First, the inhibitors of the Krebs cycle may have secondary effects. Inhibition of the uptake of malate and of the efflux of isocitrate by malonate[29] and fluorocitrate[30], respectively, has been reported. Secondly, we have pointed out some ambiguities in the way of extrapolation of the oxygen uptake[31]. In our experiments the total acetyl-CoA flux and the acetyl ratio are calculated from the oxygen uptake, or the disappearance of substrate, and the concomitant formation of ketone bodies. This method avoids the use of inhibitors of the Krebs cycle. Moreover, the mitochondria are incubated with excess substrate allowing the acetyl ratio to be measured under steady-state conditions.

Our results support the classic hypothesis[8] that the availability of oxaloacetate to citrate synthase is the main factor in the distribution of acetyl-CoA over the citric acid cycle and ketone body formation. No direct effect of the phosphate potential on the acetyl ratio was found. An indirect effect is demonstrated however which is mediated by changes in the mitochondrial redox state. Preliminary reports of this work have been published[14,32].

MATERIALS AND METHODS

Materials

Palmitylcarnitine and acetoacetate were synthesized essentially according to Bremer[33] and Hall[34], respectively. Bovine serum albumin (Fraction V) was defatted with charcoal[35] and dialyzed extensively against physiological saline in order to

remove the relatively large amounts of citrate, present in commercial albumin preparations[36]. Dialysis against a salt solution proved to be essential for the complete removal of citrate as indicated in Table I. Albumin purified by isooctane extraction and dialysis against distilled water[37] is still contaminated with citrate.

L-Carnitine chloride was a generous gift of Dr Masanobu Umehara, Otsuka Pharm. Co., Osaka, Japan. Albumin, oligomycin, rotenone, fatty acids and L-malic acid were purchased from Sigma. Enzymes, nucleotides, GSH and CoASH were supplied by Boehringer. Other chemicals were of the purest grade available. Rat liver mitochondria were isolated as described by Myers and Slater[38].

TABLE I

THE CITRATE CONTENT OF COMMERCIAL AND PURIFIED PREPARATIONS OF BOVINE SERUM ALBUMIN

Preparation	*Citrate content (nmoles/mg albumin)*
Sigma, Fraction V, 108 B-0440	44
After isooctane extraction and dialysis against distilled water[37]	32
After charcoal treatment and dialysis against 0.9% NaCl[35]	0
After 72 h dialysis against distilled water	21
After 60 h dialysis against 0.9% NaCl and 6 h against distilled water	0.1

Reaction conditions and assays

Unless indicated otherwise, incubations were carried out at 25 °C for 30 min in 1 ml of a medium containing as standard components 50 mM sucrose, 5 mM $MgCl_2$, 2 mM EDTA, 15 mM KCl and 50 mM Tris (pH 7.5)[39]. The reactions were started by addition of the mitochondria.

Oxygen uptake was measured with differential manometers with narrow capillaries and gas volumes of 4–5 ml. The following metabolites were assayed spectrophotometrically by standard enzymic methods[40]: acetoacetate, 3-hydroxybutyrate, citrate, isocitrate, pyruvate, phosphoenolpyruvate, malate, fumarate, α-oxoglutarate and acetyl-L-carnitine. Since 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) (Boehringer, Grade II) contains malate dehydrogenase (EC 1.1.1.37), malate, fumarate and 3-hydroxybutyrate were assayed according to the following procedure. First, malate is assayed with malate dehydrogenase (4 units/ml) in a medium containing 0.1 M Tris (pH 9.5), 1 mM EDTA, 2 mM NAD^+, 0.5 mM acetyl phosphate, 30 μM CoASH, 1.3 mM GSH, 0.7 unit/ml phosphate acetyltransferase (EC 2.3.1.8), 2 units/ml citrate synthase. Fumarate can then be assayed by adding 1 unit/ml fumarate hydratase (EC 4.2.1.2). Finally, hydrazine–HCl (pH 9.5) is added to the same test to a final concentration of 0.33 M and 3-hydroxybutyrate is assayed with 0.1 unit/ml 3-hydroxybutyrate dehydrogenase. Inorganic phosphate was measured according to Sumner[41]. The protein content of the mitochondria was determined by the biuret method as used by Cleland and Slater[42].

Calculations

The acetyl ratio (R_{ac}) is defined as that part of the total acetyl flux (ΣAcCoA) through the acetyl-CoA pool which is recovered as acetoacetate (Acac) and 3-hydroxybutyrate (HB)

$$R_{ac} = \frac{2(\Delta Acac + \Delta HB)}{\Sigma AcCoA} \quad (1)$$

From the scheme in Fig. 1 it can be derived that the number of oxygen atoms consumed in the formation of ketone bodies and acetylcarnitine (AcCn) during oxidation of a saturated fatty acid with an even number (n) of carbon atoms is:

$$a = (4 - 8/n)\,\Delta Acac + (3 - 8/n)\,\Delta HB + (2 - 4/n)\Delta AcCn \quad (2)$$

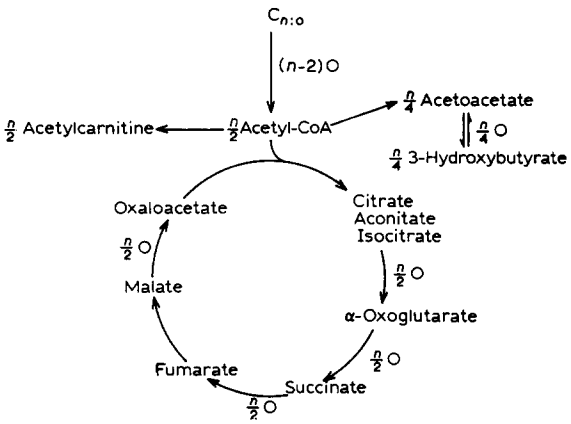

Fig. 1. Oxidation of a saturated fatty acid ($C_{n:o}$) with an even number (n) of carbon atoms. The amounts of oxygen required for the breakdown of one molecule of $C_{n:o}$ are expressed as atoms.

When malate is added to the reaction medium, intermediates of the citric acid cycle may accumulate. The oxygen consumed in the accumulation of citrate (C), isocitrate (IC), aconitate (A), α-oxoglutarate (α) and succinate (S) is:

$$b = (3 - 4/n)\,(\Delta C + \Delta IC + \Delta A) + (4 - 4/n)\Delta\alpha + (5 - 4/n)\Delta S \quad (3)$$

So the oxygen consumption for complete turns of the cycle can be represented by:

$$c = \Delta O - (a + b) \quad (4)$$

The acetyl groups incorporated into citric acid cycle intermediates and CO_2 are found as:

$$d = \Delta C + \Delta IC + \Delta A + \Delta\alpha + \Delta S + \{n/(6n - 4)\}c \quad (5)$$

The total flux of acetyl groups through the acetyl-CoA pool is:

$$\Sigma AcCoA = 2(\Delta Acac + \Delta HB) + \Delta AcCn + d \quad (6)$$

When the medium contains only small amounts of citric acid cycle intermediates and carnitine, Eqn 6 is reduced to:

$$\Sigma AcCoA = 2(\Delta Acac + \Delta HB) + \\ + \{\Delta O - (4 - 8/n)\Delta Acac - (3 - 8/n)\Delta HB\}\,\{n/(6n - 4)\} \quad (7)$$

In most conditions citrate is the only cycle intermediate accumulating in considerable amounts in the presence of added malate (*cf.* Figs 3–5). Since virtually all of the citrate accumulates outside the matrix space of the mitochondrion, citrate does not equilibrate with isocitrate and aconitate through the action of aconitate hydratase (EC 4.2.1.3). Therefore, ΔA and ΔIC can be neglected. Accumulation of α-oxoglutarate was never observed. So, in the presence of added malate and carnitine, ΣAcCoA may be expressed in good approximation by:

$$\Sigma AcCoA = 2(\Delta Acac + \Delta HB) + \Delta C + \Delta AcCn + \\ + \{\Delta O - (4 - 8/n)\Delta Acac - (3 - 8/n)\Delta HB - \\ - (2 - 4/n)\Delta AcCn - (3 - 4/n)\Delta C\}\ \{n/(6n - 4)\} \quad (8)$$

A small error may be introduced in the calculation by accumulation of succinate, which was not assayed.

To summarize some important assumptions in this calculation:

(1) Acetyl-CoA, oxaloacetate, succinyl-CoA, phosphoenolpyruvate, acetate, isocitrate, aconitate and acetone are present only in very low concentrations and the variations in these small pools can be neglected.

(2) The added fatty acid is the only source of acetyl-CoA.

(3) At zero time the only added cycle intermediate is malate or fumarate.

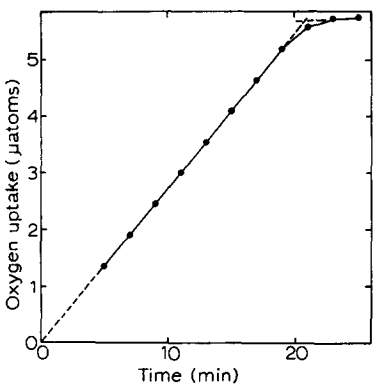

Fig. 2. Oxidation of a limited amount of octanoate. The total oxygen uptake required for the breakdown of the added octanoate is found by extrapolation of the straight line to zero time and to the deflection point. The standard reaction medium was supplemented with 30 mM glucose, 30 mM potassium phosphate (pH 7.5), 0.9 mM ADP, 6 units hexokinase, 0.8 μmole of octanoate and mitochondria (4.1 mg protein).

The method was checked by using a limited amount of octanoate (0.8 μmole) which was completely oxidized during the incubation time (Fig. 2). The oxygen uptake was 5.67 μatoms. Formation of acetoacetate and 3-hydroxybutyrate was 1.41 and 0.06 μmoles, respectively. Eqn 7 gives ΣAcCoA = 3.18 μmoles. Therefore, a good recovery of the added octanoate carbon in the measured ketone bodies and the calculated CO_2 production is observed.

RESULTS AND DISCUSSION

Addition of citric acid cycle intermediates to the medium and the acetyl ratio

Without added cycle intermediates the acetyl ratio during palmitate oxidation is close to 1 (Table II), indicating a low activity of the Krebs cycle. This low activity is probably limited by the low levels of endogenous cycle intermediates. Some variation in the acetyl ratio was observed in different preparations of mitochondria, reflecting a varying content of endogenous cycle intermediates. During incubation, the endogenous cycle intermediates may be depleted by an exchange of succinate or malate with phosphate in the medium[43].

TABLE II

EFFECTS OF CITRATE AND ALBUMIN ON KETOGENESIS

Rat liver mitochondria (6.8 mg protein) oxidized palmitate (2 μmoles) in the standard medium with 30 mM glucose, 30 mM potassium phosphate (pH 7.5), 0.9 mM ADP, 6 units hexokinase and further additions as indicated. Albumin-G and Albumin-Ch, albumin purified by the methods of Goodman[37] and Chen[35], respectively. The acetyl ratio (R_{ac}) is calculated from Eqns 1 and 7. Palmitate was added as 0.02 ml of a 0.1 M ethanolic solution.

Additions	ΔO_2	*ΔAcetoacetate*	*ΔHydroxybutyrate*	R_{ac}
	(*μmoles/mg protein per h*)			
—	0.749	0.255	0.005	0.84
Citrate (25 μM)	0.947	0.258	0.005	0.75
Citrate (125 μM)	1.117	0.250	0.011	0.69
Citrate (250 μM)	1.621	0.133	0.048	0.44
Citrate (500 μM)	2.023	0.180	0.052	0.36
Albumin-G (15 mg/ml)	1.855	0.086	0.038	0.30
Albumin-Ch (15 mg/ml)	0.805	0.272	0.005	0.83

The cycle is stimulated by addition of small amounts of citrate, resulting in a large increase of the oxygen uptake, which is mainly effected by a concurrent lowering of the acetyl ratio. β-Oxidation is also stimulated upon addition of citrate and this will contribute to the increased oxygen uptake as well. This last observation is reminiscent of the classic idea that a catalytic amount of added cycle intermediates is necessary to "spark" fatty acid oxidation[44,45].

Commercial preparations of albumin[36] and albumin purified according to Goodman[37] contain appreciable amounts of citrate (Table I). The stimulation of oxygen uptake by albumin in our experiments (Table II) and in other reports in the literature[16,32,45,46] can be satisfactorily explained by contamination with citrate of the albumin used. After extensive dialysis against physiological saline, citrate is completely removed (Table I) and the effect of albumin on the acetyl ratio is no longer observed (Table II).

Steady-state concentrations of cycle intermediates and the acetyl ratio

In agreement with the results of Lehninger[8], we find that during palmitate oxidation in State 3 conditions an extensive accumulation of citrate occurs, when malate is added to the medium (Fig. 3). When an initial malate concentration of

0.5 mM is added (Fig. 3A) malate and citrate reach a constant level of about 0.05 and 0.2 mM, respectively, within 15 min. The fall in the level of malate is accompanied by an increase in the rate of acetoacetate production. In fact, the acetyl ratio was calculated to increase from 0.2 in the first 8 min to 0.5 over the whole incubation period. This observation suggests that the lowering of the malate concentration is paralleled by a decrease of the intramitochondrial oxaloacetate concentration which limits the rate of citrate synthesis.

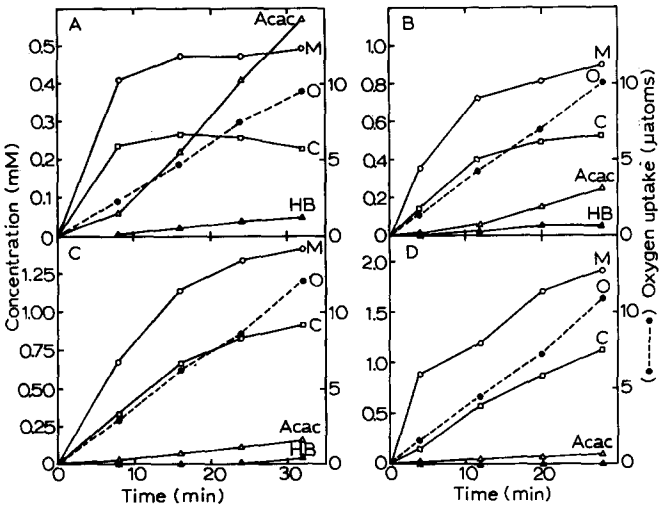

Fig. 3. Oxidation of palmitate in the presence of various concentrations of L-malate. The medium was as in Table II with 1 μmole palmitate (added as 0.2 ml of a 5 mM aqueous suspension), 3.2 mg mitochondrial protein and 0.5 mM (A), 1.0 mM (B), 2.0 mM (C) or 4.0 mM (D) L-malate. ○—○, malate disappearance; ●---●, oxygen uptake; □—□, citrate formation; △—△, acetoacetate formation; ▲—▲, 3-hydroxybutyrate formation.

Figs 3B, 3C and 3D show that increasing concentrations of added malate result in a lower acetoacetate production, whereas the time necessary to reach a constant level of citrate increases. At an initial malate concentration of 4 mM (Fig. 3D) a constant level of citrate is not reached within the incubation period and the rate of citrate production remains almost linear.

About 50 % of the malate disappearance can be accounted for by citrate formation. Fumarate was assayed in other experiments and was found to be a constant fraction of the malate concentration (20–25 %) after the first few minutes of incubation. This equilibration of the added malate with fumarate explains the initial rapid rate of disappearance of malate in Fig. 3D. Evidently, added malate has rapid access to the mitochondrial fumarate hydratase. Isocitrate, phosphoenolpyruvate and α-oxoglutarate were present only in very low concentrations (< 10 μM). Assuming that the sum of the cycle intermediates is constant, it is tentatively concluded that 15–25 % of the added malate is present as succinate in the steady state. Succinate accumulation has previously been reported by Björntorp[45].

The steady state is defined operationally in our experiments by using the following criteria: (1) A constant consumption of substrates. (2) A constant output

of products. (3) Constant levels of all intermediates. (4) Levels of the various cycle intermediates which are independent of the intermediate initially added.

From Fig. 3 it can be seen that the first three criteria are fulfilled (see also Fig. 5). The last criterium was checked by adding citrate, α-oxoglutarate or succinate instead of malate. The same steady state was reached in each case. Fig. 4 shows that at the end of the incubation period the ratio of citrate over malate + fumarate is about 2.7, independent of the intermediates initially added.

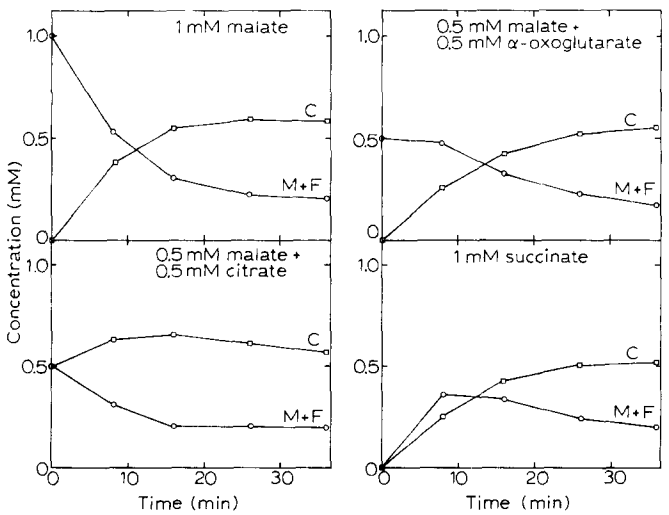

Fig. 4. Independence of the steady state concentrations of citrate and malate on the Krebs cycle intermediate added initially. The standard reaction medium was supplemented with 50 mM glucose, 50 mM potassium phosphate (pH 7.5), 0.5 mM AMP, 4.5 units/ml hexokinase, 0.72 mM palmitate (complexed with bovine serum albumin), 0.6 mM L-carnitine, 25 μM CoASH, 0.25 mM GSH and Krebs cycle intermediates as indicated. Mitochondrial protein, 4.0 mg/ml. An open Erlenmeyer flask with 12 ml of this incubation mixture was shaken vigorously in a waterbath. At the times indicated 2-ml samples were withdrawn for enzymic analyses of citrate (□—□) and malate *plus* fumarate (○—○).

Accumulation of citrate

It may be concluded that during the initial stage of State 3 oxidation of fatty acid plus malate, citrate synthesis is faster than citrate oxidation. Later, a steady state is reached in which citrate oxidation equals citrate synthesis.

Accumulation of citrate is not a specific property of fatty acid oxidation: Fig. 5 shows the time course of pyruvate *plus* malate oxidation in State 3. An extensive accumulation of citrate was observed also in this case. In agreement with the conclusion of Walter and Stucki[47] we have found that pyruvate carboxylase activity is almost completely suppressed during State 3 oxidation of pyruvate in the absence of added bicarbonate.

It should be emphasized that the citric acid cycle operating in steady-state conditions in isolated mitochondria differs principally from the *in vivo* situation. In Fig. 6 the fluxes into and out of the mitochondrial pool of cycle intermediates are outlined. During the oxidation of fatty acid *plus* malate by isolated rat liver mitochondria the metabolic fluxes are of minor importance and the pool size is virtually

constant. Therefore, a steady state is reached when the efflux of each intermediate from the matrix is compensated for by the influx of this intermediate. The rates of the successive reactions of the cycle are then necessarily equal. In the intact cell metabolic fluxes will contribute to the turnover of the mitochondrial pool of cycle intermediates and interconversions of cycle intermediates occur in the cytosol as well as in the mitochondria. In this case, a different steady state settles in which the total influx of intermediates into the mitochondrial pool (from the cytosol and net synthesis) is balanced by the efflux. Consequently, *in vivo* the rates of the various reactions of the cycle may not be equal in a steady state.

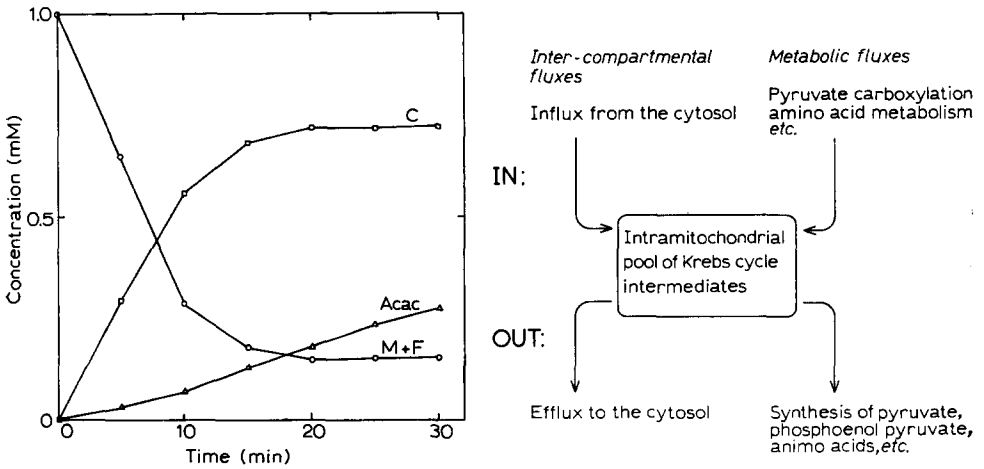

Fig. 5. Time course of the formation of ketone bodies and citrate during pyruvate oxidation in State 3. To the standard reaction medium 30 mM glucose, 30 mM potassium phosphate (pH 7.5), 1 mM AMP, 6 units/ml hexokinase, 1 mM L-malate and 4 mM pyruvate were added. Mitochondrial protein, 3.8 mg/ml. An open Erlenmeyer flask with 20 ml of this incubation mixture was shaken vigorously in a waterbath. At the times indicated 2-ml samples were withdrawn for enzymic analyses of citrate (□—□), malate *plus* fumarate (○—○) and acetoacetate (△—△).

Fig. 6. Schematic diagram of the turnover of intramitochondrial Krebs cycle intermediates.

Citrate has been attributed an important role as an acetyl carrier from the mitochondrial to the cytosolic compartment in lipogenic conditions[48-50]. Our experiments show that in isolated rat liver mitochondria oxidizing palmitate or pyruvate in the presence of malate in State 3, efflux of citrate is a rapid process. It is tempting to speculate that during lipogenesis *in vivo* a steady state is reached in which a rapid synthesis and efflux of citrate is accompanied by a rapid cleavage of citrate in the cytosolic compartment. This mechanism would effectively shunt the Krebs cycle from citrate to oxaloacetate.

McGarry and Foster[51] recently concluded from studies with isolated perfused rat liver oxidizing octanoate that in certain conditions the observed inhibition of lipogenesis is a major factor in the increase of ketogenesis. This conclusion seems to contradict the general hypothesis[5,6,17] that a lesion in the citric acid cycle is the most important factor in the induction of a ketotic state. These contrasting views can be reconciled, however, if citrate production and citrate efflux from the mitochondrial

compartment are obligatory steps in lipogenesis. Inhibition of citrate synthesis will then cause a decrease in the rate of lipogenesis.

At least two factors may regulate the observed accumulation of citrate. First, the activity of the NAD-linked isocitrate dehydrogenase (EC 1.1.1.41) may be limited by low ADP/ATP and NAD^+/NADH ratios[52]. We found, however, that addition of an uncoupler has no effect on the rate and the extent of citrate accumulation (not shown). Secondly, anion-translocation reactions may be competing with the oxidation of citrate. The actual mechanism of citrate accumulation is at the moment under investigation.

TABLE III

KETOGENESIS IN COUPLED AND UNCOUPLED MITOCHONDRIA

Rat liver mitochondria (5.7 mg protein) oxidizing 1 mM DL-palmitylcarnitine in the medium of Table II were uncoupled by addition of 0.1 mM 2,4-dinitrophenol.

		Coupled	*Uncoupled*
a. Oxygen uptake (μatoms)		4.89	3.93
b. Phosphate disappearance	(μmoles)	11.40	0.00
c. P/O ratio ($= b/a$)		2.33	0.00
d. Acetoacetate production	(μmoles)	0.75	0.60
e. 3-Hydroxybutyrate formed	(μmoles)	0.00	0.00
f. Acetyl-CoA to ketone bodies	(μmoles)	1.50	1.20
g. Acetyl-CoA to Krebs cycle	(μmoles)	0.39	0.32
h. Acetyl ratio $\{f/(f+g)\}$		0.79	0.79

TABLE IV

KETOGENESIS IN VARIOUS ENERGY STATES

To the standard reaction medium, 30 mM glucose, 30 mM potassium phosphate (pH 7.5), 0.9 mM ADP, 1 mM L-malate, 1 mM DL-palmitylcarnitine and mitochondria (6.0 mg protein) were added. Further additions as indicated. The total acetyl flux through the acetyl-CoA pool (Σ AcCoA) was calculated with Eqn 7 and R_{ac} with Eqn 1.

2,4-Dinitrophenol (μM)	ΔO_2	*ΔAcetoacetate*	*ΔHydroxybutyrate*	*ΣAcCoA*	*ΔHydroxybutyrate / ΔAcetoacetate*	R_{ac}
	(μmoles)					
0*	1.84	0.15	0.84	2.16	5.60	0.92
0	1.80	0.17	0.44	1.55	2.59	0.79
5	2.95	0.30	0.12	1.63	0.40	0.52
10	4.19	0.27	0.03	1.88	0.11	0.32
20	5.65	0.23	0.02	2.32	0.09	0.22
50	5.34	0.13	0.01	2.05	0.08	0.14
100	5.09	0.11	0.01	1.94	0.09	0.12

* Albumin (10 mg/ml) was added to this incubation.

Ketogenesis and the energy state of the mitochondrion

The addition of uncoupling concentrations of 2,4-dinitrophenol to mitochondria oxidizing palmitylcarnitine in the presence of glucose and hexokinase (EC 2.7.1.1), does not change the acetyl ratio (Table III)[14]. No 3-hydroxybutyrate was formed in

this experiment, indicating a low NADH/NAD$^+$ ratio. But in the absence of hexokinase the acetyl ratio is strongly influenced by addition of 2,4-dinitrophenol (Table IV). Increasing concentrations of the uncoupler release the respiratory control, resulting in a lower NADH/NAD$^+$ ratio (monitored as a decreased reduction of the ketone bodies). At the same time the acetyl ratio is largely decreased. Maximal reduction levels and acetyl ratios are observed in the presence of albumin, which binds palmitylcarnitine and thereby protects the mitochondria against the detergent and uncoupling effect of this compound.

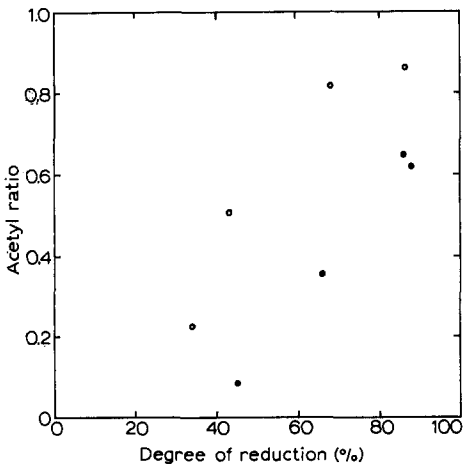

Fig. 7. Dependence of ketogenesis on the redox level of the mitochondria. The degree of reduction of the mitochondrial nicotinamide nucleotide coenzymes was varied by adding various amounts of hexokinase (0–6 units) to mitochondria (5.6 mg protein) oxidizing 1 mM DL-palmitylcarnitine in the standard reaction medium with 30 mM glucose, 30 mM potassium phosphate (pH 7.5), 0.9 mM ADP, (5 mg/ml) bovine serum albumin and 1 mM (○) or 10 mM (●) L-malate. The acetyl ratio (R_{ac}) is calculated from Eqns 1 and 7. The degree of reduction is expressed as $\{\Delta HB/(\Delta Acac+\Delta HB)\}\cdot 100\%$.

In Fig. 7 the acetyl ratio is plotted against the reduction level of the ketone bodies. In this experiment the degree of reduction was varied by changing the concentration of hexokinase. In all experiments we observed a positive correlation between the acetyl ratio and the reduction level. This correlation was found at low as well as at high concentrations of added malate.

From the experiment shown in Fig. 8 the following conclusions may be drawn:

(1) The acetyl ratio depends on the malate concentration both in the low (State 3) and in the high-energy state (State 4).

(2) The acetyl ratio is dependent on the energy state: in a low-energy state (low ATP/ADP and NADH/NAD$^+$ ratios) the acetyl ratio is low; in a high-energy state (high ATP/ADP and NADH/NAD$^+$ ratios) the acetyl ratio is high.

(3) Uncoupling has no effect on the acetyl ratio in a low-energy state.

Discrimination between possible effects of phosphate potential and reduction level on the acetyl ratio

During fatty acid oxidation in a high-energy state (State 4), a high acetyl ratio is observed, indicating an inhibition of the Krebs cycle. Physiologically the increased

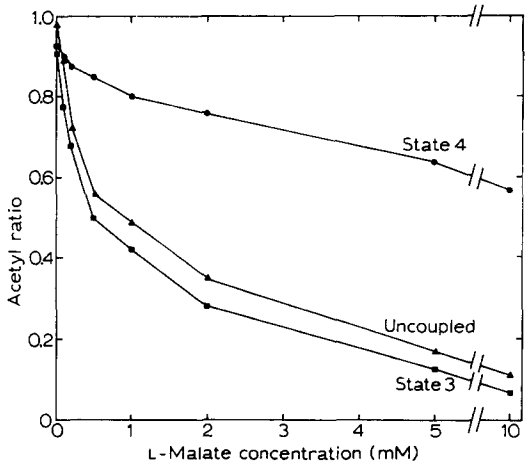

Fig. 8. The effect of added malate on the acetyl ratio in various energy states. To the standard reaction medium 40 mM glucose, 40 mM potassium phosphate (pH 7.5), 1 mM L-palmitylcarnitine and mitochondria (7.4 mg protein) were added. Further additions: State 4 (●—●), 1 mM ATP and 10 mg/ml bovine serum albumin; State 3 (■—■), 1 mM AMP, 4.5 units hexokinase and 10 mg/ml bovine serum albumin; uncoupled (▲—▲), 1 mM AMP, 4.5 units hexokinase and 20 μM pentachlorophenol. Reaction time 24 min (State 4), 20 min (State 3) or 30 min (uncoupled). The acetyl ratio is calculated from Eqns 1 and 8.

TABLE V

DISCRIMINATION BETWEEN POSSIBLE CAUSES OF INCREASED KETOGENESIS IN STATE 4

Expt 1, medium of Table IV with (5 mg/ml) bovine serum albumin. Expt 2, medium of Table II with 1 mM L-malate. Expt 3, standard reaction medium with 30 mM glucose, 0.1 mM 2,4-dinitrophenol, 1 mM DL-palmitylcarnitine and 1 mM L-malate. Expt 4, medium of Expt 3 with 10 μg oligomycin. Mitochondrial protein, 5.6 mg (Expt 1), 5.0 mg (Expt 2), 4.0 mg (Expt 3) or 4.5 mg (Expt 4).

Expt	*Additions*	ΔO_2	*ΔAceto-acetate*	*ΔHydroxy-butyrate*	*ΣAcCoA*	*ΔHydroxy-butyrate* / *ΔAcetoacetate*	R_{ac}
		(*μmoles*)					
1	—	1.78	0.11	0.71	1.88	6.45	0.87
	Hexokinase (1.5 units)	5.14	0.41	0.31	2.84	0.76	0.51
	Hexokinase (6 units)	5.54	0.19	0.10	2.51	0.53	0.23
2	—	6.32	0.08	0.02	2.34	0.25	0.09
	Rotenone (20 ng)	5.73	0.42	0.22	2.92	0.52	0.44
	Rotenone (40 ng)	2.28	0.20	0.48	1.82	2.40	0.75
3	—	5.50	0.08	0.00	2.03	0.00	0.08
	Rotenone (30 ng)	3.68	0.18	0.12	1.72	0.67	0.35
	Rotenone (50 ng)	1.60	0.10	0.41	1.34	4.10	0.76
4	—	2.88	0.10	0.02	1.17	0.20	0.21
	ATP (20 mM)	2.61	0.08	0.02	1.05	0.25	0.19
	ATP (40 mM)	2.61	0.11	0.02	1.09	0.18	0.24

acetyl ratio in State 4 can be understood as a feed-back mechanism. The complete oxidation of fatty acid in the liver is controlled by energy demand; excess fatty acid is converted into ketone bodies to serve as a fuel for extrahepatic tissues[53]. The central role of the phosphorylation state of the adenine nucleotides, expressed as the "energy charge", has been stressed by Atkinson[54]. In State 4 the high ATP/ADP ratio induces a high NADH/NAD^+ ratio. Clearly an increase in the acetyl ratio in State 4 may be caused either directly by the increased ATP/ADP ratio or indirectly *via* the effect of the increased NADH/NAD^+ ratio on the intramitochondrial level of oxaloacetate.

Table V shows the results of some experiments performed in order to discriminate between the effects of the phosphate potential and the redox level on the acetyl ratio. In Expt 1 a gradual transition from State 4 to State 3 is brought about by adding various concentrations of hexokinase (*cf.* Fig. 7). In Expts 2 and 3 low concentrations of rotenone are added to mitochondria in State 3. Rotenone inhibits the oxidation of NADH in the respiratory chain and thereby causes an increase in the NADH/NAD^+ ratio, reflected by the increased 3-hydroxybutyrate/acetoacetate ratio. In these experiments the ATP/ADP ratio is kept low by addition of hexokinase and glucose or of an uncoupler. A parallel increase in the 3-hydroxybutyrate/acetoacetate ratio and the acetyl ratio is observed. In Expt 4, in which the reduction level is kept low by addition of 2,4-dinitrophenol, addition of oligomycin and of high concentrations of ATP does not affect the acetyl ratio, indicating that the intramitochondrial ATP/ADP ratio has no direct effect on the citric acid cycle in isolated rat liver mitochondria.

In conclusion, the observed inhibition of the Krebs cycle in the high energy state can be best explained by the effect of the mitochondrial phosphate potential on the NADH/NAD^+ ratio. Our results therefore support the hypothesis[8-10] that the activity of citrate synthase is ultimately controlled by the availability of oxaloacetate.

In the ketotic liver *in vivo*[10] and in the isolated liver upon perfusion with oleate[3] an elevated mitochondrial NADH/NAD^+ ratio has been reported. The increase in the mitochondrial redox state is not accompanied by an increase in the phosphate potential of the liver. In fact the ATP/ADP ratio in the liver decreases in ketogenic conditions[5]. The discrepancy between mitochondrial redox state and phosphate potential in whole liver suggests that in the ketotic state, the cytosolic and mitochondrial phosphate potential are out of equilibrium. Indeed, as proposed earlier by Garland[55], an increase in the mitochondrial ATP/ADP ratio may be masked by a concurrent decrease of this ratio in the cytosol.

Unequal states in the mitochondrial and extramitochondrial phosphate potential may be caused by an increase of the fatty acyl-CoA level leading to an inhibition of the transport of ADP into the mitochondrial compartment. An inhibitory action of fatty acyl-CoA on the adenine nucleotide-translocating system of mitochondria has been reported[56-58]. Some implications of this inhibitory effect for the oxidation of fatty acids and the acetyl ratio in isolated rat liver mitochondria will be published elsewhere[59] (M. Lopes-Cardozo, W. J. Vaartjes and S. G. van den Bergh, unpublished). A similar but less pronounced effect of oleate was observed earlier by Wojtczak and Zaluska[60].

The metabolic consequences for the liver *in vivo* may be tentatively visualized as follows. An increased flow of free fatty acids into the liver, together with an inhibition of triglyceride synthesis[7], causes an increase in the levels of free fatty acids,

acyl-CoA and acylcarnitine. The resulting decrease in the activity of the adenine nucleotide translocator and the increase in the rate of mitochondrial ATP synthesis by β-oxidation will both increase the mitochondrial ATP/ADP ratio. Both an increase of this ratio and an increased flow of reducing equivalents through the flavoprotein–ubiquinone part of the respiratory chain will increase the mitochondrial NADH/NAD^+ ratio. Increases in the ratio of acetyl-CoA over CoA, in the phosphate potential and in the redox level will all effect a switch from pyruvate decarboxylation to pyruvate carboxylation and thereby stimulate gluconeogenesis. A drain of intermediates towards gluconeogenesis and the increase in the NADH/NAD^+ ratio will lower the mitochondrial oxaloacetate level, resulting in a reduced rate of citrate synthesis and of citrate efflux from the mitochondria. Consequently the transport of acetyl groups from the mitochondria to the cytosol towards lipogenesis slows down. Lipogenesis may also be depressed by the cooperative effects of an elevated level of fatty acyl-CoA and a decrease in cytosolic citrate on acetyl-CoA carboxylase (EC 6.4.1.2).

The increase in the rate of β-oxidation and a diminished rate of acetyl-CoA utilization in the Krebs cycle will automatically "switch on" ketogenesis.

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