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KETOGENESIS IN ISOLATED RAT LIVER MITOCHONDRIA

II. FACTORS AFFECTING THE RATE OF β-OXIDATION

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SUMMARY

1. During fatty acid oxidation by rat liver mitochondria, the rate of β-oxidation is dependent on the relative amounts of substrate and mitochondrial protein, on the energy state of the mitochondria, on the chain length and the number of double bonds of the fatty acid and on the concentration of various compounds in the reaction medium (L-carnitine, CoASH, hexokinase, albumin).

2. The rate of β-oxidation of long-chain fatty acids decreases when the ratio of albumin over fatty acid is increased. This effect is most marked in the absence of added carnitine.

3. Addition of excess hexokinase decreases the rate of β-oxidation in the presence of added carnitine.

4. Maximal rates of β-oxidation are observed with octanoate and decanoate (40–60 nmoles acetyl-CoA/min per mg mitochondrial protein at 25 °C).

5. Odd-numbered fatty acids are oxidized at a much lower rate than the even-numbered homologues. In a low-energy state propionyl-CoA accumulates; in a high-energy state in the presence of bicarbonate, Krebs-cycle intermediates accumulate.

6. L-Carnitine enhances the rate of β-oxidation of all fatty acids except butyrate. The stimulatory effect is most pronounced with odd-numbered and with long-chain fatty acids.

7. In the absence of added carnitine the rate of β-oxidation of long-chain fatty acids decreases with the chain length and increases with the number of double bonds. It is suggested that the solubility of the long-chain fatty acids in the aqueous medium is the rate-limiting factor under these conditions.

8. In the presence of carnitine and albumin, palmitate, oleate, linoleate and linolenate are all oxidized at about the same rate (25–30 nmoles/min per mg protein at 25 °C).

9. Propionyl-CoA is not formed as an intermediate during oxidation of unsaturated fatty acids.

Abbreviations: Acac, acetoacetate; AcCn, L-acetylcarnitine; ΣAcCoA, total flux through the acetyl-CoA pool; HB, D-3-hydroxybutyrate.

INTRODUCTION

Since the pioneering studies of Lehninger [1] it is known that isolated rat liver mitochondria oxidize fatty acids to ketone bodies and CO_2. Low levels of added Krebs-cycle intermediates stimulate the uptake of acetyl-CoA into the Krebs cycle and thereby diminish the rate of ketogenesis. Kennedy and Lehninger [2] compared various fatty acids with respect to the products of oxidation. They concluded that with even-numbered fatty acids the acetyl-CoA formed during oxidation of medium-chain fatty acids is preferentially incorporated into acetoacetate, whereas longer fatty acids yielded primarily CO_2. Odd-numbered fatty acids, from 7 to 17 carbon atoms, were all found to give rise preferentially to CO_2 rather than to acetoacetate. At that time sensitive enzymic assays [3] for acetoacetate and hydroxybutyrate were not available and the effects of carnitine [4] and of the mitochondrial energy state [5] on fatty acid oxidation and on ketogenesis were not yet recognized. Therefore, we decided to reinvestigate the comparative aspects of fatty acid oxidation, using a method to calculate the rate of β-oxidation from the formation of intermediates which accumulate during the incubation [5].

The present report deals with the effects of several factors on the rate of acetyl-CoA production during β-oxidation of various fatty acids. The effects of the chain length and of the number of double bonds of the fatty acid substrate and the effects of albumin, carnitine and hexokinase on the rate of β-oxidation have been investigated. In addition, even-numbered and odd-numbered fatty acids have been compared.

In the next paper of this series [6] the relationship between the rate of β-oxidation and ketogenesis is examined. In contrast to the conclusion of Kennedy and Lehninger [2] it is shown that the different rates of ketone-body synthesis observed during oxidation of various fatty acids are satisfactorily explained by the different rates of β-oxidation.

METHODS AND MATERIALS

Reaction conditions and assays

Unless indicated otherwise, incubations were carried out at 25 °C in 2 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–HCl (pH 7.5). Reactions were started by the addition of mitochondria (8–12 mg protein). O_2 uptake was measured with a Gilson respirometer. After 16–20 min, $HClO_4$ was added to a final concentration of 0.4 M and metabolites were measured spectrophotometrically in the neutralized supernatants using standard enzymic methods [5].

CoASH and CoA-derivatives were assayed fluorimetrically essentially according to Tubbs and Garland [7]; 1 mM 1,4-dithioerythritol was added to the neutralized samples. CoASH was assayed with oxoglutarate dehydrogenase (EC 1.2.4.2), isolated from pig heart, in a medium containing 2-oxoglutarate and NAD^+. The subsequent assay of acetyl-CoA was based on the liberation of CoASH from acetyl-CoA by addition of oxaloacetate and citrate synthase (EC 4.1.3.7). This assay was complicated by an NADH-oxidase activity occurring after the addition of oxaloacetate which was presumably caused by a contamination of the oxoglutarate

dehydrogenase preparation with malate dehydrogenase (EC 1.1.1.37). The acetyl-CoA values obtained by extrapolation to the time of addition of oxaloacetate agreed quite well with values obtained in the assay system with L-carnitine and carnitine acetyltransferase (EC 2.3.1.7). In our hands the assay based on the release of CoASH from acetyl-CoA with phosphate acetyltransferase (EC 2.3.1.8) also resulted in too low values due to an extensive decrease of NADH.

It is known that carnitine acetyltransferase is not fully specific for acetylcarnitine [8]; succinylcarnitine, however, is not converted [9]. CoA-derivatives assayed with L-carnitine and carnitine acetyltransferase are, therefore, designated as short-chain CoA-esters. When a precursor of propionyl-CoA was oxidized, e.g. odd-numbered fatty acids (Table I), the values obtained for acetyl-CoA (in the citrate synthase assay) and for short-chain CoA-esters differed significantly, indicating an accumulation of propionyl-CoA. Only after this report was prepared a specific assay for propionyl-CoA has been described [10]. The pellet of acid-insoluble material (mitochondria plus albumin) was washed twice with 0.4 M and 0.1 M $HClO_4$, respectively. Hydrolysis of long-chain CoA-esters was effected by a 10-min incubation at pH 11.5 and 55 °C in the presence of 10 mM 1,4-dithioerythritol [11].

Calculations

The calculation of the flux through the acetyl-CoA pool [5] (ΣAcCoA) was slightly modified and extended in order to be applicable to the oxidation of pyruvate, unsaturated and odd-numbered fatty acids. The amount of acetyl-CoA incorporated into acetoacetate (Acac), D-3-hydroxybutyrate (HB), citrate (C) and L-acetylcarnitine (AcCn) was assessed directly by measurement of these metabolites:

$$2(\Delta\text{Acac}+\Delta\text{HB})+\Delta\text{C}+\Delta\text{AcCn} = \alpha \quad (1)$$

The amount of O_2 used in the formation of these metabolites can be calculated:

$$p\alpha-\Delta\text{HB}+\Delta\text{C} = \beta \quad (2)$$

p, a substrate-specific constant, represents the average number of oxygen atoms necessary for the formation of one molecule of acetyl-CoA and varies between one (in the case of butyrate or pyruvate) and two (in the case of odd-numbered fatty acids). Obviously, in the series of even-numbered fatty acids p depends on the number of carbon atoms (n) and on the number of double bonds (d) : $p = 2(n-2-d)/n$. In addition, four oxygen atoms are taken up in the Krebs cycle for each molecule of acetyl-CoA which is completely oxidized. So the amount of acetyl-CoA which is oxidized to CO_2 can be evaluated indirectly from the total uptake of oxygen atoms (O) corrected for oxygen consumed in the formation of other metabolites:

$$(\Delta\text{O}-\beta)/(p+4) = \gamma \quad (3)$$

The total flux through the acetyl-CoA pool is:

$$\Sigma\text{AcCoA} = \alpha+\gamma \quad (4)$$

The assumptions underlying this calculation have been discussed previously [5]. All calculations were programmed and performed on a Canola 167P electronic calculator.

Materials

Hexokinase (Boehringer) was dialyzed to remove the $(NH_4)_2SO_4$ and its activity after dialysis was measured as glucose 6-phosphate production (μmoles/min at 25 °C) in the standard reaction medium supplemented with 20 mM potassium phosphate (pH 7.5), 20 mM glucose, 0.5 mM L-malate and 5 mM ATP. L-Carnitine was a generous gift of Dr Masanobu Umehara, Otsuka Pharm. Co., Osaka, Japan. 1,4-Dithioerythritol and carbonylcyanide *m*-chlorophenyl-hydrazone were purchased from Merck and Calbiochem, respectively. Palmitoyl-CoA was obtained from Serva. Other biochemicals and preparations were as previously described [5].

RESULTS AND DISCUSSION

Factors affecting the rate of palmitate oxidation

Rat liver mitochondria oxidize palmitate in the absence of carnitine and albumin at a rate which is about 80 % of the carnitine-stimulated rate. Fig. 1A shows that the oxidation rate depends on the amount of the micellar palmitate suspension added. In this experiment (with 9.3 mg mitochondrial protein) a half-maximal rate is recorded at 200 nmoles palmitate per ml. This value is, however, dependent on the amount of mitochondrial protein, the malate concentration and the ionic strength of the medium and on the method of preparation of the micellar suspension of potassium palmitate [2] (data not shown). In contrast to unsaturated and shorter fatty acids, palmitate at higher concentrations does not inhibit its own oxidation [12]. The P:O ratio was only slightly lowered upon an increase of the palmitate concentration. At 500 nmoles per ml (about 100 nmoles per mg protein)

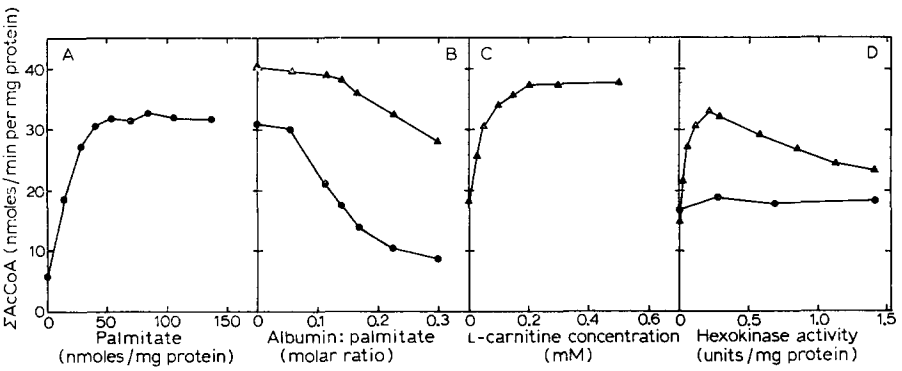

Fig. 1. The effect of various parameters on the rate of palmitate oxidation. The standard reaction medium was supplemented with 40 mM glucose, 40 mM potassium phosphate (pH 7.5), 1 mM ATP. Further additions: (A) 2 mM L-malate, 6.5 units hexokinase and palmitate as indicated, its concentration expressed relative to the amount of mitochondrial protein (9.3 mg). (B) 2 mM L-malate, 6.5 units hexokinase, 0.5 μmole palmitate/ml and varying amounts of albumin (expressed as the albumin:palmitate molar ratio). ●–●, no further additions; ▲–▲, +0.2 mM L-carnitine, 0.2 mM GSH and 20 μM CoASH. (C) 2 mM L-malate, 6.5 units hexokinase, 0.5 mM palmitate complexed to albumin (albumin:palmitate molar ratio, 0.18), 0.2 mM GSH, 20 μM CoASH and L-carnitine as indicated. (D) 0.5 mM L-malate, 0.4 mM palmitate complexed to albumin (albumin: palmitate molar ratio, 0.20) and hexokinase as indicated, its amount expressed relative to the amount of mitochondrial protein (9.3 mg). ●–●, no further additions. ▲–▲, +0.5 mM L-carnitine (Cn), 0.25 mM GSH and 25 μM CoASH.

a P:O ratio of 1.9–2.1 was measured. Obviously, the uncoupling concentration cannot be reached because of the low solubility of palmitate in the aqueous medium [12].

Addition of albumin inhibits the rate of palmitate oxidation appreciably (Fig. 1B). In the presence of CoASH and L-carnitine this inhibitory effect of albumin is smaller than in the absence of these cofactors. An inhibitory effect of albumin on palmitate oxidation was already observed by Fritz [13] and can be explained by binding of palmitate to albumin [14]. It has been found that the concentration of palmitate decreases almost linearly from 10 to 1 μM when the average molar ratio of palmitate over albumin is lowered from 6 to 3 at 37 °C [14]. This concentration range is expected to be even lower at 25 °C since palmitate is bound more tightly at lower temperatures [15].

The addition of L-carnitine to a medium supplemented with low concentrations of CoASH, albumin and hexokinase results in a maximal rate of palmitate oxidation (Fig. 1C). When the amount of added hexokinase is increased (Fig. 1D), the rate of palmitate oxidation passes through a maximum. At lower concentrations, hexokinase stimulates the oxidation rate by releasing respiratory control. Excess hexokinase lowers the rate of palmitate oxidation (Fig. 1D, upper curve). In the absence of carnitine and CoASH, excess hexokinase is not inhibitory (Fig. 1D, lower curve).

All the effects on the rate of palmitate oxidation shown in Fig. 1 are compatible with the localization and kinetics of the different palmitoyl-CoA synthetase (EC 6.2.1.3) activities demonstrated to be present in rat liver mitochondria [16]. A large part of this activity resides in the mitochondrial outer membrane. An ATP-dependent acyl-CoA synthetase with a lower affinity towards palmitate has, however, been demonstrated in the "inner-membrane plus matrix" fraction [16].

In the absence of added carnitine only palmitoyl-CoA formed within the mitochondrial inner membrane can undergo β-oxidation [4]. Van Tol and Hülsmann [16] reported a K_m of 0.18 mM for palmitate in the activation reaction in the matrix, whereas in our experiments the half-maximal oxidation rate is recorded at about 200 nmoles palmitate per ml (Fig. 1A). The agreement between these results points to a limitation of palmitate oxidation by the activation reaction in the mitochondrial matrix. The observation that in the absence of carnitine the oxidation rate declines rapidly upon the addition of albumin (Fig. 1B) shows that the unbound palmitate outside the inner membrane is in equilibrium with a palmitate pool in the matrix compartment.

In the presence of CoASH, ATP and Mg^{2+} palmitate is activated at the mitochondrial outer membrane with a maximal rate (70 nmoles/min per mg protein at 37 °C) [16] which exceeds the maximal rate of palmitate oxidation about 7-fold, (cf. Fig. 1, and assuming a Q_{10} of 2). L-Carnitine is necessary for the transport of palmitoyl-CoA to the matrix compartment [4] by the catalytic action of carnitine palmitoyl transferase (EC 2.3.1.21) [17]. A V of 40–80 nmoles/min per mg protein at 37 °C has been reported for this enzyme in rat liver mitochondria, dependent on the assay used [18, 19]. It appears, therefore, that in the presence of carnitine plus CoASH the rate of oxidation is not limited by the capacity of the enzymes involved in the synthesis and transport of palmitoyl-CoA. On the other hand, albumin (Fig. 1B) and excess hexokinase (Fig. 1D) slow down the reaction under these circumstances. For an evaluation of the rate-limiting step in vivo, more data are required on the dis-

tribution of free palmitate and palmitoyl-CoA in the liver cell and on the availability of CoASH, carnitine and ATP to the enzymes involved.

Although carnitine-independent oxidation of fatty acids is of considerable interest for the study of fatty acid oxidation in isolated rat liver mitochondria, the contribution of activating enzymes localized within the mitochondrial inner membrane to the synthesis of CoA-esters of long-chain fatty acids is probably very low in vivo. (+)-Decanoylcarnitine, an inhibitor of carnitine palmitoyltransferase, suppresses almost completely the oxidation of long-chain fatty acids [20, 21] in perfused rat liver. This strongly suggests that in vivo fatty acid activation occurs outside the mitochondrial inner membrane and that carnitine-mediated transport is an obligatory step in the catabolism of long-chain fatty acids in rat liver. The occurrence of carnitine-independent fatty acid oxidation in vivo has, however, been proposed for the liver stimulated by glucagon [22]. As shown in Fig. 1B the rate of carnitine-independent palmitate oxidation decreases when the ratio of palmitate over albumin becomes lower (i.e. at low concentrations of unbound palmitate). At the same time the stimulatory effect of carnitine increases. This suggests that in vivo the level of intracellular unbound free fatty acids is very low in liver as a result of the adsorption of long-chain fatty acids to membranous structures [23] or protein moieties [24].

The rate of β-oxidation as a function of fatty acid chain length

The rate of β-oxidation is dependent on the chain length of the fatty acid (Fig. 2A). Maximal rates are recorded for $C_{10:0}$ and $C_{13:0}$ in the series of even- and odd-numbered fatty acids, respectively. In the experiment shown in Fig. 2 equivalent amounts of carbon of the different fatty acids were added. Control experiments showed, however, that the oxidation rates are not affected by the concentration of the fatty acid substrates in the range of 0.5–2 mM. (For the longer-chain fatty acids this is only true when the ratio of fatty acid over albumin is kept constant.)

It should be noted that short- and medium-chain fatty acids of the odd-

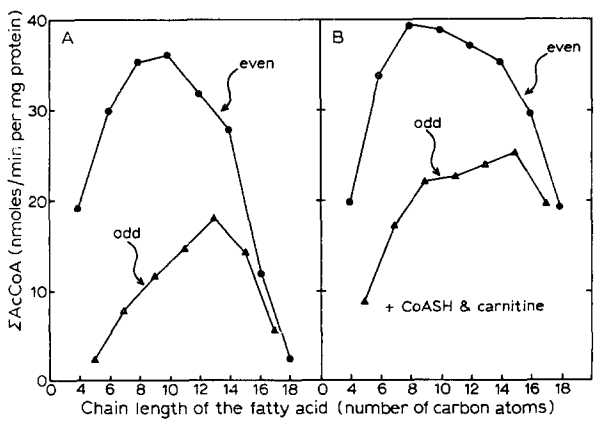

Fig. 2. The rate of β-oxidation and the chain length of the fatty acid substrate. The standard reaction medium was supplemented with 20 mM glucose, 20 mM potassium phosphate (pH 7.5), 0.5 mM ATP, 2.6 units hexokinase, 0.2 mM L-malate, 75 μM albumin and different saturated fatty acids as indicated (equivalent to 16 μatoms carbon, e.g. 1 μmole palmitate, 2 μmoles octanoate etc.). (B) 0.2 mM GSH, 20 μM CoASH and 0.5 mM L-carnitine were added.

numbered series are oxidized at much slower rates than those of the even-numbered series. β-Oxidation of odd-numbered fatty acids proceeds to propionyl-CoA which can be converted to succinyl-CoA via methylmalonyl-CoA [25]. The carboxylation of propionyl-CoA is dependent on ATP and CO_2 and is expected to be impaired under the conditions of Fig. 2 by the presence of hexokinase plus glucose, the absence of bicarbonate and the excess phosphate [26] in the medium. Indeed, accumulation of short-chain acyl-CoA, presumably propionyl-CoA (see Methods), is observed during the oxidation of odd-numbered fatty acids (Table I). The impaired oxidation rates of odd-numbered short- and medium-chain fatty acids as compared with the even-numbered series may be the result either of a depletion of mitochondrial CoASH or of a direct inhibitory effect of propionyl-CoA. The breakdown of odd-numbered fatty acids is probably limited by the deacylation rate of propionyl-CoA. This would explain the observed decrease of propionyl-CoA and the increase in the oxidation rate with increasing chain length of the odd-numbered fatty acids (Table I).

TABLE I

ACCUMULATION OF SHORT-CHAIN ACYL-CoA DURING STATE 3 OXIDATION OF ODD-NUMBERED FATTY ACIDS

20 mM glucose, 20 mM potassium phosphate (pH 7.5), 0.5 mM ATP, 0.2 mM L-malate, 2.6 units hexokinase and odd-numbered fatty acids (equivalent to 16 μatoms carbon) were added to the standard reaction medium. CoASH and CoA-esters were measured at the end of the incubation.

	$-\Delta O_2$	ΔAcac	ΔHB	ΣAcCoA	CoASH	AcCoA	Short-chain CoA-esters	Long-chain CoA-esters
	(nmoles/min per mg protein)				(nmoles per mg protein)			
Pentanoate	12.9	1.0	0.0	6.0	0.1	< 0.1	2.5	0.5
Heptanoate	22.0	1.6	0.2	10.3	0.1	0.1	2.4	0.4
Nonanoate	34.9	3.0	0.2	16.8	0.1	0.1	2.5	0.5
Undecanoate	43.1	3.6	0.5	21.0	0.2	0.2	1.8	0.8
Tridecanoate	47.9	4.4	0.4	23.6	0.1	0.3	1.7	1.3
Pentadecanoate	46.6	2.3	0.2	20.1	0.7	0.2	1.3	1.1
Heptadecanoate	30.4	0.5	0.0	11.6	1.3	0.1	0.8	1.1

Fig. 2B shows the rate of β-oxidation after addition of carnitine plus CoASH. A maximal stimulatory effect of these compounds is found with $C_{5:0}$, $C_{9:0}$ and with long-chain fatty acids. The stimulatory effect of carnitine on the oxidation of short-chain fatty acids of the odd-numbered series is in line with the effect observed in kidney [26] and can be interpreted as a release of CoASH from propionyl-CoA. Addition of carnitine does not stimulate butyrate oxidation.

The rate of oxidation of $C_{6:0}$, $C_{8:0}$ and $C_{10:0}$ is slightly enhanced by carnitine. These results imply that in the presence of CoASH and carnitine, the acyl-CoA synthetase localized in the mitochondrial outer membrane contributes to the activation of medium-chain fatty acids. This activating enzyme has indeed a broad chain-length specificity [27] (J. J. Batenburg, personal communication). It may be of interest that the stimulation by carnitine of the oxidation of $C_{10:0}$ was always less than that

of $C_{6:0}$ and $C_{8:0}$. This observation probably reflects the chain-length specificity of the enzymes catalyzing the transport of acyl-CoA through the mitochondrial inner membrane [28].

Oxidation of unsaturated fatty acids and the question of γ-oxidation

In Table II the rates of oxidation of various saturated and unsaturated long-chain fatty acids are compared, both in the presence and in the absence of carnitine plus CoASH. Stearate is oxidized at a lower rate than palmitate and unsaturated C_{18}-acids. We consider the solubility of a fatty acid to be the main factor determining the rate of its oxidation in the absence of added carnitine plus CoASH. However, this offers no explanation for the relative oxidation rates of the unsaturated C_{18}-acids.

TABLE II

STIMULATION OF THE OXIDATION OF UNSATURATED FATTY ACIDS BY CARNITINE

The standard reaction medium was supplemented with 40 mM glucose, 40 mM potassium phosphate (pH 7.5), 0.5 mM ATP, 0.2 mM L-malate, 2.6 units hexokinase and 0.5 mM fatty acid complexed to albumin (molar ratio 7 : 1). Values in parentheses represent oxidations in the presence of 1 mM L-carnitine and 100 μM CoASH.

	O_2 uptake (nmoles/min per mg protein)	Σ AcCoA
Palmitate	20.2 (35.8)	10.8 (28.9)
Stearate	10.1 (31.7)	3.9 (22.6)
Oleate	18.9 (35.4)	8.8 (27.6)
Linoleate	16.5 (37.2)	8.5 (29.3)
Linolenate	18.9 (33.9)	12.6 (27.9)

Addition of carnitine plus CoASH stimulates the rate of β-oxidation (ΣAcCoA) of long-chain fatty acids 2- to 6-fold. From this observation it is clear that the system for fatty acid activation localized at the mitochondrial outer membrane has a very high capacity with respect to saturated and unsaturated long-chain fatty acids. As discussed above, it is unlikely that this capacity is rate-limiting for the total process of fatty acid oxidation. The availability of fatty acid for the activating system may, however, become rate-limiting at lower ratios of fatty acid over albumin (Fig. 1B) or in case of $C_{18:0}$ which is poorly soluble in aqueous media at pH 7.5.

It is well-established that in rat liver mitochondria the physiologically important unsaturated long-chain fatty acids can be oxidized completely by β-oxidation. In that process the intermediates 3-*cis*- and 2-*cis*-enoyl-CoA are transformed to 2-*trans*-enoyl-CoA before hydration of the double bond occurs (for refs see ref. 29 and ref. 30). An alternative route of breakdown of unsaturated fatty acid has been proposed in which direct hydration of the 3-*cis* double bond [31] takes place. As a consequence unsaturated fatty acids would generate propionyl-CoA and synthesis of carbohydrate from unsaturated fatty acids by the classical gluconeogenic pathway would be possible. Evidence [30] brought forward in favour of this pathway has recently been questioned [29].

In our experiments the occurrence of γ-oxidation of unsaturated fatty acids

TABLE III

ACCUMULATION OF KREBS-CYCLE INTERMEDIATES DURING THE STATE 4 OXIDATION OF VARIOUS SUBSTRATES IN THE PRESENCE OF BICARBONATE

The standard reaction medium was supplemented with 30 mM glucose, 30 mM potassium phosphate (pH 7.5), 25 mM $KHCO_3$, 0.5 mM ATP and 75 μM albumin. Further additions: 1 mM nonanoate ($C_{9:0}$), 0.4 mM stearate ($C_{18:0}$) or linolenate ($C_{18:3}$) and 5 mM pyruvate. Mitochondrial protein, 7.0 mg. The low amounts of malate (M), fumarate (F) and citrate (C) formed during the incubation were measured on an Aminco–Chance dual-wavelength spectrophotometer.

Substrate	Δ(M+F+C) (nmoles/min per mg)
Pyruvate	+23.4
$C_{9:0}$	+ 3.8
$C_{18:0}$	− 0.1
$C_{18:3}$	0.0

could not be detected. When the citric acid cycle was blocked with fluorocitrate the measured O_2 uptake was always equivalent to the production of ketone bodies (data not shown), indicating that unsaturated fatty acids are completely degraded to acetyl-groups. Moreover, no formation of Krebs-cycle intermediates could be measured during State 4 oxidation of linolenate in the presence of bicarbonate (Table III) in contrast to a definite accumulation of these intermediates during oxidation of nonanoate and pyruvate. The data shown in Table III are considered to be strong evidence against a formation of propionyl-CoA during oxidation of linolenate by isolated rat liver mitochondria. In agreement with conclusions drawn from studies with bovine heart mitochondria [29] and with perfused rat liver [32] we suggest that the hypothesis of net glucose production from unsaturated fatty acids [30, 31] should be discarded.

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