A Spectrophotometric Procedure for Rapid and Sensitive Measurements of β -Oxidation

DEMONSTRATION OF FACTORS THAT CAN BE RATE-LIMITING FOR β -OXIDATION

By HARALD OSMUNDSEN and JON BREMER

Institute for Medical Biochemistry, University of Oslo, Karl Johansgate 47, Oslo, Norway

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1. A spectrophotometric direct-reading assay for measurements of β -oxidation by intact mitochondria is described. The procedure relies on the ability of ferricyanide to trap reducing equivalents generated by the acyl-CoA dehydrogenases (EC 1.3.99.3). The reduction of ferricyanide was recorded by using a dual-wavelength spectrophotometer. 2. Oxaloacetate or acetoacetate was used to stimulate the rate of β -oxidation by rotenone-blocked mitochondria. Although both were effective with rat liver mitochondria, oxaloacetate gave about 75% more stimulation. With heart or kidney mitochondria, only oxaloacetate gave marked stimulation. Acetoacetate had no stimulatory effect with heart mitochondria, but a small stimulatory effect on the rate of β -oxidation by kidney mitochondria. 3. The stoichiometry of β -oxidation-dependent reduction of ferricyanide was examined, and good correlations were found between experimental and theoretical amounts of ferricyanide reduced. 4. Ferricyanide appears as efficient a final electron acceptor as O_2 . With ferricyanide the rate of β -oxidation by heart mitochondria can be measured without interference from the oxidation of tricarboxylic acid-cycle intermediates.

Studies have shown that the oxidation of some very-long-chain fatty acids (containing 20–22 carbon atoms) proceeds very slowly (Christophersen & Bremer, 1972), and also that such acids may interfere with the oxidation of fatty acids of shorter chain lengths (Christophersen & Christiansen, 1975). For comparative studies of the oxidation of different fatty acids by tissues from different species we have found that commonly used polarographic techniques have too low sensitivity.

An alternative approach is to measure the acid-soluble radioactivity from ^{14}C -labelled fatty acids, but this is limited by the types of fatty acids that are commercially available with ^{14}C label. With this procedure it is not possible to follow directly the progress of β -oxidation (unlike polarographic techniques).

We have therefore developed a spectrophotometric procedure for measurements of β -oxidation by intact mitochondria. It appears to possess greater sensitivity and accuracy than polarographic techniques, while allowing the progress of β -oxidation to be directly monitored by recording the decrease in absorption as a function of time.

The principle of this procedure rests on the ability of ferricyanide to trap electrons generated by the acyl-CoA dehydrogenases of β -oxidation (EC 1.3.99.3). These enzymes are thought to be rate-limiting for β -oxidation (Stanley & Tubbs, 1975), and measure-

ments of the rate of ferricyanide reduction should therefore be a measure of the rate of β -oxidation. The use of a dual-wavelength spectrophotometer allows measurements of the rate of ferricyanide reduction, which are both more accurate and sensitive than polarographic measurements.

Experimental

Materials

Antimycin A (type III), rotenone (grade II), *cis*-oxaloacetic acid (grade 1), cytochrome *c* (type III), methyl acetoacetate and Hepes* were from Sigma Chemical Co., St. Louis, MO, U.S.A. Fatty acids were from E. Merck, Darmstadt, Germany, and were of analytical grade. [U - ^{14}C]Palmitate was from The Radiochemical Centre, Amersham, Bucks., U.K. All other reagents were of analytical grade, or of highest purity available, and were from Merck, or May and Baker, Dagenham, Essex, U.K.

Lithium acetoacetate was prepared from methyl acetoacetate by the procedure of Hall (1962). Acylcarnitines were prepared by the procedure of Bremer (1968), and [U - ^{14}C]palmitoylcarnitine was prepared as described by Bremer & Wojtczak (1972). Hypo-

* Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

glycine had been prepared from ackee seeds by Mr. D. Billington, Department of Pharmacological Sciences, University of Newcastle upon Tyne, U.K., by the procedure of Kean (1974).

Mitochondria were prepared from livers or hearts of male albino Wistar rats (150–200 g) by using an isolation medium containing 300 mM-mannitol, 10 mM-Hepes and 1 mM-EDTA. Guinea-pig kidney mitochondria were prepared in the same medium from 400 g male albino animals. The mitochondria were otherwise prepared as described by Myers & Slater (1957) and by Davis (1967).

Methods

Haas (1937) used ferricyanide for the manometric determination of reduced glutathione, as well as NADH, both of which are autoxidized by ferricyanide. Ferricyanide has similarly been used by Quastel & Wheatley (1938) to measure the oxidation of substrates by various tissue preparations in the absence of O_2 . On accepting a reducing equivalent (one electron), ferricyanide is reduced to ferrocyanide with concordant production of one proton:

In early manometric work ferricyanide reduction was measured by evolution of CO_2 from added HCO_3^- , caused by the proton generation. Ferricyanide, however, possesses an absorption peak at 420 nm, which on reduction disappears. It is therefore well suited for spectrophotometric measurements of flavoprotein-catalysed reactions, and has been widely used in this capacity (see Hatefi & Stiggall, 1970).

Ferricyanide does not penetrate the inner mitochondrial membrane (Mitchell & Moyle, 1969; Klingenberg, 1970). It has been suggested by Klingenberg (1970) that the major site of ferricyanide reduction by intact mitochondria is cytochrome *c*. This is facilitated by the localization of cytochrome *c* at the outer face of the mitochondrial inner membrane (Klingenberg, 1968).

Klingenberg (1970) has shown that ferricyanide reduction generated by the oxidation of succinate by intact rat liver mitochondria is powerfully inhibited by antimycin A, whereas the reduction caused by *sn*-glycerol 3-phosphate oxidation was not inhibited. This difference in antimycin A sensitivity is, according to Klingenberg (1970), due to the localization of succinate dehydrogenase at the inner side of the mitochondrial inner membrane. The flow of electrons from the enzyme therefore has to pass through the antimycin A-sensitive connexion between cytochromes *b* and *c*₁ before reaching cytochrome *c*. *sn*-Glycerol 3-phosphate dehydrogenase (EC 1.1.99.5), however, being located at the outer surface of the inner membrane, allows transfer of electrons from the

reduced flavin prosthetic group directly to ferricyanide.

It is now well documented that the enzymes of β -oxidation are located in the mitochondrial matrix (Garland *et al.*, 1967). Considerations of the respiratory chain would therefore predict that reducing equivalents generated at the level of both acyl-CoA dehydrogenases and β -hydroxyacyl-CoA dehydrogenases (EC 1.1.1.35) could be tapped at cytochrome *c*, by using ferricyanide as the electron acceptor (see Scheme 1).

Measurements of β -oxidation-dependent reduction of ferricyanide

Unless stated to the contrary we have used the following assay system for routine measurements of β -oxidation by reduction of ferricyanide, using rat liver or guinea-pig kidney mitochondria. The assay medium contained 130 mM-KCl, 10 mM-Hepes, 0.1 mM-EDTA, 1 mM-KCN, 0.5 mM- $K_3Fe(CN)_6$, 1 mM-ADP, 1 mM- P_i , 0.3 mg of cytochrome *c* and 4.5 mg of defatted bovine serum albumin, pH 7.2. After addition of mitochondria (up to 2 mg of protein), 3 μ g of rotenone was added, followed by addition of acylcarnitine (20–100 μ M, depending on chain length) and 10 mM-oxaloacetate or 10 mM-acetoacetate. The final assay volume was 3.0 ml, contained in a cuvette of 1 cm light-path.

When measuring β -oxidation by rat heart mitochondria the assay medium was identical, except that 300 mM-mannitol replaced 130 mM-KCl.

The pH of solutions was adjusted with KOH solution, and the pH of solutions of ADP, oxaloacetate, acylcarnitines and albumin was adjusted with solid K_2CO_3 .

The reduction of ferricyanide was followed with an Aminco DW-2 spectrophotometer operated in the dual-wavelength mode. The wavelength pair used was 420 and 475 nm, the latter being isosbestic with respect to ferricyanide. Any reduction of mitochondrial flavoproteins which might be manifested at 475 nm (Chance, 1966) did not interfere with the assay, as the use of 510 nm as reference wavelength did not significantly alter rates of ferricyanide reduction. A bandpass of 1.5 nm was used as a routine. The measurements were carried out at 20°C. An extinction coefficient of ferricyanide at 420 nm of 1.05 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ (Connelly *et al.*, 1968) has been assumed throughout this work.

Measurements of β -oxidation by using [U - ^{14}C]palmitoylcarnitine

The oxidation of [U - ^{14}C]palmitoylcarnitine was followed under conditions identical with those

Scheme 1. Paths of electron flow from β -oxidation to ferricyanide

A diagrammatic presentation of the paths likely to be involved in the transport of reducing equivalents from acyl-CoA dehydrogenases (EC 1.3.99.3) (1) and β -hydroxyacyl-CoA dehydrogenases (EC 1.1.1.35) (2) to ferricyanide by intact mitochondria. The sites of inhibition by rotenone and antimycin A are indicated. Abbreviations: Cyt b , cytochrome b ; Cyt c , cytochrome c ; Cyt c_1 , cytochrome c_1 .

described for measurements of reduction of ferricyanide, except that ferricyanide, KCN and cytochrome *c* were excluded when O_2 was used as the final acceptor for reducing equivalents. The reaction mixtures were contained in small vessels thermostatically maintained at $20^\circ C$, and magnetically stirred. To each reaction mixture (3 ml final volume) was added $20 \mu M$ -palmitoylcarnitine (specific radioactivity 1330 d.p.m./nmol).

At various time-intervals samples (0.20 ml) were withdrawn and added to 0.20 ml of ice-cold 5% (v/v) $HClO_4$. After centrifugation at 38000g for 10 min, 0.20 ml portions of the supernatants were removed, and added to 10 ml of Diluene (Packard, Downers Grove, IL, U.S.A.) scintillation fluid. The radioactivity was measured in a Packard model 3375 liquid-scintillation counter, adjusted to measure ^{14}C with 90% efficiency.

Protein determination

Proteins were determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard.

Sonication of rat liver mitochondria

Mitochondria (about 50 mg of protein/ml), suspended in the isolation medium at $0-4^\circ C$, were disrupted for three periods of 1 min (with 1 min cooling between each period) by using an MSE sonicator.

Kinetic analysis of experimental data

Where relevant, apparent K_m and V_{max} were determined by fitting experimental data to a Michaelis-Menten hyperbola by using program HYPER (Cleland, 1963). Computations were carried out with the NORD-10 computer of the Pre-clinical Institutes, University of Oslo.

Results

Effect of rotenone on acylcarnitine-induced reduction of ferricyanide

In the absence of rotenone all mitochondrial preparations showed an appreciable degree of endogenous reduction of ferricyanide. This must primarily be caused by NAD- or NADP-linked reactions as the addition of $3 \mu g$ of rotenone to the assay mixture dramatically diminished the rate of endogenous reduction (Fig. 1). In the absence of rotenone the addition of $20 \mu M$ -palmitoylcarnitine caused a marked stimulation of reduction of ferricyanide (Fig. 1). In the presence of rotenone the addition of palmitoylcarnitine alone stimulated reduction to about 15–20% of the rate obtained in the absence of rotenone (after subtraction of endogenous rates) (Fig. 1). This is consistent with the view that rotenone inhibits β -oxidation through NADH generated by the β -hydroxyacyl-CoA dehydrogenase reaction (Bremer & Wojtczak, 1972).

Bremer & Wojtczak (1972) have also demonstrated that rotenone inhibition of β -oxidation in liver mitochondria can be overcome by the addition of oxaloacetate or acetoacetate. In line with these findings, we have observed that the addition of 10 mM-oxaloacetate or 10 mM-acetoacetate gave a dramatic stimulation of acylcarnitine-dependent reduction of ferricyanide by rotenone-blocked rat liver mitochondria (Fig. 1). Acylcarnitine-dependent reduction of ferricyanide by rat heart mitochondria could only be stimulated by oxaloacetate (Fig. 1). The lack of stimulation by acetoacetate is presumably due to the lack of acetyl-group acceptors and to the very small amount of β -hydroxybutyrate dehydrogenase (EC 1.1.1.30) in this tissue (Williamson *et al.*, 1971). Oxaloacetate is here fulfilling the dual capacity of being acceptor for both reducing equivalents (from NADH) and acetyl-groups.

Guinea-pig kidney mitochondria also showed a dramatic stimulation by 10 mM-oxaloacetate, whereas

Fig. 1. *Effects of rotenone, oxaloacetate and acetoacetate on acylcarnitine-dependent reduction of ferricyanide by intact mitochondria*

The effects of palmitoylcarnitine and oxaloacetate on the rate of reduction of ferricyanide by guinea-pig kidney mitochondria in the absence (a) and presence (b) of rotenone are shown. Similar results were also obtained with liver and heart mitochondria (not shown). The effect of acetoacetate on the rate of palmitoylcarnitine-dependent reduction by guinea-pig kidney mitochondria (c), the effects of acetoacetate (d) and oxaloacetate (e) on the rates of palmitoylcarnitine-dependent reduction by rat liver mitochondria, and similarly the effects of oxaloacetate (f) and acetoacetate (g) with rat heart mitochondria are also shown. Palmitoylcarnitine was always added at a concentration of $20 \mu M$, and oxaloacetate and acetoacetate were added each at 10 mM. Rotenone ($3 \mu g$), when present [in all traces, except (a)], was always added after addition of mitochondria. Control experiments showed that the addition of oxaloacetate or acetoacetate alone gave no significant stimulation of reduction of ferricyanide (not shown). The numbers in parentheses represent the rates of reduction of ferricyanide expressed as nmol reduced/min per mg of protein. The reduction of ferricyanide was measured at 420 nm, with 475 nm as the reference wavelength, as described in the Experimental section. Abbreviations: AcAc, acetoacetate; PCn, palmitoylcarnitine; OxAc, oxaloacetate.

Fig. 2. Effect of various concentrations of acetoacetate (a) and oxaloacetate (b) on the palmitoylcarnitine-dependent reduction of ferricyanide by rat liver mitochondria (●) and rat heart mitochondria (○)

Palmitoylcarnitine was always added at a concentration of 20 μ M, and acetoacetate and oxaloacetate at concentrations indicated in the Figure. These data were obtained in one experiment, but are representative of results obtained in two other experiments. The rates of reduction of ferricyanide were otherwise measured as described in the Experimental section.

acetoacetate only caused about 10% stimulation (Fig. 1). This may similarly be explained by a low β -hydroxybutyrate dehydrogenase activity (Williamson *et al.*, 1971), and the possible need for acetyl-group acceptors to stimulate β -oxidation. The effect of various concentrations of oxaloacetate and aceto-

acetate on palmitoylcarnitine-dependent reduction of ferricyanide is shown in Fig. 2.

Effect of antimycin A on acylcarnitine-dependent reduction of ferricyanide

By analogy with the antimycin A-sensitive reduction of ferricyanide induced by succinate oxidation (Klingenberg, 1970), acylcarnitine-dependent reduction of ferricyanide should also be blocked by antimycin A (see Scheme 1). Fig. 3 shows the effect of antimycin A on succinate-, *sn*-glycerol 3-phosphate- and palmitoylcarnitine-dependent reduction of ferricyanide by rat liver mitochondria. With rat heart mitochondria (Fig. 3), however, palmitoylcarnitine-dependent reduction of ferricyanide was only inhibited by about 50% by antimycin A, whereas the succinate-dependent reduction was about 90% blocked. This percentage difference may only be apparent, as the antimycin A-resistant reduction may represent the maximal capacity of an antimycin A-independent electron pathway. Other acylcarnitines (butyrylcarnitine, octanoylcarnitine and myristoylcarnitine) showed similar antimycin A-sensitivity (results not shown).

Effect of the mitochondrial protein concentration and the concentrations of ferricyanide and cytochrome c on the rate of reduction of ferricyanide by acylcarnitines

Fig. 4 shows that the rate of reduction of ferricyanide was proportional to the mitochondrial protein concentration up to 0.3 mg of protein/ml: 0.1 mM-ferricyanide was sufficient to give nearly maximal rate of reduction, higher concentrations having only a small stimulatory effect. Concentrations higher than 1 mM were inhibitory (Fig. 4).

The addition of low concentrations of cytochrome c caused only a small further stimulation of the reduction of ferricyanide, although higher concentrations were inhibitory (Fig. 4).

These results indicate that endogenous cytochrome c will sustain practically maximal rates of reduction of ferricyanide.

Effect of administration of hypoglycin to rats on rates of reduction of ferricyanide by isolated liver mitochondria

Rats that had been deprived of food for 24 h were given 80 mg of hypoglycin/kg body wt. by intraperitoneal injection. After a further 20 h liver mitochondria were isolated, and the rates of reduction of ferricyanide caused by selected acylcarnitines were compared with rates obtained from rats injected with 0.9% NaCl. Typical results are presented in Table 1.

Comparison of rates of β -oxidation with O_2 or ferricyanide as terminal electron acceptor

The rates of β -oxidation with O_2 or ferricyanide as electron acceptors, under otherwise identical conditions, were measured by following the acid-soluble radioactivity formed from the oxidation of [U- ^{14}C]-palmitoylcarnitine. Results showing the generation of acid-soluble radioactivity as a function of time, with heart and liver mitochondria, are presented in Fig. 5.

An initial burst of acid-soluble radioactivity was consistently observed (see Fig. 5). This may be due to an initial very fast rate of β -oxidation during the time-interval when the removal of reducing equivalents from the acyl-CoA dehydrogenases is not rate-limiting. As soon as the capacity of the electron-transport system has been saturated a slower steady rate of β -oxidation results.

Stoichiometry of reduction of ferricyanide by acylcarnitines

To investigate whether the reduction of ferricyanide, as measured spectrophotometrically, was solely due to electrons originating from the acyl-CoA dehydrogenases, the stoichiometry of the reduction caused by a defined quantity of acylcarnitine was investigated. Results obtained with myristoylcarnitine are presented in Fig. 6. Similar results were obtained with palmitoylcarnitine and hexanoylcarnitine (results not shown).

Effect of acylcarnitine carbon-chain length on the rate of reduction of ferricyanide

Fig. 7 shows the rate of reduction of ferricyanide as a function of chain length of the acylcarnitine. Results obtained from polarographic measurements of the rate of O_2 uptake by the same mitochondrial preparation are also shown. The acylcarnitine concentrations were chosen so as to give the same number of acetyl groups as 40 μ M-palmitoylcarnitine.

Discussion

Source of the reducing equivalents

In the absence of rotenone, the electrons flowing to cytochrome *c* during oxidation of acylcarnitines originate from the activities of both the acyl-CoA dehydrogenases and β -hydroxyacyl-CoA dehydrogenases, the former having FAD as prosthetic groups (Beinert, 1962), the latter requiring NAD (Green & Allman, 1968). The inhibitory effect of rotenone on acylcarnitine-dependent reduction of ferricyanide and subsequent stimulation by acetoacetate or oxaloacetate is quite analogous to the relief of rotenone inhibition of O_2 uptake caused by the oxidation of acylcarnitine, which has been

discussed by Bremer & Wojtczak (1972) and Bremer & Davis (1972).

In the absence of rotenone, however, there is on addition of oxaloacetate still stimulation of the rate of reduction of ferricyanide (see Fig. 1). After subtraction of endogenous reduction of ferricyanide, the rate is about 100% faster than that obtained in the combined presence of rotenone and oxaloacetate. The reason for this difference is probably that oxaloacetate stimulates the production of NADH by the tricarboxylic acid cycle.

After the addition of acylcarnitine to rotenone-blocked mitochondria there is still stimulation of the rate of reduction of ferricyanide (Fig. 1). This may represent the energy-dependent reoxidation of NADH through the action of the transhydrogenase (EC 1.6.1.1), and subsequent utilization of NADPH by the enoyl-CoA reductase (EC 1.3.1.8) (Podack & Seubert, 1972). A limited reoxidation of NADH could allow a slow flux through the acyl-CoA dehydrogenases. It is also possible that reoxidation of NADH through a leak in the rotenone block contributes to this rate. Rotenone-insensitive β -oxidation has previously been described by Bremer & Wojtczak (1972).

The electrons flowing to ferricyanide after the addition of acylcarnitine and oxaloacetate (or acetoacetate) to rotenone-blocked mitochondria (Fig. 1) are likely to arise almost solely from the activity of the acyl-CoA dehydrogenases. In rat liver mitochondria this electron flow is about 95% blocked by antimycin A. This effect is quite analogous to that of antimycin A on succinate-dependent reduction of ferricyanide (Fig. 3), and dissimilar to the insensitivity of *sn*-glycerol 3-phosphate-dependent reduction to antimycin A (Fig. 3). By analogy with the arguments of Klingenberg (1970), we therefore suggest that the source of the reducing equivalents is located within the mitochondria matrix (or on the inner face of the inner mitochondrial membrane).

With sonicated mitochondria in a medium supplemented with 1 mM-NAD⁺ and 1 mM-CoA, palmitoylcarnitine-dependent stimulation of reduction of ferricyanide was still observed (Fig. 3). The reaction was now, however, insensitive to antimycin A. We take this to indicate that ferricyanide is now reduced by direct interaction with reduced flavoproteins.

The use of liver mitochondria from rats previously given hypoglycin may serve to prove further that the source of reducing equivalents is in fact acyl-CoA dehydrogenases. A hypoglycin metabolite is known to inhibit the butyryl-CoA dehydrogenase (EC 1.3.99.2) specifically and irreversibly (Osmundsen & Sherratt, 1975). We would therefore expect the mitochondria from these rats to be unable to catalyse the butyrylcarnitine-dependent reduction of ferricyanide, and the rates induced by other short-chain-length carnitines to be severely inhibited. By analogy with polarographic measurements (Osmundsen &

Sherratt, 1975) we would expect the rate of palmitoyl-carnitine-dependent reduction of ferricyanide to be about 60% inhibited relative to control rates. The results presented in Table 1 show exactly the predicted pattern of inhibition. We take this to show that the source of reducing equivalents from rotenone-blocked mitochondria is the acyl-CoA dehydrogenases, and that the rate of reduction of ferricyanide is a direct measurement of the rate of acyl-CoA flux through these enzymes. These results also suggest that the acyl-CoA dehydrogenases under these con-

ditions are also rate-limiting for β -oxidation. The measurement of the rate of reduction of ferricyanide is therefore a measurement of the flux through β -oxidation.

The stoichiometry of reduction of ferricyanide shows a good correlation between experimental and theoretical values (Fig. 6). This strongly indicates that the reducing equivalents flowing during oxidation of acylcarnitines in the presence of rotenone originate almost exclusively from the activity of the acyl-CoA dehydrogenases.

Fig. 4. Effects of various concentrations of mitochondrial protein, ferricyanide and cytochrome *c* on rates of acylcarnitine-dependent reduction of ferricyanide

The rates of myristoylcarnitine-dependent reduction of ferricyanide as a function of added quantity of mitochondrial protein (*a*), the concentration of ferricyanide (*b*) and the added quantity of cytochrome *c* (*c*) are shown. This experiment was carried out with rat liver mitochondria. Myristoylcarnitine (20 μ M) was always included in the assay, and the reduction of ferricyanide was stimulated by the addition of 10 mM-oxaloacetate. Other assay details are described in the Experimental section.

Fig. 3. Effect of antimycin A on the succinate- (*a*), *sn*-glycerol 1-phosphate- (*b*) and palmitoylcarnitine-dependent (*c*) reduction of ferricyanide by rat liver mitochondria

Traces showing the effect of antimycin A on palmitoylcarnitine- (*d*) and succinate-dependent (*e*) reduction by rat heart mitochondria are also shown. Trace (*f*) shows the lack of effect of antimycin A on palmitoylcarnitine-dependent reduction by sonicated rat liver mitochondria. Palmitoylcarnitine was always used at a concentration of 20 μ M, *sn*-glycerol 3-phosphate at 20 mM, and succinate at 10 mM. NAD⁺ and CoA were, when sonicated mitochondria were used, each added to a concentration of 1 mM (*f*). Oxaloacetate to a concentration of 10 mM and 6 μ g of antimycin A were added to each sample. In traces (*c*) and (*d*) the time of addition of palmitoylcarnitine has not been indicated, but may be taken to be represented by the origin of the traces. The numbers in parentheses represent the rates of reduction of ferricyanide expressed as nmol reduced/min per mg of mitochondrial protein. Other assay details are described in the Experimental section. Abbreviations: Ant A, antimycin A; Glyc-3-P, *sn*-glycerol 3-phosphate; OxAc, oxaloacetate; AcAc, acetoacetate.

Table 1. *Effect of hypoglycin treatment on the rates of acylcarnitine-dependent reduction of ferricyanide*
The effect of intraperitoneal administration of hypoglycin to rats, before isolation of liver mitochondria, on the rates of reduction of ferricyanide induced by selected acylcarnitines was measured. Butyrylcarnitine and hexanoylcarnitine were added to a concentration of $100\mu\text{M}$, and octanoylcarnitine and palmitoylcarnitine to a concentration of $20\mu\text{M}$. The rates tabulated are those obtained after addition of 10mM -acetoacetate. Other assay details are described in the Experimental section. The tabulated values represent the mean values $\pm$ S.E.M. from two separate experiments.

Acylcarnitine derivative	Rate of ferricyanide reduction (nmol/min per mg of protein)		Inhibition (%)
	Hypoglycin	Control	
Butyryl-	0 ± 0	16 ± 2	100 ± 0
Hexanoyl-	15 ± 2	45 ± 4	66 ± 8
Octanoyl	32 ± 3	53 ± 4	40 ± 2
Palmitoyl-	18 ± 3	48 ± 3	62 ± 3

It has to our knowledge not previously been possible to follow selectively the activity of acyl-CoA dehydrogenases in intact mitochondria by using a rate assay, although Flatmark & Pedersen (1975) have described a procedure for flavoprotein titration by progressive reduction induced by increasing concentrations of acylcarnitines.

Kinetic parameters of stimulation of β -oxidation by oxaloacetate or acetoacetate

Acetoacetate stimulates the palmitoylcarnitine-dependent reduction of ferricyanide by rotenone-blocked rat liver mitochondria with apparent K_m and V_{max} of $0.12 \pm 0.03\text{mM}$ (mean $\pm$ S.D. of two experiments) and $38.1 \pm 2.6\text{nmol}$ of ferricyanide reduced/min per mg of protein. The corresponding K_m and V_{max} for oxaloacetate were $0.93 \pm 0.18\text{mM}$ and $78.1 \pm 4.3\text{nmol}$ of ferricyanide reduced/min per mg of protein. It is noteworthy that the kinetic parameters for the oxaloacetate-stimulated process in rotenone-blocked rat heart mitochondria are similar

Fig. 5. *Comparison of rates of β -oxidation with ferricyanide or O_2 as the final acceptor of reducing equivalents*
The production of acid-soluble radioactivity from $[\text{U-}^{14}\text{C}]$ palmitoylcarnitine from rat liver mitochondria (a) and rat heart mitochondria (b) was followed as described in the Experimental section. Results obtained with ferricyanide as acceptor are indicated by $\bullet$ and with O_2 by $\circ$. Each curve is based on measurements from two separate experiments. The incubation mixtures contained $3\mu\text{g}$ of rotenone, and 10mM -oxaloacetate was added 1 min before the addition of $[\text{U-}^{14}\text{C}]$ palmitoylcarnitine.

Fig. 6. Stoichiometry of acylcarnitine-dependent reduction of ferricyanide

This Figure shows a representative trace obtained from recording of the reduction of ferricyanide induced by a limiting quantity of acylcarnitine. This trace shows results obtained by the addition of 12 nmol of myristoylcarnitine (MyrCn), followed by subsequent addition of 3 nmol of myristoylcarnitine. The theoretical quantity of ferricyanide reduced by β -oxidation of 12 nmol of myristoylcarnitine, under the present assay conditions, is 144 nmol. The reduction of ferricyanide was measured as described in the Experimental section. This experiment was carried out with rat liver mitochondria.

to those of liver mitochondria, the K_m and V_{max} , being 1.1 ± 0.1 mM and 74.4 ± 2.7 nmol/min per mg respectively. These kinetic parameters, which are derived from the data presented in Fig. 2, are likely to reflect the properties of the carriers for acetoacetate and oxaloacetate, and not those of β -hydroxybutyrate dehydrogenase or malate dehydrogenase (EC 1.1.1.37) and citrate synthetase (EC 4.1.3.7) respectively. We base this conclusion on the much lower K_m values obtained with isolated preparations of these enzymes (see Lehninger *et al.*, 1960; Shepherd & Garland, 1969; England, 1969). Bremer & Davis (1972) have similarly observed that oxaloacetate is a better stimulator of rotenone-blocked β -oxidation than is acetoacetate.

Comparison of rates of β -oxidation with O_2 or ferricyanide as final electron acceptors

Because oxaloacetate suppresses the rate of O_2 uptake by respiring mitochondria (presumably because NADH is used to form malate), polarographic

Fig. 7. Rate of reduction of ferricyanide as a function of acylcarnitine carbon-chain length

Typical results obtained from measurements of reduction of ferricyanide brought about by acylcarnitines of different carbon-chain length are shown. The acylcarnitine-dependent reduction was stimulated by the addition of 10 mM-acetoacetate. The measurements of rates of reduction of ferricyanide were otherwise carried out as described in the Experimental section. O_2 uptake was measured on the same mitochondrial preparation, under experimental conditions identical with those described in the legend to Table 2. (a) Respiratory rate and (b) rates of reduction of ferricyanide before ($\square$) and after the addition of acetoacetate ($\bullet$), as a function of carbon-chain length. All acylcarnitines had a fully saturated acyl group, except the C_{22} acid, which here is cetoylelcarnitine (the $C_{22:1}$ derivative).

techniques are not suitable when comparing β -oxidation under these conditions. We have instead followed the formation of acid-soluble radioactivity as a function of time. These results show that the rates of β -oxidation are similar with O_2 or ferricyanide in

liver mitochondria (Fig. 5). With heart mitochondria, however, the use of ferricyanide gives a markedly higher rate of β -oxidation. The reason for this may be that electrons from β -oxidation in heart mitochondria can access ferricyanide by pathways not present in liver mitochondria. The observed differences in β -oxidation-sensitivity to antimycin A could support this suggestion (see Fig. 3).

These results also strongly suggest that ferricyanide is as efficient an acceptor of electrons generated by isolated mitochondria as is O_2 .

Comparison of rates of O_2 uptake with rates of ferricyanide reduction

Table 2 shows typical rates of reduction of ferricyanide by rotenone-blocked rat liver mitochondria, obtained in the presence of oxaloacetate or acetoacetate by using three different acylcarnitines. The rates of O_2 uptake (in the presence of 10mM-malonate) are also listed. These rates are numerically comparable, because the rate of reduction of ferricyanide should be divided by 2 (2 mol of ferricyanide is reduced/mol of acyl-CoA oxidized by the acyl-CoA dehydrogenase). The O_2 uptake should similarly be halved because the measured rate is the sum of two O_2 -consuming reactions.

These data suggest that there is a close correlation between the acetoacetate-stimulated rate of hexanoylcarnitine oxidation and the rate of O_2 uptake

obtained from hexanoylcarnitine. With lauroylcarnitine and palmitoylcarnitine the acetoacetate-stimulated rate of reduction is higher than the rate of O_2 uptake. This difference is, however, in each case almost identical in magnitude. It is possible that this difference is similar in nature to that observed when measuring the oxidation of [U- ^{14}C]palmitoylcarnitine (see Fig. 3). Because hexanoylcarnitine is oxidized more slowly there is likely to be a numerically smaller difference between the acetoacetate-stimulated rate of hexanoylcarnitine oxidation and the rate of oxidation obtained by measurement of O_2 uptake. The use of acetoacetate in the ferricyanide system makes it also unlikely that the differences in oxidation rates are due to acetyl-group entry into the tricarboxylic acid cycle, as no cycle intermediates have been added.

The oxaloacetate-stimulated rates of acylcarnitine carnitine oxidation are, for lauroylcarnitine and palmitoylcarnitine, about twice the rates obtained from measurements of O_2 uptake (Table 2). Here, however, β -oxidation is stimulated by facilitated entry of acetyl-groups into the tricarboxylic acid cycle. In the polarographic system this possibility is blocked by the presence of malonate. One component of the oxaloacetate-stimulated velocity will, as with acetoacetate, also be due to the additional stimulation observed when ferricyanide is used (see Fig. 3).

A surprising finding was, however, that the oxaloacetate-stimulated rate of hexanoylcarnitine oxidation is about 50% of the rate obtained in the presence of acetoacetate (i.e. opposite to what is the case with medium- and long-chain-length acylcarnitines). The reason for this difference is not clear. It is, however, possible that oxaloacetate might inhibit the transport of hexanoylcarnitine into the matrix.

Rates of reduction of ferricyanide as a function of the carbon-chain length of acylcarnitines

The rates of reduction of ferricyanide obtained with acylcarnitines of different chain lengths show a pattern of variation similar to that obtained from measurements of O_2 uptake (Fig. 7).

It is notable that the rotenone-insensitive rates of reduction vary in a manner similar to the stimulated rate, suggesting that the former is likely to represent a mechanism involving the acyl-CoA dehydrogenases.

The extent of stimulation after addition of acetoacetate increases with increasing chain length. This phenomenon may be brought about by greater matrix accumulation of NADH by the longer acylcarnitines, giving a greater inhibition of β -oxidation. On addition of acetoacetate there is consequently relatively more stimulation of β -oxidation as the NADH block is removed.

Table 2. *Comparison of rates of acylcarnitine-dependent reduction of ferricyanide with rates of O_2 uptake*

Typical results are shown for rates of reduction of ferricyanide after addition of 10mM-oxaloacetate or 10mM-acetoacetate with rat liver mitochondria. The rates of O_2 uptake were obtained by using the same mitochondrial preparation. Palmitoylcarnitine and lauroylcarnitine were always added to a concentration of 20 μ M, and hexanoylcarnitine to a concentration of 100 μ M. Rates of reduction of ferricyanide were otherwise measured as described in the Experimental section. O_2 uptake was measured by using a Clark-type oxygen electrode. The reaction mixture (2.5 ml) was at 20°C, and had a composition identical with that described for measurements of β -oxidation with O_2 as the final electron acceptor, modified to contain 10mM-malonate.

Acylcarnitine derivative	Rate of ferricyanide reduction (nmol/min per mg of protein)		Rate of oxygen uptake (ng-atoms/min per mg of protein)
	Oxaloacetate	Acetoacetate	
Palmitoyl-	64	46	35
Lauroyl-	71	50	39
Hexanoyl-	13	25	23

Application of measurements of rates of reduction of ferricyanide to routine measurements of β -oxidation

Measurements of β -oxidation can, with this spectrophotometric procedure, be carried out with very small amounts of protein (less than 1 mg; see Fig. 4). This is considerably less than the amounts used in most polarographic measurements (2–3 mg or more).

Ferricyanide has in our standard assay been included at a concentration of 0.5 mM, which is well below the concentration at which it becomes inhibitory (Fig. 4). This concentration should ensure an adequate supply of the final electron acceptor for most assay purposes.

Although most of our mitochondrial preparations would catalyse reduction of ferricyanide without added cytochrome *c*, we have chosen to include 0.3 mg of cytochrome *c* in the assay. Added cytochrome *c* usually causes a slight stimulation of reduction of ferricyanide (Fig. 4), and this should also protect differences due to a variable loss of endogenous cytochrome *c*.

The inclusion of 3 μ g of rotenone to the assay has been found highly desirable, owing to the dramatic decrease in endogenous reduction of ferricyanide. Care must, however, be taken to add the rotenone after the mitochondria to avoid inactivation of rotenone by ferricyanide (Walter & Lardy, 1964). Rotenone also prevents the reduction of ferricyanide by NADH formed by the β -hydroxyacyl-CoA dehydrogenase, and by NADH-generating reactions of the tricarboxylic acid cycle, making the assay specific for the acyl-CoA dehydrogenases of β -oxidation.

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