Stimulation of Oxidation of Mitochondrial Fatty Acids and of Acetate by Acetylcarnitine

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1. Acetylcarnitine added in catalytic amounts to kidney mitochondria produces an active oxidation of endogenous fatty acids. 2. In conditions of mitochondrial 'aging', under which acetate is not oxidized, acetylcarnitine also promotes the oxidation of this exogenous substrate. 3. Dinitrophenol completely abolishes the action of acetylcarnitine. 4. Carnitine is ineffective both in the oxidation of endogenous fatty acids and of exogenous acetate. 5. The action of acetylcarnitine is shared, though to a smaller extent, by pyruvate. 6. The mechanism of acetylcarnitine action has been interpreted by considering that the readily oxidizable acetyl group of acetylcarnitine can supply the initial investment of energy needed to start fatty acid oxidation.

Carnitine has been shown to enhance the oxidation of long-chain fatty acids by homogenates of different tissues (Fritz, 1955; Fritz & McEwen, 1959) and also by isolated mitochondria (Fritz, Kaplan & Yue, 1962).

Bremer (1962*a*,*b*) has observed that the carnitine esters of some fatty acids are rapidly oxidized by mitochondria, and Bode & Klingenberg (1964) have demonstrated that muscle mitochondria, which were unable to oxidize free fatty acids, can rapidly oxidize their corresponding esters with carnitine.

Though carnitine, which influences the oxidation of added long-chain fatty acids, has no effect on the oxidation of acetate, acetylcarnitine stimulates the oxidation of acetate by heart-muscle mitochondria (Fritz & Yue, 1964). In this regard Bremer (1962b) found that in kidney mitochondria, with acetylcarnitine as substrate, the net oxygen uptake was somewhat greater than that expected from the disappearance of acetylcarnitine alone. Bremer (1962*b*) put forward the hypothesis that acetylcarnitine could stimulate the oxidation of some endogenous substrates, perhaps lipids.

In the present paper we report that acetylcarnitine does in fact enhance the oxidation of endogenous fatty acids of kidney mitochondria and, under suitable conditions, the oxidation of exogenous acetate.

MATERIALS AND METHODS

Chemicals. DL-Carnitine and acetyl-DL-carnitine were kindly supplied by the Labaz Co. (Brussels, Belgium). [1-^{14}C]Acetyl-DL-carnitine was prepared from [1-^{14}C]acetyl chloride and unlabelled carnitine according to the method of Bremer (1962*a*). Hexokinase was type III from Sigma Chemical Co., St Louis, Mo., U.S.A.

Preparation of kidney mitochondria. Rat kidneys were homogenized in 0·25M-sucrose–2mM-EDTA solution, pH 7·4, at 0°. Nuclei and cell debris were removed by centrifugation at 900*g* for 5min. Mitochondria were sedimented at 4500*g* for 10min., washed once with 0·25M-sucrose–2mM-EDTA solution and resedimented at 12500*g* for 10min. Mitochondria were finally suspended in 0·25M-sucrose at a concentration of 30–40mg. of protein/ml. When required, the mitochondrial preparation was stored at 0° for about 5hr. Mitochondrial protein content was measured by the biuret method, as described by Gornall (1949).

Oxygen uptake. Oxygen uptake was measured in small Warburg flasks attached to differential manometers, shaken in a bath at 25°.

In a final volume of 1ml. the standard medium contained: KH_2PO_4–KOH buffer, pH 7·4 (20mM); $MgCl_2$ (5mM); KCl (15mM); sucrose (60mM); tris–HCl buffer, pH 7·4 (25mM); glucose (35mM); ADP (1mM); hexokinase [43 Kunitz & McDonald (1946) units]. The reaction was started by the addition of mitochondria, corresponding to 3–4mg. of mitochondrial protein, in a volume of 0·1ml.

Determination of $^{14}CO_2$. At the end of the incubation period, the NaOH in the centre well of each flask was quantitatively transferred to a centrifuge tube containing 2ml. of 5% (w/v) $BaCl_2$–0·5% NH_4Cl solution, and the precipitated $BaCO_3$ was washed three times with water, twice with acetone and once with ether, before being dried and weighed. After transfer to plastic planchets of 1cm.^{2} area, it was counted at infinite thickness by using an end-window Geiger–Müller tube TGC-2/1B84 (Tracerlab automatic counter).

Each 1μc of [1-^{14}C]acetyl-DL-carnitine converted into $^{14}CO_2$ by the persulphate oxidation method of Abraham & Hassid (1957) gave 340×10^3 total counts (i.e. counts/min. at infinite thickness × mg. of $BaCO_3$ produced).

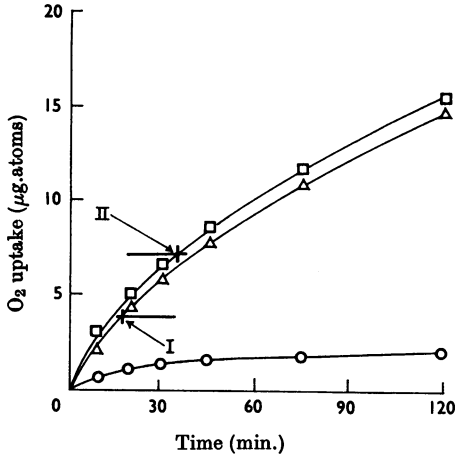

Fig. 1. 'Aged' kidney mitochondria (stored for 5 hr. at 0°), containing 3·8 mg. of protein, were incubated at 25° in 1 ml. of test medium of the following composition: KH_2PO_4–KOH buffer, pH 7·4 (20 mM); $MgCl_2$ (5 mM); KCl (15 mM); sucrose (60 mM); tris–HCl buffer, pH 7·4 (25 mM); glucose (35 mM); ADP (1 mM); hexokinase [43 Kunitz & McDonald (1946) units]. ○, Succinate (1 mM); △, succinate (1 mM) + acetyl-DL-carnitine (1 mM); □, succinate (1 mM) + pyruvate (1 mM). Maximum I is the maximum O_2 uptake expected for the oxidation of succinate (to oxaloacetate) (2 μg.-atoms of O for 1 μmole of succinate) + acetyl-DL-carnitine (2 μg.atoms of O for 0·5 μmole of acetyl-L-carnitine. Maximum II is the maximum O_2 uptake expected for the oxidation of succinate (to oxaloacetate) (2 μg.atoms of O for 1 μmole of succinate) + pyruvate (5 μg.atoms of O for 1 μmole of pyruvate). The results derived from a typical experiment are shown.

Determination of mitochondrial free fatty acids. The contents of each Warburg flask were extracted with the lipid solvent propan-2-ol–*n*-heptane–N-H_2SO_4 (40:10:1, by vol.) and subjected to thin-layer chromatography according to the method of Garland & Randle (1963).

Free fatty acids, after elution from the plate, were assayed spectrophotometrically by the method of Duncombe (1963).

RESULTS

Fig. 1 and Table 1 show that acetylcarnitine in the presence of succinate (malate may replace succinate), produced a large extra oxygen uptake, much greater than that expected for the complete oxidation of both the succinate and the acetyl group of acetylcarnitine.

A similar effect was given when acetylcarnitine was replaced by pyruvate.

As shown in Table 1 the evolution of $^{14}CO_2$ from [1-^{14}C]acetyl-DL-carnitine accounted only for a fraction of the extra oxygen uptake. It is necessary to conclude that some endogenous substrate was responsible for the extra oxygen uptake. From the values in the last column of Table 1 it is very likely that endogenous free fatty acids of the mitochondria constituted the endogenous substrate, since the presence of 1 μmole of acetylcarnitine or pyruvate caused a large decrease in the content of free fatty acids.

2,4-Dinitrophenol greatly decreased the oxygen uptake by kidney mitochondria incubated with succinate and acetylcarnitine (1 μmole). Such inhibition was considerably diminished when acetylcarnitine was added in larger amounts (20 μmoles).

Table 1. *Stimulation of mitochondrial fatty acid oxidation by acetylcarnitine and by pyruvate*

All values are referred to 3·8 mg. of mitochondrial protein and to 120 min. of incubation. Experimental conditions are given in the legend of Fig. 1. All vessels contained 1 μmole of succinate. 2,4-Dinitrophenol was added at 0·1 mM concentration. The final volume was 1 ml. Mitochondrial free fatty acid content is expressed as μmoles of palmitic acid. The results derived from a typical experiment are shown.

	O_2 uptake (μg.atoms)		$^{14}CO_2$ evolution				Mitochondrial free fatty acids (μmole)	
			Dinitrophenol absent		Dinitrophenol present			
Additions	Dinitrophenol absent	Dinitrophenol present	(counts/min.)	(μmoles)	(counts/min.)	(μmoles)	Dinitrophenol absent	Dinitrophenol present
None	3·71	1·99					0·091	0·149
DL-Carnitine (1 μmole)	3·79	2·05					0·089	0·145
Acetyl-DL-carnitine (1 μmole)	13·34	3·85	98600*	0·29	136000	0·40	0·059	0·146
Pyruvate (1 μmole)	14·60	4·61					0·065	0·150
Acetyl-DL-carnitine (20 μmoles)	23·81	18·79	74820†	2·15	135720	3·90	0·052	0·141

* [1-^{14}C]Acetyl-DL-carnitine with radioactivity of 1 μc/1 μmole.

† [1-^{14}C]Acetyl-DL-carnitine with radioactivity of 2 μc/20 μmoles.

Table 2. *Effect of acetylcarnitine on acetate oxidation*

All values are referred to 3·8 mg. of mitochondrial protein and to 180 min. incubation at 25°. The conditions are the same as in Table 1. The final volume was 1 ml. Expt. 1: 'aged' kidney mitochondria; Expt. 2: fresh kidney mitochondria. The results derived from a typical experiment are shown.

Expt. no.	Additions	O_2 uptake (μg.atoms)		Mitochondrial free fatty acids (μmole)	
		Dinitrophenol absent	Dinitrophenol present	Dinitrophenol absent	Dinitrophenol present
1	None	3·1	2·2	0·100	0·139
	Acetate (20 μmoles)	4·5	2·3	0·095	0·147
	Acetate (20 μmoles) + acetyl-DL-carnitine (1 μmole)	30·4	3·7	0·032	0·144
2	None	11·3	2·4	—	—
	Acetate (20 μmoles)	30·6	2·5	—	—
	Acetate (20 μmoles) + acetyl-DL-carnitine (1 μmole)	31·5	3·8	—	—

In either case, the evolution of $^{14}CO_2$ from [1-^{14}C]-acetyl-DL-carnitine was invariably increased in the presence of dinitrophenol.

Carnitine had no effect on either oxygen uptake or on fatty acid disappearance.

It is emphasized that the stimulating action of acetylcarnitine was most pronounced in mitochondria 'aged' for 5 hr. at 0°. Such mitochondria showed a lowered acceptor (ADP) control of respiration.

In agreement with these findings (see Table 2), acetylcarnitine and pyruvate also stimulated the oxidation of added acetate. Carnitine again was ineffective.

Dinitrophenol abolished, as might be expected, the oxygen uptake due to oxidation of free acetate.

With fresh mitochondria the oxidation of acetate, even in the presence of hexokinase, proceeded at a very high rate and it was not significantly stimulated by acetylcarnitine or pyruvate (see Table 2).

The oxidation of added acetate proceeded together with a parallel oxidation of endogenous fatty acids (see Table 2).

DISCUSSION

Acetylcarnitine enhances the oxidation of endogenous fatty acids by kidney mitochondria, as well as the oxidation of exogenous acetate. Such activation is shared by pyruvate when it is added, like acetylcarnitine, in a catalytic amount.

This action of acetylcarnitine and of pyruvate is more evident in 'aged' mitochondria, which show a lowered acceptor control of respiration, and in the presence of a hexokinase–glucose trap.

Fritz & Yue (1964) have observed an analogous stimulatory effect of acetylcarnitine on the oxidation of acetate in fresh heart mitochondria in the absence of the hexokinase–glucose trap and in the presence of AMP as phosphate acceptor.

The results reported in the present paper were obtained with acetyl-DL-carnitine.

If we assume that acetyl-L-carnitine is the active isomer (Bremer, 1962*a*), it appears that the action of this ester is quantitatively higher than that of an equimolar amount of pyruvate. This may be attributed to the fact that free carnitine, gradually available to mitochondria on oxidation of the acetyl group of acetylcarnitine, could create the proper conditions for enhancement of the activation of fatty acids (see below).

In this regard, Hülsmann, Siliprandi, Ciman & Siliprandi (1964) have observed that acetoacetate and β-hydroxybutyrate oxidation is stimulated by carnitine. This effect was perhaps due to a sparing action of carnitine on the CoA available for thiolysis of acetoacetyl-CoA, and for α-oxoglutarate oxidation.

The decrease in the mitochondrial free fatty acids does not fully account for the extra oxygen uptake. However, it is probable that the pool of free fatty acids in mitochondria reflects a dynamic state, in which free fatty acids are both leaving and entering. Previous work (Rossi, Sartorelli, Tatò & Siliprandi, 1964) showed that, during 'aging' of mitochondria, phospholipids are rapidly hydrolysed to lysophospholipids. We cannot, however, exclude the possibility that some other endogenous substrates, e.g. amino acids, are also undergoing oxidation.

It is also possible that acetylcarnitine, or pyruvate, could, by forming acetyl-CoA, remove endogenous oxaloacetate, thus releasing the inhibition of succinate dehydrogenase (Chappell, 1961). However, the possible activation of succinate oxidation consequent on the removal of oxaloacetate could not account for the large extra oxygen uptake consistently observed under the conditions of our experiments. The oxygen uptake in the presence of 1 μmole of succinate and 1 μmole of acetylcarnitine (see Fig. 1 and Table 1) is at least 3–4 times as great as

the summation expected for the complete oxidation of succinate to oxaloacetate and of the acetyl group of acetylcarnitine to carbon dioxide. The extra oxygen uptake is explainable, as Bremer (1962*a*,*b*) also postulated, only by assuming the oxidation of some endogenous substrate. The disappearance of mitochondrial free fatty acids, as demonstrated here, enable us to identify the endogenous substrates that are being oxidized.

The same results were obtained when succinate was replaced by malate.

The addition of dinitrophenol, which suppresses the activation of endogenous fatty acids or of exogenous acetate, allows an evaluation of the authentic oxidation of the acetyl group of acetylcarnitine. As can be deduced from the $^{14}CO_2$ evolution from [1-^{14}C]acetyl-DL-carnitine, dinitrophenol increases the oxidation of the acetyl group of acetylcarnitine, very probably by eliminating the competition with endogenous fatty acids for the CoA. This observation completes and clarifies that of Bremer (1962*a*) with regard to the strong inhibitory effect of dinitrophenol on the oxygen uptake by kidney mitochondria with acetylcarnitine as substrate.

In effect dinitrophenol does not inhibit, but stimulates, the oxidation of the acetyl group of acetylcarnitine. Apparently the decrease of oxygen uptake is due to the complete inhibition of the oxidation of endogenous free fatty acids 'sparked' by acetylcarnitine and not to the oxidation of acetylcarnitine itself.

A tentative explanation of the present findings is summarized in Scheme 1.

It is postulated that the availability of intramitochondrial ATP or 'high-energy' intermediates, necessary for the activation of acetate or fatty acids, is critical in 'aged' mitochondria. It is postulated that acetylcarnitine and pyruvate can stimulate the oxidation of acetate or fatty acids in 'aged' mitochondria because they are oxidized by the citric acid-cycle pathway and make available an increased rate of production of either 'high-energy' intermediates of oxidative phosphorylation or ATP. It must be assumed that such ATP is produced by the oxidation of acetylcarnitine, or pyruvate, at a rate higher than the hexokinase system can remove it, or that intramitochondrial ATP is produced in a site not completely accessible to added hexokinase. Otherwise it is necessary to assume that some 'high-energy' mitochondrial precursor of ATP might be involved in the activation of acetate or fatty acids.

Since only a small amount of acetylcarnitine is required to produce the effects described, it is necessary to assume that this quantity is sufficient to supply the initial amount of ATP needed to start fatty acid oxidation. The latter process, once started, then proceeds autocatalytically, since it provides more than enough ATP for activation.

Free carnitine, on the other hand, is inactive because it does not contribute, as acetylcarnitine does, to ATP formation.

Conceivably, the carnitine moiety, remaining after the metabolism of acetylcarnitine, may be available as an acyl acceptor. Thus carnitine could 'spare' the endogenous mitochondrial CoA supply, which might be a rate-limiting factor in fatty acid oxidation (Hülsmann, Siliprandi, Ciman & Siliprandi, 1964). In this regard, carnitine esters of fatty acids may be considered an acyl reservoir, analogous to the phosphate reserve of phosphocreatine.

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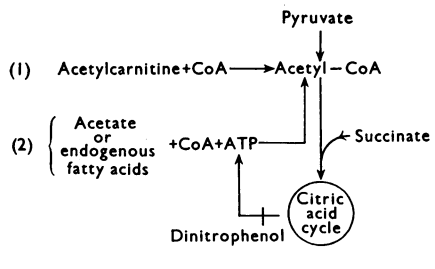

Scheme 1.

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