4-Methyl-2-oxopentanoate Oxidation by Rat Skeletal-Muscle Mitochondria

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1. Oxidative decarboxylation of 4-methyl-2-oxopentanoate (2-oxoisocaproate) by mitochondria of rat skeletal muscle showed biphasic kinetics. Two apparent K_m values of 9.1 μM and 0.78 mM were established. In broken mitochondria the rate of oxidation was lower and only the higher apparent K_m value was found. 2. Isovalerylcarnitine inhibited 4-methyl-2-oxopentanoate oxidation in the presence and absence of carnitine, but isovaleryl-CoA had no inhibitory effect. 3. Addition of ADP enhanced 4-methyl-2-oxopentanoate oxidation. Malate, succinate and 2-oxoglutarate additionally increased the rate of oxidation, but in the absence of ADP succinate and 2-oxoglutarate inhibited. 4. Addition of rotenone and simultaneous addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and valinomycin markedly decreased 4-methyl-2-oxopentanoate oxidation. 5. These observations indicate that the branched-chain 2-oxo acid dehydrogenase complex is situated on the inner side of the mitochondrial inner membrane. 6. In mitochondria and homogenates CO_2 was only produced by oxidative decarboxylation of 4-methyl-2-oxopentanoate. In intact muscle oxidation of this oxo acid proceeds more to completeness. 7. The physiological significance of intermediate formation during oxidation of branched-chain amino acids is discussed.

Branched-chain amino acids play an important role in the protein balance of the organism (Goldberg & Chang, 1978) and act as substrates for energy production during starvation and exercise (Paul & Adibi, 1978a). During rest they are mainly stored in skeletal muscle (Goldberg & Chang, 1978), which has also the highest capacity for transamination of all tissues (Shinnick & Harper, 1976). However, the main part of the capacity of rat to decarboxylate branched-chain oxo acids is present in liver.

Johnson & Connelly (1972) reported that the branched-chain 2-oxo acid dehydrogenase complex is situated on the outer surface of the mitochondrial inner membrane, but their results have subsequently been questioned (Bremer & Davis, 1978; Van Hinsbergh *et al.*, 1978b). If the complex was situated inside the mitochondrion, its activity in skeletal muscle might be underestimated, since it might be affected by a limitation in the distal part of the oxidation route (Tanaka *et al.*, 1976). Such a limitation could exist, since no activity of hydroxymethylglutaryl-CoA lyase was detected in skeletal-muscle homogenates of the rat (McGarry & Foster, 1969).

The flux through the branched-chain 2-oxo acid dehydrogenase in skeletal-muscle mitochondria and homogenates can be stimulated by the addition of carnitine (Paul & Adibi, 1978b; Van Hinsbergh *et al.*, 1978a,b). This enhancement is accompanied by a stoichiometric accumulation of isovalerylcarnitine

(Van Hinsbergh *et al.*, 1978b). Short-circuiting the oxidation route of 4-methyl-2-oxopentanoate (2-oxoisocaproate) by carnitine has enabled us to re-evaluate the localization of the branched-chain 2-oxo acid dehydrogenase complex in rat skeletal muscle, and to examine the effects of tricarboxylic acid-cycle intermediates and the redox state of the mitochondrion on the oxidative decarboxylation of 4-methyl-2-oxopentanoate by muscle mitochondria. The oxidation of 4-methyl-2-oxopentanoate in homogenates and mitochondria was compared with that in intact muscle to investigate to what extent a limitation in the further oxidation route exists in muscle.

Experimental

Materials

Chemicals were obtained or prepared as described previously (Van Hinsbergh *et al.*, 1978a,b). The rats were starved for 18–24 h before they were killed. Homogenates (10%, w/v; 600 g/5 min supernatant) and mitochondria were prepared from quadriceps femoralis muscles as described previously (Van Hinsbergh *et al.*, 1978c).

Methods

Oxidation rates were measured in a medium containing 10 mM-potassium phosphate, 75 mM-Tris/HCl (pH 7.4), 25 mM-sucrose, 35 mM-KCl, 1 mM-EDTA,

5mM-MgCl₂, 1mM-2-oxoglutarate, 1mM-NAD⁺, 25 μ M-cytochrome *c*, 0.1mM-CoA, 5mM-ADP and 2mM-L-carnitine (unless otherwise mentioned). L-[1-¹⁴C]-Leucine, 4-methyl-2-oxo[1-¹⁴C]pentanoate or 3-methyl-2-oxo[1-¹⁴C]butanoate was used as substrate (sp. radioactivity 100 μ Ci/mmol). Incubation occurred in a final volume of 1.0ml containing 0.8–1.2mg of homogenate protein or 0.10–0.15mg of mitochondrial protein at 37°C for 30min. ¹⁴CO₂ was trapped in 0.2ml of ethanolamine/ethylene glycol (1:2, v/v). Reactions were terminated by the addition of 0.2ml of 3M-HClO₄, and the vials were incubated for 75min at 0°C. Radioactivity of ¹⁴CO₂ was measured with 0.4% Omnifluor in toluene/methanol (2:1, v/v).

Transamination rates were determined by chemical decarboxylation of the oxo acids with ceric sulphate (Shinnick & Harper, 1976). Incubation time and conditions were identical with those for the determination of oxidative decarboxylation, but the final volume was 0.2ml. The 4-methyl-2-oxopentanoate formed was decarboxylated by addition of 0.6ml of 0.2M-ceric sulphate in 2M-H₂SO₄. The resulting ¹⁴CO₂ was bound to 0.2ml of 1M-Hyamine hydroxide 10-X in methanol, which was injected into the centre well immediately before addition of the ceric sulphate solution. After 90min incubation at 37°C radioactivity of ¹⁴CO₂ was determined with 0.4% Omnifluor in toluene/methanol (2:1, v/v). Samples containing various amounts of 4-methyl-2-oxo[1-¹⁴C]pentanoate were used to correct for the efficiency of decarboxylation by ceric sulphate.

Assays of cytochrome *c* oxidase activity and protein concentration were described previously (Van Hinsbergh *et al.*, 1978c).

Results

The effect of ADP on the oxygen consumption during oxo acid oxidation was measured with an oxygen electrode and with 1mM-2-oxoglutarate as substrate, since the oxygen consumption during 4-methyl-2-oxopentanoate oxidation is very small. At 37°C and in the standard medium (without ADP and L-carnitine) oxygen consumption was 63 $\pm$ 3 and 389 $\pm$ 54 ng-atoms/min per mg of protein before and after addition of ADP, respectively. The ADP/O ratio was 2.6–2.9, which indicates an appropriate coupling. After depletion of ADP the oxidation rate still exceeded the rate before ADP addition. This is due to contaminating ATPase activity, which probably originates from fragments of sarcotubular membranes, myofibrils and/or submitochondrial particles. Also the oxidation rate before addition of ADP is partly caused by this ATPase activity, since the mitochondria were isolated in a medium containing ATP.

The effects of various cofactors on the oxidative decarboxylation of 4-methyl-2-pentanoate also suggests that the mitochondria used in our experiments are intact (Table 1). Addition of thiamin pyrophosphate and omission of CoA and NAD⁺ had no significant effect on the activity of oxidative decarboxylation of 4-methyl-2-oxopentanoate. When the mitochondria were broken by freezing and thawing, the activity decreased considerably and was markedly dependent on these cofactors.

Concentration-dependence of leucine transamination and 4-methyl-2-oxopentanoate decarboxylation in rat skeletal-muscle homogenates and mitochondria is given in Fig. 1. When the rates with 0.5mM-L-

Table 1. *Effects of cofactors on the oxidative decarboxylation of 4-methyl-2-oxopentanoate by intact and broken mitochondria of rat muscle*

Oxidative decarboxylation of 0.5mM-4-methyl-2-oxo[1-¹⁴C]pentanoate by rat skeletal-muscle mitochondria was measured in standard medium, containing 0.1mM-CoA, 1mM-NAD⁺, 25 μ M-cytochrome *c*, 5mM-ADP, 2mM-L-carnitine and 1mM-2-oxoglutarate as cofactors. Thiamin pyrophosphate was added at 1mM. Mitochondria were disrupted by freezing and thawing three times after dilution with 0.25M-sucrose/10mM-Tris/HCl (pH 7.4)/0.2mM-EDTA to 0.50–0.75 mg of protein/ml. Values represent means $\pm$ s.d. of the activities relative to those without addition or omission: numbers of experiments are given in parentheses. Activities were 1.30 $\pm$ 0.54 nmol of ¹⁴CO₂/min per mg of protein in intact mitochondria and 0.27 $\pm$ 0.15 nmol of ¹⁴CO₂/min per mg of protein in broken mitochondria (means $\pm$ s.d. of 18 and 9 experiments respectively).

Addition (+) or omission (–)	Activity (% of control)	
	Intact mitochondria	Broken mitochondria
None	100	100
+Thiamin pyrophosphate	98 $\pm$ 3 (3)	197 $\pm$ 4 (3)
–CoA	93 $\pm$ 7 (4)	—
–CoA and NAD ⁺	95 $\pm$ 6 (4)	53 $\pm$ 9 (3)
–L-Carnitine	51 $\pm$ 14 (14)	79 $\pm$ 16 (6)

Fig. 1. Effect of substrate concentration on transamination and oxidative decarboxylation of leucine. Transamination and oxidative decarboxylation of L-[1-¹⁴C]leucine by (a) homogenates (600g supernatant) and (b) mitochondria of rat skeletal muscle were measured in the presence of 5 mM-ADP, 1 mM-2-oxoglutarate and 2 mM-L-carnitine. Transamination was determined from the total amount of ¹⁴CO₂ after chemical decarboxylation of the resulting 4-methyl-2-oxopentanoate. Details are given in the Experimental section. At all concentrations transamination (○) and oxidative decarboxylation (●) proceeded linearly for a 30 min period. Data represent the means $\pm$ s.d. for three experiments. Note the difference in scale.

leucine are expressed on the basis of cytochrome *c* oxidase activity, the mean rates of transamination and oxidative decarboxylation were 0.76 ± 0.19 and 0.096 ± 0.011 nmol $\cdot$ min⁻¹ $\cdot$ (μ mol of cytochrome *c* $\cdot$ min⁻¹)⁻¹ with homogenates and 0.66 ± 0.30 and 0.125 ± 0.012 nmol $\cdot$ min⁻¹ $\cdot$ (μ mol of cytochrome *c* $\cdot$ min⁻¹)⁻¹ with mitochondria, respectively (means $\pm$ s.d., four experiments). The rate of transamination markedly exceeds that of oxidative decarboxylation in both mitochondria and homogenates of rat skeletal muscle. The oxidation rates with homogenates might be somewhat underestimated because of the presence of endogenous branched-chain amino acids or pyruvate, which is inhibitory (Van Hinsbergh *et al.*, 1978a). Oxidative decarboxylation appears to be maximal at 0.2–0.3 mM-leucine in mitochondria and homogenates. These values are comparable with those previously reported for rat skeletal-muscle homogenates (Paul & Adibi, 1976).

Oxidation of 4-methyl-2-oxopentanoate showed biphasic kinetics. At low substrate concentrations an increase in oxidative decarboxylation was observed, comparable with that with leucine. At concentrations above 0.15 mM an additional increase in ¹⁴CO₂ pro-

duction from 4-methyl-2-oxo[1-¹⁴C]pentanoate was noted (Fig. 2). Two apparent *K_m* values of 9.1 ± 4.7 μ M and 0.78 ± 0.40 mM (means $\pm$ s.d. of five experiments) could be calculated. Similar *K_m* values were observed when CoA and NAD⁺ were omitted from the medium, which excludes the possibility that the biphasic kinetics could be caused by the contamination of broken mitochondria. The biphasic kinetics of decarboxylation were not observed with leucine (Fig. 1), since under our experimental conditions mitochondria produced about 40 nmol of 4-methyl-2-oxopentanoate/ml during a 30 min oxidation period at 1.0 mM-leucine. Only one Michaelis constant was found for 4-methyl-2-oxopentanoate in studies with the purified branched-chain oxo acid dehydrogenase complex of rat and ox liver (Parker & Randle, 1978a,b; Connelly *et al.*, 1968), and in those with rat liver mitochondria (Wohlhueter & Harper, 1970). When we disrupted the mitochondria by freezing and thawing, not only the rate of oxidative decarboxylation decreased (Table 1), but also the pattern of a high affinity at low 4-methyl-2-oxopentanoate concentrations disappeared (Fig. 2). This latter phenomenon may suggest that an accumulation of 4-methyl-

Fig. 2. Effect of substrate concentration on oxidative decarboxylation of 4-methyl-2-oxopentanoate by rat skeletal-muscle mitochondria

Oxidative decarboxylation of 4-methyl-2-oxo[1- 14 C]-pentanoate was determined with intact (●) and broken (▲) mitochondria in the presence of 5 mM-ADP, 1 mM-2-oxoglutarate and 2 mM-L-carnitine as described in the Experimental section. Mitochondria were disrupted by freezing and thawing three times after dilution with 0.25 M-sucrose/10 mM-Tris/HCl (pH 7.4)/0.2 mM-EDTA to 0.50–0.75 mg of protein/ml. Inset graphs represent Lineweaver-Burk plots of 4-methyl-2-oxopentanoate (S) oxidation by intact (●) and broken (▲) mitochondria.

2-oxopentanoate inside the mitochondria favours oxidative decarboxylation or that two enzymes with branched-chain oxo acid dehydrogenase activity with different affinities and stabilities exist.

A prerequisite for the first suggestion is that the branched-chain 2-oxo acid dehydrogenase complex is situated at the inner surface of the inner membrane of the mitochondrion. Isovaleryl-CoA inhibits the partially purified branched-chain 2-oxo acid dehydrogenase complex (Parker & Randle, 1978a), but this molecule cannot penetrate through the mitochondrial inner membrane. Isovalerylcarnitine can be transported into the matrix, where it will be converted into isovaleryl-CoA (Van Hinsbergh *et al.*, 1978b; Paul & Adibi, 1978b). Fig. 3 shows that isovaleryl-CoA has no effect on the oxidation of 4-methyl-2-oxopentanoate by intact rat muscle mitochondria, and slightly stimulated in the presence of L-carnitine. Isovalerylcarnitine, however, inhibited 4-methyl-2-oxopentanoate oxidation. This inhibition increased with increasing isovalerylcarnitine concentrations.

These data suggest that the branched-chain 2-oxo acid dehydrogenase complex is located at the inner side of the mitochondrial inner membrane. This is

Fig. 3. Effects of isovaleryl-CoA and isovalerylcarnitine on oxidative decarboxylation of 4-methyl-2-oxopentanoate by rat skeletal-muscle mitochondria

Oxidative decarboxylation of 0.5 mM-4-methyl-2-oxo[1- 14 C]pentanoate was determined in the presence of 5 mM-ADP, 1 mM-2-oxoglutarate and 0.1 mM-CoA as described in the Experimental section. The effects of different concentrations of isovaleryl-CoA or isovaleryl-L-carnitine were measured in the presence and absence of L-carnitine: ●, isovaleryl-CoA; ○, isovaleryl-L-carnitine; ▲, isovaleryl-CoA + 2 mM-L-carnitine; △, isovaleryl-L-carnitine + 2 mM-L-carnitine. Data represent the means of four experiments $\pm$ s.d. of the oxidation rates relative to those without addition of isovaleryl ester. Without addition the oxidation rates were 2.1 ± 0.7 and 1.0 ± 0.5 nmol of $^{14}\text{CO}_2$ /min per mg of protein in the presence and absence of L-carnitine respectively.

further supported by the observation that oxidative decarboxylation is affected by the metabolic state of the mitochondrion. Addition of ADP causes an increase in the rate of $^{14}\text{CO}_2$ production from 4-methyl-2-oxo[1- 14 C]pentanoate by rat skeletal-muscle mitochondria (Table 2). In the presence of ADP, addition of malate, succinate and 2-oxoglutarate slightly stimulated this rate, whereas palmitoyl-L-carnitine did not. Under these conditions pyruvate and 3-methyl-2-oxobutanoate at 1 mM concentration decreased oxidation of 4-methyl-2-oxopentanoate by 74 and 51 %, respectively. Without ADP, succinate and 2-oxoglutarate were inhibitory, whereas malate was not.

Table 2. *Effect of metabolic state and co-substrates on 4-methyl-2-oxopentanoate oxidation by rat skeletal-muscle mitochondria*

Rat skeletal-muscle mitochondria were prepared as described in the Experimental section, but ATP was omitted from the media. $^{14}\text{CO}_2$ production from 0.1 mM-4-methyl-2-oxo[1- ^{14}C]pentanoate was determined after a 15 min incubation period in the medium described in the Experimental section. 2-Oxoglutarate was only added when indicated. Relative values are expressed as percentages (means $\pm$ S.D.) of the rate without additions (0.37 ± 0.12 nmol of $^{14}\text{CO}_2$ /min per mg of protein) for the numbers of experiments indicated in parentheses.

Addition	Relative oxidation rate (%)	
	-ADP	+5 mM-ADP
None	100 (4)	152 $\pm$ 6 (4)
L-Malate (1 mM)	127 $\pm$ 13 (3)	174 $\pm$ 1 (3)
Succinate (1 mM)	68 $\pm$ 17 (3)	172 $\pm$ 9 (3)
2-Oxoglutarate (1 mM)	74 $\pm$ 12 (4)	201 $\pm$ 7 (4)
2-Oxoglutarate (1 mM)+rotenone (4 μM)	—	14 $\pm$ 4 (3)
Palmitoyl-L-carnitine (20 μM)	91 $\pm$ 3 (3)	116 $\pm$ 2 (3)
Palmitoyl-L-carnitine (20 μM)+rotenone (4 μM)	—	11 $\pm$ 3 (2)
Carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone (1 μM)+valinomycin (1 μM)	42 $\pm$ 3 (3)	67 $\pm$ 7 (3)

Table 3. *Oxidation of ^{14}C -labelled 4-methyl-2-oxopentanoate by various preparations of rat skeletal muscle*

Oxidation of 0.1 mM-4-methyl-2-oxopentanoate (1- ^{14}C - or U- ^{14}C -labelled) was determined at similar specific radioactivities. The incubation system for 600g supernatants and mitochondria of quadriceps muscle is given in the Experimental section. When indicated 15 mM-bicarbonate was added and only 60 mM-Tris was used. Incubation was then performed under O_2/CO_2 (19:1). Quarter diaphragm and intact soleus and extensor digitorum longus muscles were incubated in 5 ml of Krebs-Ringer buffer as described by Odessey & Goldberg (1972). Pairs of muscles of one rat were compared. Oxidation rates are expressed as nmol of 4-methyl-2-oxopentanoate oxidized to $^{14}\text{CO}_2$ /min per mg of protein for 600g supernatants and mitochondria, and per g of tissue for intact muscle. Values represent means $\pm$ S.D. of the numbers of experiments indicated.

System	No. of expts.	Label	Oxidation rate		Ratio $\frac{\text{U-}^{14}\text{C}}{\text{1-}^{14}\text{C}}$
			1- ^{14}C	U- ^{14}C	
Mitochondria	5		0.86 $\pm$ 0.27	0.15 $\pm$ 0.04	0.182 $\pm$ 0.023
Mitochondria + HCO_3^-	5		1.05 $\pm$ 0.26	0.18 $\pm$ 0.05	0.173 $\pm$ 0.006
Mitochondria + HCO_3^- - carnitine	3		0.89 $\pm$ 0.06	0.15 $\pm$ 0.01	0.163 $\pm$ 0.004
600g Supernatant	3		0.038 $\pm$ 0.014	0.007 $\pm$ 0.002	0.180 $\pm$ 0.003
600g Supernatant + HCO_3^-	3		0.037 $\pm$ 0.019	0.007 $\pm$ 0.004	0.182 $\pm$ 0.017
Intact muscle					
Diaphragm	5		1.6 $\pm$ 0.5	0.82 $\pm$ 0.10	0.53 $\pm$ 0.10
Soleus	6		1.1 $\pm$ 0.4	0.53 $\pm$ 0.30	0.52 $\pm$ 0.20
Extensor digitorum longus	5		0.75 $\pm$ 0.5	0.33 $\pm$ 0.18	0.48 $\pm$ 0.13

Elevation of the redox state of the mitochondrion by the addition of rotenone inhibited oxidative decarboxylation by more than 90%. The presence of 1 mM-NAD $^+$ in the incubation system had no effect on this inhibition. $^{14}\text{CO}_2$ production from 4-methyl-2-oxo[1- ^{14}C]pentanoate was also inhibited by the simultaneous addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and valinomycin. The effects of these substances alone were smaller and rather variable.

In mitochondria and homogenates (600g supernatants) of rat skeletal muscle, $^{14}\text{CO}_2$ production from 4-methyl-2-oxo[1- ^{14}C]pentanoate was about

six times that from [U- ^{14}C]4-methyl-2-oxopentanoate, when similar specific radioactivities were used (Table 3). Therefore $^{14}\text{CO}_2$ production from [U- ^{14}C]4-methyl-2-oxopentanoate represented only α -decarboxylation of the substrate. Neither the addition of malate instead of 2-oxoglutarate (cf. Dohm *et al.*, 1976), nor the addition of bicarbonate to the incubation system, nor the omission of carnitine, changed this ratio in our system. Analysis of the acid-soluble products after oxidation of [U- ^{14}C]4-methyl-2-oxopentanoate showed that no labelled tricarboxylic acid-cycle intermediates or acetylcarnitine accumulated in the incubation system. This indicated also a

Table 4. *Oxidative capacity of rat skeletal-muscle mitochondria*

Oxidative decarboxylation of branched-chain 1-¹⁴C-labelled oxo acids was determined as described in the Experimental section. Data on oxidation of [1-¹⁴C]pyruvate and 2-oxo[1-¹⁴C]glutarate were obtained from Bookelman (1978). [1-¹⁴C]Palmitate oxidation was assayed as described by Van Hinsbergh *et al.* (1978c). The oxidative-decarboxylation rate of oxo acids was expressed in nmol of ¹⁴CO₂/min per mg of protein, and the oxidation rate of palmitate in nmol of [1-¹⁴C]palmitate degraded to ¹⁴CO₂ and ¹⁴C-labelled acid-soluble products/min per mg of protein. Values represent the means $\pm$ S.D. of the numbers of experiments indicated. Concentrations of the substrates are given in parentheses.

Substrate	No. of expts.	Oxidation rate
4-Methyl-2-oxopentanoate (0.1 mM)	13	0.72 $\pm$ 0.26
4-Methyl-2-oxopentanoate (0.5 mM)	13	1.41 $\pm$ 0.68
3-Methyl-2-oxobutanoate (0.1 mM)	7	4.00 $\pm$ 1.02
3-Methyl-2-oxobutanoate (0.5 mM)	4	9.03 $\pm$ 3.12
Pyruvate (1 mM) + L-malate (1 mM)	12	216 $\pm$ 54
2-Oxoglutarate (1 mM)	6	248 $\pm$ 32
Palmitate (120 μ M) + L-malate (0.5 mM)	16	3.96 $\pm$ 1.41

limited degradation of the 4-methyl-2-oxopentanoate molecule in mitochondria and homogenates. On the contrary, comparison of the production of ¹⁴CO₂ from 1-¹⁴C- and U-¹⁴C-labelled 4-methyl-2-oxopentanoate in intact diaphragm, soleus and extensor digitorum longus muscles of the rat shows that 4-methyl-2-oxopentanoate oxidation proceeds more to completeness in intact muscle (Table 3).

For comparison the rates at which skeletal-muscle mitochondria of 18h-starved rats oxidize various oxo acids and palmitate are given in Table 4. The specific activity of the branched-chain 2-oxo acid dehydrogenase was higher with 3-methyl-2-oxobutanoate than with 4-methyl-2-oxopentanoate, but both activities and the oxidative capacity for palmitate were markedly lower under the nearly physiological substrate concentrations used than the activities of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase.

Discussion

From the rates of leucine oxidation by homogenates and mitochondria of rat skeletal muscle expressed on the basis of cytochrome *c* oxidase activity, it can be concluded that the branched-chain 2-oxo acid dehydrogenase activity is localized in the mitochondria, as it is in kidney and liver (Dawson *et al.*, 1967; Wohlhueter & Harper, 1970; Johnson & Connelly, 1972). From studies with ferricyanide on bovine liver mitochondria Johnson & Connelly (1972) concluded that the branched-chain 2-oxo acid dehydrogenase complex would be situated on the outer surface of the mitochondrial inner membrane. In their studies CoA and NAD⁺ were required for maximal activity. Since oxidation of 4-methyl-2-oxopentanoate by intact mitochondria of rat liver, heart and skeletal muscle was unaffected by the addition of CoA and NAD⁺ (Noda & Ichihara, 1974; Bremer & Davis, 1978; Van Hinsbergh *et al.*,

1978a), the conclusion of Johnson & Connelly (1972) has subsequently been questioned. The effects of isovaleryl-CoA and isovalerylcarnitine on the oxidative decarboxylation of 4-methyl-2-oxopentanoate reported in the present paper confirm the suggestion that the branched-chain 2-oxo acid dehydrogenase complex is situated inside the mitochondrion (Bremer & Davis, 1978; Van Hinsbergh *et al.*, 1978b).

Evidence for the location of the complex inside the mitochondrion is further strengthened by the inhibitory effects of rotenone and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone together with valinomycin. Addition of these last two substances causes a collapse of the potential over the mitochondrial inner membrane (Green, 1977). This might deteriorate the mitochondria or impair the uptake of 4-methyl-2-oxopentanoate, comparable with what happens to the uptake of pyruvate (Halestrap, 1978). In any case, the decrease would only occur when the branched-chain 2-oxo acid dehydrogenase complex is situated inside the mitochondrion.

Tricarboxylic acid-cycle intermediates and adenine nucleotides affect the rate of ketogenesis from 4-methyl-2-oxopentanoate by rat liver mitochondria (Noda & Ichihara, 1974) and homogenates (Krebs & Lund, 1976). An explanation of these effects was mainly sought in the availability of oxaloacetate and in the carboxylation of 3-methylcrotonyl-CoA. Under our experimental conditions rat skeletal-muscle mitochondria did not further oxidize isovaleryl-CoA, but converted it into isovalerylcarnitine (Van Hinsbergh *et al.*, 1978b). This means that the effects of tricarboxylic acid-cycle intermediates and ADP reflect a direct action on the uptake or decarboxylation of 4-methyl-2-oxopentanoate by these mitochondria. The inhibition by 2-oxoglutarate in the absence of ADP cannot be due to competition, as with pyruvate and 3-methyl-2-oxobutanoate, because it would then also inhibit in the presence of ADP. It seems more probable that an increase of the succinyl-

CoA (3-carboxypropionyl-CoA) concentration leads to this inhibition, since both succinate and 2-oxoglutarate are inhibitory in the absence of ADP, whereas L-malate is not, as in heart mitochondria (Bremer & Davis, 1978). It is uncertain whether succinyl-CoA itself, or sequestration of CoA, decreases the activity of oxidative decarboxylation.

Partially purified or solubilized rat liver 4-methyl-2-oxopentanoate dehydrogenase showed a single K_m value ranging from 15 to $32\mu\text{M}$ (Parker & Randle, 1978a; Danner *et al.*, 1978). With rat liver mitochondria the apparent K_m was $200\mu\text{M}$ (Wohlhueter & Harper, 1970). With rat heart mitochondria, half-maximal rate of oxidation was obtained with 0.1 mM 4-methyl-2-oxopentanoate (Bremer & Davis, 1978). Studies on skeletal muscle only considered substrate-dependence of 4-methyl-2-oxopentanoate oxidation by intact muscle (Odessey & Goldberg, 1972; Buse *et al.*, 1975). With rat skeletal-muscle mitochondria we observed two K_m values. The biphasic kinetics and particularly the high affinity at low 4-methyl-2-oxopentanoate concentration may reflect accumulation of 4-methyl-2-oxopentanoate in the mitochondrion, since it disappeared after freezing and thawing. However, the activity of 4-methyl-2-oxopentanoate dehydrogenase and other oxo acid dehydrogenases is markedly decreased by this procedure (Van Hinsbergh *et al.*, 1978b; Araki, 1977; Table 1). Therefore a second explanation might be that the low K_m value reflects a branched-chain 2-oxo acid dehydrogenase which is comparable with the solubilized liver enzyme, and the higher K_m value the ability of another more stable oxo acid dehydrogenase to decarboxylate 4-methyl-2-oxopentanoate.

Scheme 1 summarizes the oxidation pathway of

leucine in rat skeletal muscle. Transamination mainly proceeds in the cytoplasm (Taylor & Jenkins, 1966; Paul & Adibi, 1976), but our data show that a considerable part may be associated with the mitochondria. This was also found by Cappuccino *et al.* (1978). Oxidative decarboxylation occurs within the mitochondrion. Data on the further oxidation of isovaleryl-CoA are conflicting. No hydroxymethylglutaryl-CoA lyase could be detected in rat skeletal-muscle homogenates (McGarry & Foster, 1969), and $^{14}\text{CO}_2$ was only liberated from [U- ^{14}C]leucine by α -decarboxylation of 4-methyl-2-oxopentanoate in homogenates and slices of gastrocnemius muscle of the rat (Paul & Adibi, 1976). This latter observation was confirmed with homogenates and mitochondria of quadriceps muscle under our experimental conditions. On the other hand, in intact muscle 4-methyl-2-oxopentanoate oxidation proceeds more to completeness. This was also observed by Odessey & Goldberg (1972) and Buse *et al.* (1975). The discrepancy might be explained by loss of enzyme activity or by alterations in the mitochondrial environment during homogenization (cf. Krebs & Lund, 1976).

During perfusion of rat skeletal muscle with 4-methyl-2-oxopentanoate, isovalerylcarnitine accumulated (Spydevold *et al.*, 1976). Therefore it might be that skeletal muscle, depending on its metabolic state, releases intermediates of leucine oxidation, i.e. 4-methyl-2-oxopentanoate and isovalerate, into the blood stream to be used in other tissues. Release of isovalerate could be of special interest in man, since human liver has a limited capacity to decarboxylate branched-chain oxo acids (Khatra *et al.*, 1977). By release of intermediates,

Scheme 1. Leucine oxidation in rat skeletal muscle

skeletal muscle may obtain part of the energy of branched-chain amino acids, whereas the carbon skeleton remains available for the organism to be used for ketogenesis or gluconeogenesis in liver.

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