Leucine Degradation in Cell-Free Extracts of Skeletal Muscle

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Since skeletal muscle is the major site in the body for oxidation of leucine, isoleucine and valine, the pathway and control of leucine oxidation were investigated in cell-free preparations of rat muscle. Leucine was found to be transaminated to 4-methyl-2-oxopentanoate, which was then oxidatively decarboxylated. On differential centrifugation 70–80% of the transaminase activity was recovered in the soluble fraction of the cell, and the remaining amount in the mitochondrial fraction. The transaminase, from both fractions had similar pH optima and both were markedly inhibited by Ca^{2+} . Thus changes in cellular Ca^{2+} concentration may regulate transaminase activity. Both transaminases had a much higher affinity for 2-oxoglutarate than for pyruvate. Therefore the utilization of amino groups from leucine for the biosynthesis of alanine in muscle [Odessey, Khairallah & Goldberg (1974) *J. Biol. Chem.* **249**, 7623–7629] *in vivo* involves transamination with 2-oxoglutarate to produce glutamate, which is then transaminated with pyruvate to produce alanine. The dehydrogenase activity assayed by the decarboxylation of methyl-2-oxo[1- ^{14}C]pentanoate was localized exclusively in the fraction containing mitochondria and required NAD^+ , CoA and thiamin pyrophosphate for optimal activity. Measurements of competitive inhibition suggested that the oxo acids of leucine, isoleucine and valine are all decarboxylated by the same enzyme. The enzyme activity was decreased by 90% upon freezing or sonication and was stimulated severalfold by Mg^{2+} , K^+ and phosphate ions. In addition, it was markedly inhibited by ATP, but not by non-metabolizable analogues. This observation suggests that splitting of ATP is required for inhibition. The oxidative decarboxylation of 4-methyl-2-oxopentanoate by the dehydrogenase appears to be the rate-limiting step for leucine oxidation in muscle homogenates and also in intact tissues. In fact, rat muscles incubated with [1- ^{14}C]leucine release 1- ^{14}C -labelled oxo acid into the medium at rates comparable with the rate of decarboxylation. Intact muscles also released the oxo acids of [1- ^{14}C]valine or [1- ^{14}C]isoleucine, but not of other amino acids. These findings suggest that muscle is the primary source of the branched-chain oxo acids found in the blood.

Skeletal muscle rapidly degrades leucine, isoleucine and valine, and this tissue is probably the major site for the degradation of these amino acids in the organism (Odessey & Goldberg, 1972; Chang & Goldberg, 1978a,b; Adibi, 1976; Goldberg & Chang, 1978). Diverse physiological conditions such as starvation, diabetes, and hypophysectomy markedly enhance the catabolism of these amino acids by isolated muscles (Goldberg & Odessey, 1972; Odessey, 1973; Buse *et al.*, 1973, 1975; Goldberg & Chang, 1978). The oxidation of leucine may provide appreciable ATP to the muscle in starvation (Chang & Goldberg, 1978b) and leads to decreased oxidation of glucose (Chang & Goldberg, 1978c). The degradation of isoleucine and valine probably does not provide significant energy, but can

supply intermediates in the tricarboxylic acid cycle and the carbon skeletons used in glutamine synthesis (Chang & Goldberg, 1978b,c). In addition, the rapid degradation of the branched-chain amino acids provides the amino groups of the alanine and glutamine that are synthesized and released by muscle in large amounts (Odessey *et al.*, 1974; Chang & Goldberg, 1978a; Felig, 1975; Ruderman, 1975). The metabolism of leucine in muscle is also of special interest since this amino acid, and no other one, somehow promotes protein synthesis and retards protein breakdown in isolated muscle (Fulks *et al.*, 1975; Buse & Reid, 1976; Goldberg & Chang, 1978).

Most biochemical studies of the degradative pathway for branched-chain amino acids have focused on the liver and kidney (Aki *et al.*, 1968; Ichihara *et al.*, 1967), although physiological evidence (Odessey & Goldberg, 1972; Goldberg & Odessey,

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1972; Odessey, 1973; Miller, 1961; Goldberg & Chang, 1978; Buse *et al.*, 1973, 1975; Buse & Reid, 1976) indicates that the rate and control of this process in such tissues differ markedly from those in muscle. Previous studies of incubated rat diaphragm (Odessey & Goldberg, 1972; Goldberg & Odessey, 1972; Odessey, 1973; Buse *et al.*, 1973) have indicated that either transamination or decarboxylation is the rate-limiting step in leucine degradation. The activity of one of these reactions is stimulated by starvation, diabetes and hypophysectomy (Goldberg & Odessey, 1972; Odessey, 1973; Goldberg & Chang, 1978; Buse *et al.*, 1973). Although several studies have been published on the leucine transaminase activity in skeletal muscle (Ichihara & Koyama, 1966; Young 1970; Adibi *et al.*, 1975), these investigations have generally failed to correlate this activity with the degradation rate for leucine by the intact muscles, nor have they determined whether this reaction actually is the rate-limiting step. In non-muscle tissues the second enzyme in the pathway for leucine catabolism is 2-oxo acid dehydrogenase, but most workers until recently (Sketcher *et al.*, 1974; Shinnick & Harper, 1976) have failed to detect this activity in muscle homogenates (Connelly *et al.*, 1968; Dancis *et al.*, 1961; Wohlheuter & Harper, 1970).

To clarify the biochemical mechanisms controlling leucine oxidation and alanine synthesis in muscle, the properties of the transamination and decarboxylation reactions were examined in cell-free preparations of skeletal muscle. By using separate assays for these two reactions, we have investigated their subcellular distribution, the influence of ions and other factors on these activities, and the rate-limiting reaction in the degradative pathway.

Materials and Methods

Rats (CD strain) were purchased from the Charles River Laboratories and were provided with Purina laboratory chow and water *ad libitum*. Generally muscles from rats weighing 60–100g were used so that data would be comparable with earlier studies on incubated diaphragms (Odessey & Goldberg, 1972; Goldberg & Odessey, 1972). However, no significant difference in the rates of CO₂ production were observed when larger animals were used (Odessey, 1973).

Materials

The dithionium salt of CoA, NAD⁺, the sodium salt of thiamin pyrophosphate, dithiothreitol, sodium salt of 2-oxoglutaric acid, pyruvic acid, succinic acid, sodium malate, ATP, ADP, AMP, cyclic AMP, GTP, CTP, UTP, L-amino acid oxidase (type IV) and cytochrome *c* were obtained from Sigma Chemical Co., (St. Louis, MO, U.S.A.). Adenosine 5'-[α -

methylene]diphosphate, adenosine 5'-[β -methylene]diphosphate and adenosine 5'-[β -imido]diphosphate were obtained from Boehringer Mannheim (New York, U.S.A.). L-Leucine was purchased from Calbiochem (Los Angeles, CA, U.S.A.) and L-[1-¹⁴C]leucine was from Schwartz/Mann (Orangeburg, NY, U.S.A.). All reagents were of analytical grade.

Preparation of tissue homogenates

A modification of the homogenizing buffer of Zak *et al.* (1970) was found suitable for gentle homogenization of skeletal muscle. Rats were killed by cervical dislocation, and the muscles of the hindlimb including gastrocnemius, soleus and biceps femoris were excised. The tendons and interstitial fat were carefully removed, and the muscle was minced and washed as described (Zak *et al.*, 1970). The tissue was further washed and resuspended in the standard buffer at 4°C, which contained 0.25M-sucrose, 50mM-KCl, 5mM-MgCl₂, 5mM-EGTA and 50mM-Tris/HCl (pH 7.8). The muscle was then homogenized in a motor-driven loose-fitting (clearance 0.5–1.0mm) glass homogenizer (Kontes Glass Co., Vineland, NJ, U.S.A.) at a speed of about 50 rev./min. The homogenate was transferred to a tight-fitting (clearance 0.1mm) Ten-Broeck all-glass homogenizer (A. H. Thomas, Philadelphia, PN, U.S.A.) and further homogenized with three or four strokes of the pestle. The volume of the homogenate was adjusted to give a final concentration of about 100mg of tissue/ml. This concentration of the homogenate was constant for all enzyme determinations in any experiment.

Cell fractionation

The muscle homogenate was centrifuged at 800g for 5min at 4°C to remove unbroken cells and myofibrils. After the supernatant was decanted, the pellet was re-extracted in standard buffer and centrifuged as above. To isolate mitochondria, both 800g supernatants were centrifuged at 10000g for 10min. The 10000g supernatant of the second 800g fraction was discarded. The 10000g pellet from both fractions were combined, washed once and resuspended in buffer B (0.25M-sucrose/50mM-KCl/10mM-MgCl₂/0.1mM-EDTA/50mM-Tris/HCl, pH 7.8). Since the mitochondrial enzyme activity was not stable during storage (see the Results section), the 10000g pellets were prepared fresh for each experiment.

Preparation of ¹⁴C-labelled 4-methyl-2-oxopentanoate

4-Methyl-2-oxo[1-¹⁴C]pentanoate was prepared by a modification of the method of Meister (1951). The reaction mixture consisted of 2mM-[1-¹⁴C]-leucine (1.0mCi/mm δ l), L-amino acid oxidase

(1.0 unit) and catalase (12000 units) in 10ml of 10mM-Tris-HCl, pH 7.5. The mixture was gassed with O_2/CO_2 (19:1) in a 25ml flask and incubated at 37°C in a gyratory shaker. After 2h the reaction was terminated by addition of 1ml of 1M-perchloric acid. The protein precipitate was removed by centrifugation and the supernatant further acidified with 0.5ml of 6M-HCl. The supernatant was extracted with three equal volumes of freshly opened anaesthetic-grade diethyl ether. (Use of chloroform as the extractant gave similar results. The use of anhydrous ether resulted in very low yields of ^{14}C -labelled oxo acid, possibly because of the presence of peroxide in this reagent, which can cause the decarboxylation of the oxo acids.) The ether extracts were pooled and re-extracted with 3×3ml of 0.01M-NaOH. The NaOH solution was acidified with 20μl of 6M-HCl, and N_2/CO_2 (19:1) was bubbled through the solution for 15min to drive off radioactive CO_2 . Any remaining $^{14}CO_2$ was removed under reduced pressure for 15min at room temperature.

By using this procedure about 75–90% of the initial radioactivity was recovered as 1- ^{14}C -labelled oxo acid. Purity was established by t.l.c. in 2-methylpropan-2-ol/ethyl methyl ketone/water/formic acid (40:40:10:0.24, by vol.) (Fink *et al.*, 1963) and in propionic acid/water/butan-1-ol (5:7:10, by vol.). At least 95% of the radioactivity was confined to a spot identified as 2-oxo acid by u.v. fluorescence after reaction with *o*-phenylenediamine (Wieland & Fischer, 1949). Chromatography of the 2,4-dinitrophenylhydrazone in 3-methylbutan-1-ol/aq. 0.25M- NH_3 (20:1, v/v) (Dancis *et al.*, 1960) showed the 4-methyl-2-oxo[1- ^{14}C]pentanoate to be 99% pure. In addition, at least 97% of the 4-methyl-2-oxo[1- ^{14}C]pentanoate could be recovered as $^{14}CO_2$ after incubation for 1h with 2M- H_2O_2 , which decarboxylates 2-oxo acids. Finally, the radioactive product could be completely vacuum-evaporated from a frozen acid solution, which is consistent with the low boiling point of 4-methyl-2-oxopentanoate (Meister, 1951).

Enzyme assays

To assay enzyme activities, 0.5ml of cell extract was first added to 0.5ml of assay medium and kept at 4°C. When the cell extract was prepared in standard buffer, the assay medium consisted of 1.2mM- NAD^+ , 0.2mM-CoA, 1.0mM-dithiothreitol, 0.133mM-thiamin pyrophosphate, 5.0mM-2-oxoglutarate, 1mM-NaHCO₃ and 10mM-Tris/HCl, pH 7.8. The concentration of free Mg^{2+} in these mixtures has been calculated (Portzehl *et al.*, 1964) to be approx. 2.0mM. When buffer B was used to prepare the cell extract, the assay medium also included 60mM- K_2HPO_4 and 1.1mM- $CaCl_2$.

Transaminase activity was measured as the sum of the $^{14}CO_2$ and 4-methyl-2-oxo[1- ^{14}C]pentanoate produced from [1- ^{14}C]leucine. Except where noted

the reaction was started by the addition of 20μl of 0.5mM-[1- ^{14}C]leucine (10μM final concn.; 5μCi/mol) to 1ml of the assay mixture in 20ml specimen vials with self-sealing caps. After 10–20min at 27°C, the reaction was terminated by injection of 0.5ml of 0.5M- H_2SO_4 . The $^{14}CO_2$ evolved was collected in polyethylene cups containing 0.3ml of phenethylamine (Odessey & Goldberg, 1972). Then the 4-methyl-2-oxo[1- ^{14}C]pentanoate formed during incubation was measured as $^{14}CO_2$ liberated after addition of H_2O_2 . The vial was restoppered with a fresh cup of phenethylamine and injected with 0.5ml of 30% H_2O_2 to decarboxylate the 4-methyl-2-oxo[1- ^{14}C]pentanoate. The $^{14}CO_2$ evolved upon addition of peroxide was collected and radioactivity determined as before. This technique also provided information on the dehydrogenase activity of the extracts since it involved measurements of total $^{14}CO_2$ generation and the production of the intermediate 4-methyl-2-oxo[1- ^{14}C]pentanoate. Dehydrogenase activity was also assayed directly by measuring $^{14}CO_2$ production from purified 4-methyl-2-oxo[1- ^{14}C]pentanoate. 4-Methyl-2-oxo[1- ^{14}C]pentanoate (10μM final concn.) was added to the reaction mixture, which was incubated as described for leucine. After injection of H_2SO_4 , the $^{14}CO_2$ was collected and radioactivity determined.

The protein content of the homogenate was measured by the biuret method after precipitation with 10% (w/v) trichloroacetic acid. Bovine serum albumin (Sigma, fraction V) was used as the standard (Gornall *et al.*, 1949). Cytochrome *c* reductase was assayed by the method of Green & Ziegler (1963). Succinate dehydrogenase was assayed as described by Arrigoni & Singer (1962). Results presented in the Tables and Figures are typical of those obtained in two or more independent experiments. Calculations of the K_m and V_{max} values for the transaminase and dehydrogenase were made using a non-linear curve-fitting method (Cleland, 1967). Statistical significance was determined by using Student's *t* test.

Chromatography

For identification of isovaleryl hydroxamate, samples were run on cellulose thin layer plates (Baker Flex) in 4-methylbutan-1-ol/acetic acid/water (4:1:5, by vol.) and butan-1-ol/acetic acid/water (4:1:5, by vol.). Spots of the hydroxamate were located by spraying with 5% (w/v) $FeCl_3$ in a solution of 95% (v/v) ethanol and 0.3M-perchloric acid.

Determination of isovaleryl-CoA

After incubation, samples were deproteinized with 1M-perchloric acid, and isovaleryl-CoA (1mg/ml) was added as a carrier. The samples were extracted five times with an equal volume of chloroform (this treatment removes 99% of the 4-methyl-2-oxopentanoate). Hydroxylamine (final concn. 0.37M) was added, the

pH was adjusted to 7.0 and the mixture was incubated at 40°C for 10 min. A sample was taken for radioactivity measurement and assayed for hydroxamate (Notari & Munson, 1969). To determine the purity of the sample, the remaining mixtures were reacidified and extracted five times with 2 vol. of ethyl acetate. The volume of the samples was diminished and they were chromatographed as described.

Results

The rate of leucine transamination (i.e. the total production of 4-methyl-2-oxo[1-¹⁴C]pentanoate and ¹⁴CO₂) in the muscle homogenates was linear for approximately 10 min and then declined (Fig. 1a). At the low leucine concentration (10 μM) used in these experiments, the initial rate of substrate utilization has been calculated to be 1.5 %/min. Thus depletion of substrate may have contributed to the diminishing rates of reaction in incubations longer than 10 min. In the 10000g supernatant fraction, which contains most of the transaminase activity, but negligible dehydrogenase activity (see below), the rate of transamination was also linear for 10–20 min and then declined (Fig. 1b).

In contrast with the transamination of leucine, the rate of ¹⁴CO₂ production from [1-¹⁴C]leucine appeared to increase with time (Fig. 1a). The rate of ¹⁴CO₂ production between 30 and 60 min was 75 % greater than the initial rate in several experiments. Since this increase occurred concomitantly with an accumulation of oxo acid, it appears likely that leucine was not directly decarboxylated, but was first converted into its oxo acid, which was subsequently decarboxylated.

The production of ¹⁴CO₂ from 4-methyl-2-oxo-[1-¹⁴C]pentanoate was linear for about 30 min (Fig. 1c). After 30 min this rate declined, unlike CO₂ production from leucine, which increased with time (Fig. 1a). The rate of decarboxylation of 4-methyl-2-oxopentanoate by muscle homogenates was severalfold faster than the rate of decarboxylation of leucine. These findings are consistent with the assumption that the degradation of leucine occurs by transamination and subsequent decarboxylation of its oxo acid. Furthermore the initial rate of transamination was severalfold greater than the rate of CO₂ production, such that the intermediate 4-methyl-2-oxopentanoate accumulated. Thus decarboxylation of 4-methyl-2-oxopentanoate appeared to be the rate-limiting step in leucine degradation under these conditions (see below). To examine the dependence of the reaction on enzyme concentration, the dehydrogenase and transaminase were measured separately in the isolated 10000g supernatant and pellet fractions. All activities varied linearly with protein concentration over a range from 1/4 to 4 times the usual amount assayed.

Fig. 1. Transamination and decarboxylation of [1-¹⁴C]-leucine in muscle homogenates (a), transamination of [1-¹⁴C]leucine in 10000g supernatant (b) and decarboxylation of 4-methyl-2-oxo[1-¹⁴C]pentanoate in homogenates (c)

Muscle homogenates were prepared in buffer B and incubated with 10 μM-[1-¹⁴C]leucine as described in the text. Transaminase activity represents the sum of the ¹⁴CO₂ and the 4-methyl-2-oxo[1-¹⁴C]pentanoate production, assayed as described in the Materials and Methods section. ●, ¹⁴CO₂ production; ■, oxo acid production; ○, total ¹⁴C transaminated. (b) The 10000g supernatant was prepared from muscles homogenized in standard buffer and assayed with 100 μM-[1-¹⁴C]leucine. (c) Muscle homogenates prepared in standard buffer were incubated with 10 μM-4-methyl-2-oxo[1-¹⁴C]pentanoate. Reactions were terminated by injection of H₂SO₄, and the ¹⁴CO₂ liberated from the medium was collected as described in the Materials and Methods section.

Subcellular distribution of enzymes for leucine oxidation

The results in Table 1 indicate that the transaminase and dehydrogenase activities were distributed in

Table 1. Subcellular distribution of leucine transaminase and 4-methyl-2-oxopentanoate dehydrogenase activities

Muscles were homogenized in standard buffer and then centrifuged at 800g for 5 min. The supernatant was saved, and the pellet was resuspended in standard buffer and re-centrifuged. The supernatants from two successive washings were assayed as well as the final washed 800g pellet. Sample of the original 800g supernatant was then centrifuged at 10000g for 10 min, and the supernatant and the pellet resuspended in standard buffer were also assayed as described in the Materials and Methods section. In a separate experiment, the 10000g supernatant fraction was prepared as described. A sample was taken and centrifuged at 150000g for 60 min. Both supernatants were assayed for transaminase activity as described with [1-¹⁴C]leucine (1.0 mM). For measurement of the oxidation of 4-methyl-2-oxopentanoate, 10000g supernatants and pellets were prepared in buffer B. Each value represents the mean of assays performed in triplicate.

Fraction	CO ₂ production from:					Succinate dehydrogenase activity (% of total)
	Transamination		[1- ¹⁴ C]Leucine		4-Methyl-2-oxo[1- ¹⁴ C]pentanoate (pmol/min)	
	(pmol/min)	(pmol/min per mg of protein)	(pmol/min)	(pmol/min per mg of protein)		
Whole homogenate	208	22	11.5	1.2	—	—
800g supernatant	168	53	4.3	1.3	—	—
800g wash (1)	34	43	3.8	4.8	—	—
800g wash (2)	8	11	0.5	0.7	—	—
800g pellet	34	6	11.0	2.1	—	—
10000g supernatant	142	53	0.4	0.2	4	21
10000g pellet	39	53	15.3	26.2	72	79
10000g supernatant	2363	139				20
150000g supernatant	2140	126				1

different fractions of the muscle homogenate obtained by centrifugation. When homogenates were prepared with standard buffer, most of the transaminase activity was soluble since 70–80% of the activity remained in the supernatant after successive centrifugations for 20 min at 10000g or 150000g for 60 min (Table 1). The remaining 20–30% of the activity was recovered in the 10000g pellet. This pellet fraction has been reported to consist mainly of mitochondria (Zak *et al.*, 1970) and was found to contain a higher specific activity of cytochrome *c* reductase than the supernatant.

Unlike the transaminase, dehydrogenase activity (assayed by the decarboxylation of 4-methyl-2-oxopentanoate or leucine) was found mainly in the 10000g mitochondrial pellet and not in the 10000g supernatant (Table 1). The transaminase and dehydrogenase activities retained in the 800g pellets even after two washings probably are associated with mitochondria entrapped within the myofibrillar complexes or within incompletely disrupted cells.

The 10000g pellet contained 80% of the succinate dehydrogenase activity (Table 1) and thus included most of the mitochondria. This crude centrifugal fraction undoubtedly contains other intracellular membranes as well. However, if either the transaminase or the dehydrogenase were actually localized in a ‘microsomal’ fraction, significant activity should also be found in the fraction sedimenting between 10000 and 150000g. This result was not obtained (Table 1). Thus the enzyme activities in the 10000g pellet are probably localized in the muscle mitochondria. This conclusion is consistent with the enzyme distribution reported in other tissues (Ichiyama & Koyama, 1966; Aki *et al.*, 1968). Although further investigation with specific membrane markers will be necessary to prove a mitochondrial location, the activities of the dehydrogenase and transaminase in the 10000g pellet will henceforth be referred to as mitochondrial subject to the caveat stated above.

It is interesting that the mitochondrial pellet had 3-fold the dehydrogenase activity of the 800g supernatant from which it was isolated. Furthermore, the sum of the ¹⁴CO₂ production by the 800g supernatant, washes and pellet exceeded that of the parent homogenate. By contrast, the total transaminase activities of the 800g and the 10000g pellets were approximately equal to the activity of the parent fraction. These results also suggest that the supernatant fraction contains an inhibitory factor(s) for the mitochondrial dehydrogenase.

Properties of the transaminase and dehydrogenase reactions

(a) Cofactor requirements. Production of 4-methyl-2-oxopentanoate from leucine by the dialysed supernatant or washed mitochondrial pellets decreased by

Table 2. *Kinetic constants for leucine transamination with different substrates*

Transaminase activities of mitochondrial and 10000g supernatant fractions were assayed in triplicate in standard buffer (pH 7.8). When oxo acid concentrations were varied, leucine was present at 0.1 mM. Leucine concentrations were varied in the presence of 2.5 mM-oxoglutarate. In most cases the mean activities at four concentrations of substrate (ranging from 0.1 to 10 mM) were used. Mitochondrial transaminase activity was assayed at three pyruvate concentrations. The K_m and V_{max} were calculated by a non-linear regression analysis (Cleland, 1967).

Substrate	Supernatant activity		Mitochondrial activity	
	K_m (mM)	V_{max} (pmol/min per mg of protein)	K_m (mM)	V_{max} (pmol/min per mg of protein)
2-Oxoglutarate	0.10 ± 0.01	1030 ± 18	0.15 ± 0.01	2990 ± 300
Pyruvate	3.4 ± 1.8	140 ± 33	0.44 ± 0.08	2070 ± 86
Leucine	0.37 ± 0.07	1440 ± 220	0.48 ± 0.06	16600 ± 400

Table 3. *Cofactor requirements for leucine decarboxylation and 4-methyl-2-oxopentanoate dehydrogenase*

Muscles were homogenized and dialysed for 2 h in standard buffer and assayed for $^{14}\text{CO}_2$ production from [1- ^{14}C]leucine and 4-methyl-2-oxo[1- ^{14}C]pentanoate. Washed 10000g pellets were suspended in buffer B and assayed for dehydrogenase activity. DL-Carnitine hydrochloride (Sigma) was added at a concentration of 1 mM. NADH concentration was 0.25 mM and isovaleryl-CoA was 0.2 mM. For these last two experiments, media contained no added phosphate or Ca^{2+} , and 4-methyl-2-oxo[1- ^{14}C]pentanoate was present at a concentration of 20 μM . Each value is the mean ± S.E.M. of measurements made in triplicate. * Significantly different from control ($P < 0.01$).

Compound omitted (-) or added (+)	Activity (% of control)	
	Leucine decarboxylation in homogenate	Dehydrogenase in 10000g pellet
None	100	100
-Dithiothreitol	75 ± 2*	—
-NAD ⁺	96 ± 2	53 ± 3*
-CoA	99 ± 10	10 ± 2*
-Thiamin pyrophosphate	95 ± 10	66 ± 13*
-HCO ₃ ⁻	85 ± 1*	—
-Oxoglutarate	32 ± 3*	114 ± 18
+DL-Carnitine	99 ± 2	87 ± 9
+NADH	—	65 ± 11*
+Isovaleryl-CoA	—	80 ± 5*

97% in the absence of 2-oxoglutarate. In addition, ^{14}C -labelled oxo acid production was not stimulated by incubation with O_2 , nor was deamination inhibited by N_2 . These data argue against the existence of an L-amino acid oxidase in muscle. Thus transamination is the major and probably the only pathway for leucine deamination in muscle homogenates.

Both mitochondrial and supernatant transaminases were found to have a K_m of about 0.4 mM for leucine (Table 2) and a K_m of about 0.1 mM for oxoglutarate. By contrast, the K_m of the supernatant transaminase for pyruvate was over 3 mM and saturating concentrations of pyruvate supported only one-tenth of the maximal rate of leucine transamination. Such high concentrations of pyruvate (2.5 mM) inhibited the transamination of leucine with 2-oxoglutarate. The K_m of the mitochondrial transaminase for pyruvate was lower than that of the supernatant enzyme, but still higher than the K_m for 2-oxoglutarate (Table 2). Since the possibility existed that the reaction of pyruvate with the particulate transaminase was due to its conversion into 2-oxoglutarate, the activity was remeasured in the presence of arsenite (1 mM) and NaCN (1 mM) to block pyruvate dehydrogenase. Under these conditions, the activity was barely detectable at 1 mM-pyruvate, whereas 2.5 mM-pyruvate supported a rate of transamination that was only 5% of the maximal rate with 2-oxoglutarate. In addition, detergent treatment, which stimulates transamination with 2-oxoglutarate 2–3-fold (Table 6), did not enhance transamination with pyruvate. These data suggest that pyruvate is probably not a co-substrate for leucine transamination, but high concentrations may catalyse a small amount of non-specific transamination, with other transaminases present in the cell. Oxaloacetate (results not shown) was only 10% as effective as 2-oxoglutarate in stimulating transamination. Thus, of the major oxo acids in muscle, oxoglutarate is the preferred amino acceptor for leucine transamination.

CoA, NAD⁺ and thiamin pyrophosphate were necessary for maximal activity of 4-methyl-2-oxopentanoate dehydrogenase in the washed 10000g pellet (Table 3). The concentrations of these cofactors used in all other experiments were at least 2 times that required to give maximal dehydrogenase activity. The coenzymes were not required for the

decarboxylation of leucine in dialysed crude homogenates, probably because of a failure of the dialysis to remove completely these cofactors. The addition of carnitine, a cofactor in leucine oxidation subsequent to the decarboxylation step (Solberg & Bremer, 1970), had no significant effect on the decarboxylation of leucine in whole homogenates or on the dehydrogenase activity of washed mitochondrial pellets. The dehydrogenase activity is

inhibited by the end products of the reaction, NADH and isovaleryl-CoA. Under optimal conditions, the dehydrogenase activity was found to have a K_m for 4-methyl-2-oxopentanoate that varied from 20 to 30 μM (Fig. 2). Similar experiments on whole-muscle homogenates yielded a V_{max} of 55 pmol/min per mg of protein, which was still 8-fold slower than the maximal rate of leucine transamination.

(b) *Stoichiometry.* To determine the reaction products and stoichiometry, isolated mitochondria pellets were incubated with 4-methyl-2-oxo[U- ^{14}C]pentanoate prepared as described for 4-methyl-2-oxo[1- ^{14}C]pentanoate. The $^{14}\text{CO}_2$ liberated was measured. After incubation the reaction medium was analysed for [^{14}C]isovaleryl-CoA as described in the Materials and Methods section. The reaction product after conversion into the hydroxamate ran identically with isovaleryl hydroxamate in the two solvent systems utilized. In addition, the amount of labelled isovaleryl-CoA accumulated during the reaction (after correction for the blank) accounted for 92% of the amount expected from the amount of $^{14}\text{CO}_2$ production.

(c) *Substrate specificity.* Substrate specificity of 4-methyl-2-oxopentanoate dehydrogenase was investigated indirectly by measuring the ability of various oxo acids to inhibit the decarboxylation of 4-methyl-2-oxo[1- ^{14}C]pentanoate. The oxo acids were present at 10 times the concentration of 4-methyl-2-oxopentanoate. As shown in Table 4, the two branched-chain oxo acids derived from isoleucine and valine competitively inhibited decarboxylation of 4-methyl-2-oxopentanoate. Furthermore, the low inhibitory constants (K_i) were approximately equal to the K_m of the dehydrogenase for 4-methyl-2-

Fig. 2. Kinetics of 4-methyl-2-oxopentanoate dehydrogenase in isolated mitochondria
Isolated mitochondria were incubated in buffer B without added phosphate or Ca^{2+} . 4-Methyl-2-oxo[1- ^{14}C]pentanoate was added at the concentrations shown and incubated for 30 min. $^{14}\text{CO}_2$ was collected as described. Each point represents the mean of three measurements. Calculations using the non-linear curve fitting (Cleland, 1967) indicated that $K_m = 24 \pm 5 \mu\text{M}$ and $V_{\text{max}} = 119 \pm 6 \text{ pmol/h per mg}$.

Table 4. Effect of unlabelled oxo acids on the decarboxylation of 1- ^{14}C -labelled branched-chain oxo acids

Mitochondria were isolated in standard buffer with Mg^{2+} concentration lowered to 0.5 mM. Assay buffer (containing 0.4 mM-amino-oxacetate to prevent transamination of the oxo acids) was added and the Mg^{2+} was adjusted to 5 mM. The mixture was preincubated at 27°C for 1 h. The reaction was started by the addition of the appropriate unlabelled oxo acid (200 μM) followed by the addition of 4-methyl-2-oxo[1- ^{14}C]pentanoate at the concentration shown. After 5 min the reaction was terminated by the addition of 1 M-perchloric acid and the $^{14}\text{CO}_2$ was collected. When pyruvate and 2-oxoglutarate were studied NaCN (1 mM) was also added. This compound had no effect on the kinetic parameters of the control. The kinetic parameters were estimated by non-linear regression analysis (Cleland, 1967) of five concentrations of radioactive substrate (varied from 10 to 200 μM) assayed in triplicate in at least two independent experiments. *Significant effect of addition ($P < 0.01$).

Substrate	Addition	K_m (μM)	V_{max} (pmol/min per mg)	K_i (μM)
4-Methyl-2-oxo[1- ^{14}C]pentanoate	—	25 ± 4	154 ± 9	—
	4-Methyl-2-oxopentanoate	191 ± 58*	170 ± 36	30 ± 10
	3-Methyl-2-oxopentanoate	175 ± 41*	146 ± 24	33 ± 9
	—	23 ± 5	471 ± 34	—
	Pyruvate	24 ± 4	517 ± 47	—
3-Methyl-2-oxo[1- ^{14}C]pentanoate	2-Oxoglutarate	26 ± 8	422 ± 49	—
	—	30 ± 3	116 ± 13	—
	4-Methyl-2-oxopentanoate	194 ± 30*	131 ± 22	36 ± 7
	3-Methyl-2-oxopentanoate	195 ± 63*	128 ± 26	36 ± 12

Table 5. *Effects of Ca^{2+} , Mg^{2+} and phosphate on leucine transamination by supernatant and mitochondrial fractions*
The 10000g supernatant fraction was prepared and dialysed for 2h in standard buffer containing 0.1 mM-EDTA without added Mg^{2+} . The 10000g pellets were also washed in standard buffer without Mg^{2+} . To these mixtures MgCl_2 , CaCl_2 and K_2HPO_4 were added as indicated. The lower ($\leq 2\mu\text{M}$) Ca^{2+} concentrations were obtained by using Ca^{2+} /EGTA buffers (Portzehl *et al.*, 1964). Each value represents the mean $\pm$ S.E.M. of assay performed in triplicate.

Ion concn. (M)			Transaminase activity (%)	
Mg^{2+}	Ca^{2+}	PO_4^{3-}	Supernatant	Mitochondrial
0	0	0	100 $\pm$ 5	100 $\pm$ 4
0	0	3×10^{-2}	101 $\pm$ 7	99 $\pm$ 2
5×10^{-3}	0	0	101 $\pm$ 3	100 $\pm$ 5
5×10^{-3}	1.9×10^{-8}	0	82 $\pm$ 10	—
5×10^{-3}	1.7×10^{-7}	0	60 $\pm$ 19	—
5×10^{-3}	3.5×10^{-7}	0	25 $\pm$ 11	—
5×10^{-3}	1.6×10^{-6}	0	13 $\pm$ 7	—
5×10^{-3}	1.0×10^{-4}	0	5 $\pm$ 0	—
5×10^{-3}	0.50×10^{-3}	0	5 $\pm$ 0	—
0	0.5×10^{-3}	0	10 $\pm$ 1	109 $\pm$ 15

Table 6. *Effect of Ca^{2+} on leucine transamination in detergent-disrupted mitochondria*

Mitochondria were isolated in standard buffer. The mitochondria were diluted to 0.3 mg/ml with 50 mM-Tris/HCl buffer, pH 7.8, containing 1 mM- NaHCO_3 , 2.5 mM-2-oxoglutarate and 1.0 mM-sodium arsenite. When present, free Ca^{2+} was 0.5 mM and Triton X-100 was 5 mg/ml. Transaminase activity was assayed by adding [$1\text{-}^{14}\text{C}$]leucine (0.4 mM) and incubating for 10 min. Each value represents the mean $\pm$ S.E.M. of triplicate samples. * $P < 0.01$ versus control; ** $P < 0.01$ versus detergent alone.

	Activity (pmol/min per mg of protein)	Effect of Ca^{2+} (%)	Effect of detergent (%)
Control	2815 $\pm$ 750	—	—
+ Ca^{2+}	3298 $\pm$ 286	+17	—
+Triton X-100	7411 $\pm$ 119*	—	+163
+Both	4012 $\pm$ 328**	-46	+22

oxopentanoate. It thus appears likely that all these branched-chain oxo acids probably can serve as substrates for the same enzyme.

To test this hypothesis, the decarboxylation of 3-methyl-2-oxo[$1\text{-}^{14}\text{C}$]valerate, the oxo acid of valine, was studied. The K_m for this substrate was not significantly different from its K_i calculated from the inhibition of 4-methyl-2-oxopentanoate oxidation (Table 4). In addition, both 4-methyl-2-oxopentanoate and 3-methyl-2-oxopentanoate also completely inhibited the decarboxylation of 3-methyl-2-oxovalerate, the oxo acid of valine. The calculated K_i for 4-methyl-2-oxopentanoate is not significantly different from its K_m . Since the branched-chain oxo acids competitively inhibit the oxidation of each other with affinities that are apparently equal to their

affinities as substrates for the dehydrogenase, it is most likely that the site of inhibition and the catalytic site are one and the same. These findings are most simply accounted for by a single dehydrogenase that catalyses the decarboxylation of all three branched-chain oxo acids. This enzyme appears distinct from the dehydrogenases that oxidize pyruvate or 2-oxoglutarate, since these compounds did not inhibit the decarboxylation of 4-methyl-2-oxopentanoate (Table 4).

(d) *Influence of ions.* Neither phosphate nor Mg^{2+} ions had any significant effect on the transamination of leucine by 10000g supernatants or pellets (Table 5). However, Ca^{2+} ions inhibited markedly the activity of the supernatant transaminase. The soluble transaminase was sensitive to very low concentrations of Ca^{2+} . Thus an EGTA-buffered solution of $0.02\mu\text{M}$ - Ca^{2+} resulted in an 18% inhibition and $2\mu\text{M}$ in an 87% inhibition of transaminase activity. These results (Table 5) indicate a half-maximal inhibition at approximately $0.2\mu\text{M}$.

Unlike the soluble enzyme, the transaminase from the mitochondrial fraction was insensitive to exogenously added Ca^{2+} ions (Table 5). However, since mitochondria contain high amounts of calcium, the experiment was repeated with mitochondrial pellets disrupted with a high concentration of Triton X-100 (5 mg/ml; 17 mg/mg of protein). As shown in Table 6, addition of Ca^{2+} resulted in a 50% inhibition of transaminase activity in detergent-treated preparations. Thus, both soluble and particulate forms of the transaminase are inhibited by Ca^{2+} . Table 6 also shows that, in the absence of Ca^{2+} , addition of detergent stimulated transamination almost 3-fold. This effect appears to be caused by a lowering of the free Ca^{2+} concentration near the active site rather than removal of a permeability

barrier, since in the presence of Ca^{2+} the detergent has no significant stimulatory effect. This conclusion is supported by additional experiments showing that, in the absence of Ca^{2+} , Triton X-100 addition still stimulates activity 2–3-fold even with saturating concentration of leucine (5.0mM) and oxoglutarate (2.5mM).

Both Mg^{2+} and Ca^{2+} were necessary for maximal decarboxylation of 4-methyl-2-oxo[1- ^{14}C]pentanoate by the washed mitochondrial pellet (Table 7). The dehydrogenase was maximally active at 5mM- Mg^{2+} . Ca^{2+} ions had much smaller effects on the dehydrogenase activity (Table 7). Saturating concentrations of Ca^{2+} ions (0.5mM) increased the activity only 15–30%, both in the presence and in the absence of Mg^{2+} . Table 7 also shows that K^{+} ions had a significant stimulatory effect. Phosphate ions also stimulated the dehydrogenase activity (Table 7); 30mM concentrations of phosphate resulted in maximal activity but 2.5mM-phosphate had no effect. The addition of L-malate, which is known to stimulate

a number of mitochondrial transport systems (Chappell, 1968), in the presence of phosphate actually inhibited dehydrogenase activity.

(e) *Stability and pH-dependence.* The relative stabilities of the transaminase and dehydrogenase differed dramatically. Dehydrogenase activity was decreased 90% by sonication. Freezing (for 1 day) and thawing decreased dehydrogenase activity by 90%, and suspending the 10000g pellets in glycerol failed to preserve this activity. By contrast, sonication of the whole homogenates or freezing the 10000g supernatant for 1 month had no significant effect on the transaminase activity. Freezing, however, did result in a 30% loss of transaminase activity from the pellet.

The decarboxylation of leucine in whole homogenates and washed 10000g pellets had a pH optimum of 7.8. The same pH optimum was also found for the dehydrogenase (assayed with 4-methyl-2-oxo[1- ^{14}C]pentanoate), but the transaminase activity was maximal at a higher pH (8.2–8.6). This difference in pH optima was reproducible and is considered significant. The correspondence between the pH optimum for decarboxylation of leucine and that for oxidation of 4-methyl-2-oxopentanoate supports the earlier conclusion that the rate of leucine oxidation in muscle extracts is limited by the dehydrogenase activity.

(f) *Effects of nucleotide phosphates.* The cofactor requirements and subcellular distribution of the branched-chain oxo acid dehydrogenase resemble those of pyruvate dehydrogenase. Since pyruvate dehydrogenase is regulated by adenine nucleotides via phosphorylation (Chiang & Sactor, 1975; Linn *et al.*, 1969), the effects of ATP and ADP on dehydrogenase activity were tested. To test the effects of various nucleotide concentrations, appropriate regenerating systems were added to maintain constant concentrations of these nucleotides during the incubations (Table 8). The regenerating systems alone had no effect on enzyme activity. Under these conditions, 0.2mM-ATP inhibited dehydrogenase activity by almost 90%. A plot of inhibition versus oxo acid concentrations (R. Odessey & A. L. Goldberg, unpublished results) showed inhibition was purely non-competitive. An equal concentration of ADP inhibited only 50% (Table 8). It is still unclear whether ADP directly inhibits 4-methyl-2-oxopentanoate dehydrogenase, since subsequent analysis has shown that the glucose/hexokinase regenerating system allowed some synthesis of ATP from ADP by the mitochondria. Other nucleotides, including AMP, cyclic AMP, GTP, CTP and UTP, had no significant effect on this activity. In addition, analogues of ATP in which the oxygen atoms of the phosphate ester bonds were substituted by methylene or imido groups did not significantly inhibit activity (Table 8). Unlike the dehydrogenase, leucine trans-

Table 7. *Effects of ions on 4-methyl-2-oxopentanoate dehydrogenase*

To test the effects of bivalent cations, the washed 10000g pellets were suspended in buffer B and assay medium from which Mg^{2+} and Ca^{2+} had been omitted and substituted with the bivalent cations indicated (at the same concentration as the ion for which it was substituted). Samples without Mg^{2+} or Ca^{2+} also contained 5mM-EGTA and 5mM-EDTA. To test the effect of K^{+} removal, sodium salts were substituted for potassium salts. When both Na^{+} and K^{+} were present, the assay mixture was supplemented with 20mM-NaCl. Similarly, phosphate concentrations in the buffer were varied as indicated. Each assay was performed in triplicate.

Ion added	Concentration (mM)	Activity (%)
Mg^{2+} and Ca^{2+}	5.0	100
Ca^{2+} only	0.5	15
Mg^{2+} only	5.0	77
Mn^{2+} and Ca^{2+}	5.0	26
Mn^{2+} and Mg^{2+}	0.5	75
None	—	8
K^{+} only	50	100
K^{+} and Na^{+}	50	100
Na^{+} only	20	19
Phosphate only	50	100
	2.5	50
Phosphate and L-malate	40	75
	2	
None	—	50
Phosphate and arsenate	30	9
	1.2	

Table 8. *Effects of nucleotides on 4-methyl-2-oxopentanoate dehydrogenase and leucine transaminase activities of muscle homogenates*

Washed 10000g pellets and supernatants were prepared in buffer B from homogenates of muscles from fed rats. Phosphocreatine (2mM) and creatine kinase (30 units/ml) were used as the ATP-regenerating system. Glucose (20mM) and hexokinase (30 units/ml) were used as the ADP-regenerating system. Transaminase activity of 10000g supernatants was also assayed. The assay medium contained oxoglutarate (2mM) and EGTA (2mM), but no added Ca^{2+} or phosphate ions. There was no significant difference between the inhibition of transamination by the ADP-regenerating system with or without ADP. The regenerating system alone had no significant effect on the dehydrogenase activity. Each value represents the mean $\pm$ s.e.m. of assays run in triplicate. *Significant effect of nucleotide: $P < 0.01$.

Additions	Concentration (mM)	Activity (%)	
		Dehydrogenase	Transaminase
None	—	100	100
ATP+regenerating system for ATP	0.2	16 $\pm$ 4*	100 $\pm$ 4
Regenerating system alone	—	100 $\pm$ 2	99 $\pm$ 3
ADP alone	—	—	85 $\pm$ 15
ADP+regenerating system for ADP	0.2	51 $\pm$ 12*	41 $\pm$ 10*
Regenerating system alone	—	100 $\pm$ 6	74 $\pm$ 4*
Adenosine 5'-[β -methylene]diphosphate	0.2	109 $\pm$ 6	56 $\pm$ 23
Adenosine 5'-[α -methylene]diphosphate	0.2	101 $\pm$ 2	80 $\pm$ 9
Adenosine 5'-[β -imido]diphosphate	0.2	93 $\pm$ 8	100 $\pm$ 10
Cyclic AMP	1.0	91 $\pm$ 6	—
GTP	0.2	93 $\pm$ 4	103 $\pm$ 4
CTP	0.2	81 $\pm$ 9	105 $\pm$ 3
UTP	0.2	93 $\pm$ 5	97 $\pm$ 5

aminase was not significantly inhibited by ATP and ADP (Table 8). (For unknown reasons the ADP-regenerating system and adenosine 5'-[β -methylene]diphosphate by themselves decrease leucine transamination.)

Rate-limiting step for leucine degradation in intact muscles

These studies together indicated that in muscle extracts leucine transamination occurs much more rapidly than the decarboxylation of 4-methyl-2-oxopentanoate. Additional experiments were therefore performed to test whether in the intact muscle the decarboxylation of 4-methyl-2-oxopentanoate is also the rate-limiting step in the degradative pathway. If so, rat muscle incubated with [^{14}C]leucine would be expected to accumulate or to release appreciable amounts of 4-methyl-2-oxo[^{14}C]pentanoate. Although the rat diaphragm or soleus muscles did not contain significant 4-methyl-2-oxo[^{14}C]pentanoate at the end of incubation, these muscles released this compound into the medium (Fig. 3). In fact, when leucine was present at 0.1 mM, the rate of 4-methyl-2-oxopentanoate release approached or was similar to the rate of its decarboxylation. At 1.0 mM-leucine, 4-methyl-2-oxopentanoate release was about 30% of its rate of degradation. This result was not caused by leakage of oxo acid from the cut muscle fibres of the diaphragm, since similar or even greater 4-methyl-2-oxopentanoate production was found with the soleus muscle, which,

Fig. 3. *Production of CO_2 and 4-methyl-2-oxopentanoate from [^{14}C]leucine by incubated rat muscles*

Rat quarter diaphragms (b) or soleus muscles (a) were incubated with 0.1 mM-[^{14}C]leucine as described by Odessey & Goldberg (1972). Measurements of $^{14}\text{CO}_2$ and 4-methyl-2-oxo[^{14}C]pentanoate are described in the Materials and Methods section. —, $^{14}\text{CO}_2$ production; ----, 4-methyl-2-oxo[^{14}C]pentanoate release. Each point represents the mean $\pm$ s.e. of measurements from four different animals.

unlike the diaphragm, can be removed from the animal with both tendons intact. Similarly, the production of oxo acid cannot result from leakage or secretion of an aminotransferase by the muscle since no such activity was detectable in the medium after removal of the muscle. Thus the dehydrogenase appears to be the rate-limiting step relative to the transaminase in the degradative pathway in intact muscle as in the homogenates.

Table 9. *Production of $^{14}\text{CO}_2$ and $1\text{-}^{14}\text{C}$ -labelled oxo acids from various amino acids by diaphragm*

Quarter diaphragms from rats deprived of food for 2 days were incubated with 0.1 mM-amino acids as in Fig. 3. At the end of the 90 min incubation, CO_2 was collected (Odessey & Goldberg, 1972). The medium-containing flask was resealed. The oxo acids were decarboxylated by injection of 30% H_2O_2 , and $^{14}\text{CO}_2$ was collected and counted for radioactivity, as described in the Materials and Methods section. All amino acids were studied at the same specific radioactivity. Each point represents the mean $\pm$ S.E.M. of measurements from six different animals, and data were calculated relative to the precursor specific radioactivity in the medium. For unknown reasons, the rate of oxo acid release from the branched-chain amino acids varied appreciably on different days. NS, not significantly different from background.

Amino acid	$10^{10} \times ^{14}\text{C}$ recovered (mol/mg of muscle)	
	CO_2	$1\text{-}^{14}\text{C}$ -labelled oxo acid
[$1\text{-}^{14}\text{C}$]Leucine	5.19 ± 0.24	0.82 ± 0.4
[$1\text{-}^{14}\text{C}$]Valine	2.69 ± 0.16	1.18 ± 0.10
[$1\text{-}^{14}\text{C}$]Isoleucine	4.71 ± 0.07	1.20 ± 0.16
[$\text{U-}^{14}\text{C}$]Tyrosine	NS	NS
[$\text{U-}^{14}\text{C}$]Lysine	NS	NS
[$\text{U-}^{14}\text{C}$]Phenylalanine	NS	NS
[$\text{U-}^{14}\text{C}$]Alanine	1.91 ± 0.24	0.49 ± 0.014

In related studies, diaphragm muscles were incubated with [$1\text{-}^{14}\text{C}$]valine and [$1\text{-}^{14}\text{C}$]isoleucine. After 90 min, appreciable amounts of radioactivity were recovered in the incubation medium as the corresponding oxo acids, 2-oxoisovalerate and 3-methyl-2-oxopentanoate respectively (Table 9). [Production of branched-chain oxo acids by muscles has also been observed by Shinnick & Harper (1976) and Chang & Goldberg (1978a).] Thus, for all three branched-chain amino acids, decarboxylation appears to be the rate-limiting enzyme in muscle. In addition, these experiments together suggest that the branched-chain oxo acids normally found in the blood (Dancis *et al.*, 1960) are derived primarily from skeletal muscle (Table 9). For unknown reasons, the actual rate of release of the 2-oxo acids was highly variable, and in different experiments the rate of release of $1\text{-}^{14}\text{C}$ -labelled oxo acids from the three branched-chain amino acids ranged between 15% and 47% of the rate of $^{14}\text{CO}_2$ production by diaphragms. Appreciable release of the branched chains was observed with muscles from both fed and starved rats (Table 9) in which the rate of oxo acid decarboxylation is increased.

By contrast, when the diaphragm was incubated with [$\text{U-}^{14}\text{C}$]tyrosine, -phenylalanine, -glycine, -serine, or -lysine, insignificant label was recovered in the medium as the respective oxo acid. These amino acids are also not converted into intermediates of the tricarboxylic acid cycle and do not undergo significant oxidation in skeletal muscle (Chang & Goldberg, 1978a; Odessey & Goldberg, 1972). When the muscle was incubated with [$1\text{-}^{14}\text{C}$]alanine, production of [$1\text{-}^{14}\text{C}$]pyruvate occurred at about one-third the rate of $^{14}\text{CO}_2$ production. [This result is in accord with the large amount of alanine transaminase in skeletal muscle (Young, 1970).]

These experiments indicate that the dehydrogenase is rate-limiting for oxidation of the branched-chain amino acids; other evidence also indicates that the dehydrogenase and not subsequent steps regulate the

Table 10. *Effect of starvation on oxidation of leucine and the release of 4-methyl-2-oxopentanoate by rat diaphragm*

Rat quarter diaphragms were excised and incubated in Krebs-Ringer bicarbonate buffer for 90 min in the presence of leucine (0.1 mM) and glucose (5 mM) as described previously (Odessey & Goldberg, 1972). $^{14}\text{CO}_2$ was collected after injection of 1 M-perchloric acid. After $^{14}\text{CO}_2$ was collected (Odessey & Goldberg, 1972), the flask was restoppered and injected with 1 M- H_2O_2 to decarboxylate the oxo acid and the $^{14}\text{CO}_2$ was collected in like manner. Each point represents the mean $\pm$ S.E.M. of five pieces of tissue. * $P < 0.01$ (versus fed).

Substrate	Condition	$10^{10} \times ^{14}\text{CO}_2$ production (mol/mg of muscle)	$10^{10} \times ^{14}\text{C}$ -labelled oxo acid release (mol/mg of muscle)
[$1\text{-}^{14}\text{C}$]Leucine	Fed	1.02 ± 0.09	0.71 ± 0.03
	Starved	$3.29 \pm 0.43^*$	$0.53 \pm 0.03^*$
Ratio starved/fed		3.2	0.75
[$\text{U-}^{14}\text{C}$]Leucine	Fed	0.57 ± 0.06	—
	Starved	$2.20 \pm 0.28^*$	—
Ratio starved/fed		3.9	

rate of leucine catabolism in intact muscle. As shown previously, muscles from rats deprived of food catabolize the branched-chain amino acids more rapidly than those from fed animals (Goldberg & Odessey, 1972; Buse *et al.*, 1973). Table 10 shows that deprivation of food causes a 3-fold stimulation of $^{14}\text{CO}_2$ production from leucine whether it is uniformly labelled or labelled only in the carboxy group. Thus an increase in activity leading to the initial decarboxylation can account completely for the change observed. Note also that deprivation of food resulted in decreased release of 1- ^{14}C -labelled oxo acid, presumably due to a decrease in its intracellular concentration. The cross-over theorem (Chance *et al.*, 1958; Rolleston, 1972) states that, for any irreversible reaction occurring in a segment of a metabolic pathway containing no branch points, regulation of that enzyme can be inferred if the flux changes in the direction opposite to that of the substrate concentration. Up to the generation of 3-hydroxy-3-methylglutaryl-CoA, the pathway of leucine degradation appears to be unbranched, and the dehydrogenase is certainly irreversible. Thus the fall in oxo acid release in muscles from animals deprived of food with an increase in leucine degradation indicates that the dehydrogenase is the regulated step.

Discussion

Skeletal muscle synthesizes *de novo* and releases into the circulation large amounts of the gluconeogenic precursor, alanine (Ruderman, 1975; Felig, 1975). The amino groups found in alanine are derived primarily, if not exclusively, from the branched-chain amino acids, while the carbon

skeleton probably arises from pyruvate produced by muscle glycolysis (Odessey *et al.*, 1974; Chang & Goldberg, 1978a). The present studies suggest, however, that the amino groups released by leucine transamination are not transferred directly to pyruvate. The soluble leucine transaminase, which represents 80% of the total transaminase activity (Table 1), has a very high K_m (<3mM) for pyruvate and a low V_{max} . (Table 2). Since normal pyruvate concentration in resting muscle is about 0.1mM (Arese *et al.*, 1965; Wahren *et al.*, 1973), the rate of pyruvate transamination by this enzyme is probably negligible. Instead the catabolism of leucine probably first gives rise to glutamate via transamination with 2-oxoglutarate. The 2-oxoglutarate concentration in resting muscle is about 0.1–0.2mM (Arese *et al.*, 1965), which is about the K_m of the transaminase at physiological leucine concentrations (Table 2). The mitochondrial leucine transaminase activity also preferentially uses 2-oxoglutarate as a co-substrate (Table 3). These data suggest that *in vivo* 2-oxoglutarate is the amino acceptor for approximately 95% of the leucine transaminated in resting muscle.

Therefore the synthesis of alanine in this tissue most likely occurs by an initial transamination of leucine with 2-oxoglutarate and subsequent transamination of the resulting glutamate with pyruvate, as shown in Fig. 4. In fact, skeletal muscle has long been known to contain large amounts of glutamate-pyruvate transaminase (Young, 1970). The transfer of the amino groups of valine and isoleucine to alanine probably occurs in a similar fashion, since all three branched-chain amino acids share the same transaminases (Ichihara & Koyama, 1966; Young, 1970). In fact, addition of all the branched-chain amino acids results in an increased production of glutamate,

Fig. 4. Probable pathway for transfer of amino groups from branched-chain amino acids to alanine and glutamine in skeletal muscle

which by itself can stimulate alanine production (Chang & Goldberg, 1978a). Transamination of leucine, isoleucine and valine with 2-oxoglutarate can also account for the increased glutamine production in muscle on addition of these amino acids and the ability of NH_4^+ to promote glutamine release at the expense of glutamate and alanine production (Chang & Goldberg, 1978a; Ruderman, 1975) (Fig. 4).

Soluble and mitochondrial isoenzymes of leucine transaminase have been previously reported in pig heart (Ichihara & Koyama, 1966; Aki *et al.*, 1968) and rat liver (Aki *et al.*, 1967). Results in Table 1 suggest a similar distribution in skeletal muscle. The soluble activity remained in the supernatant and its activity was not diminished after centrifugation of 150000g for 60min, which should be sufficient to sediment any microsomal-associated enzyme. The particulate activity was associated with the mitochondrial fraction which contained 80% of the succinate dehydrogenase activity, a mitochondrial marker (Table 1). Although this assignment is not definitive in the absence of further purification of these fractions and of data on the distribution of markers for other subcellular components, the most likely conclusion from the present findings (Table 1) is that branched-chain amino acid aminotransferase in muscle exists primarily in the cytosol but also in the mitochondria. This conclusion is in accord with earlier observations (Ichihara & Koyama, 1966; Aki *et al.*, 1967, 1968) on the subcellular distribution of this enzyme in heart and liver, which were based on evidence similar to that obtained in Table 1.

In any case, the activity of the enzymes in both fractions is exquisitely sensitive to the concentration of free Ca^{2+} . Table 5 shows that $0.2\mu\text{M-Ca}^{2+}$, which is in the range of intracellular concentration (Ebashi & Endo, 1968), causes a 50% inhibition, and higher concentrations can inhibit by 90%. The enzyme in the particulate fraction also is stimulated 2–3-fold by removal of Ca^{2+} (Table 6). However, since mitochondria contain most of the cellular calcium, which is very difficult to remove from intact structures, this effect is only seen in detergent-disrupted particles. These findings raise the possibility that factors which change the intracellular calcium concentrations may affect branched-chain amino acid metabolism. For example, the findings in Table 6 predict that the rate of transamination in contracting skeletal muscle will be significantly lower than the rate in resting skeletal muscle. This possibility merits further investigation.

The present results clearly demonstrate the existence of branched-chain oxo acid dehydrogenase in extracts of skeletal muscle. Accordingly, the decarboxylation of 4-methyl-2-oxo[1- ^{14}C]pentanoate obeyed Michaelis-Menton kinetics (Fig. 2) and required NAD^+ , CoA and thiamin pyrophosphate

(Table 3). The enzyme also produces stoichiometric amounts of CO_2 and isovaleryl-CoA. Furthermore, this process was inhibited by arsenite (Table 7), which binds to dihydrolipoyl residues of the dehydrogenase complexes. As substrates for the dehydrogenase, the oxo acids of all three branched-chain amino acids compete with each other (Table 4). Furthermore, the K_i values for the oxo acids of leucine and valine are not significantly different from the K_m values. These findings are most simply accounted for by postulating a single dehydrogenase that can catalyse the decarboxylation of all three branched-chain oxo acids. This conclusion is consistent with the findings of Danner *et al.* (1978), who have partially purified a branched-chain oxo acid dehydrogenase from rat liver and have shown co-purification of an activity with all three oxo acids with K_m values similar to those reported in Table 4.

Previous failures (Connelly *et al.*, 1968; Dancis *et al.*, 1961; Wohlheuter & Harper, 1970; Goedde & Keller, 1967; Johnson & Connelly, 1968) to observe significant dehydrogenase activity in this tissue may have resulted from overly destructive methods of cell disruption. We have found that sonication markedly decreases dehydrogenase activity, and Sketcher *et al.* (1974) have reported that Polytron homogenization also destroys this activity. It should also be noted that optimal pH and cofactor concentrations for this enzyme in the muscle (Table 3) differ from those in the liver (Connelly *et al.*, 1968; Wohlheuter & Harper, 1970). Thus comparisons of enzyme activities in different tissues (Connelly *et al.*, 1968; Wohlheuter & Harper, 1970; Goedde & Keller, 1967; Dancis *et al.*, 1960) under conditions optimal for the liver extracts may therefore be misleading.

Several pieces of evidence suggest that the oxidative decarboxylation of 4-methyl-2-oxopentanoate is the rate-limiting step in the oxidation of leucine by muscle. (1) Under the present conditions, the initial rate of transamination was severalfold greater than that of decarboxylation, such that 4-methyl-2-oxopentanoate built up in these homogenates (Fig. 1) and washed mitochondria. (2) At saturating substrate concentrations, the dehydrogenase was 8-fold less active than leucine transamination. (3) The addition of 2-oxoglutarate to muscle homogenates stimulated transaminase activity to a much greater extent than CO_2 production from leucine (Table 3). (4) The pH optimum for $^{14}\text{CO}_2$ production from [1- ^{14}C]leucine in whole homogenates and washed mitochondrial pellets was identical with that found for the decarboxylation of 4-methyl-2-oxopentanoate and different from that of the transaminase reactions.

Although the relative activity of the transaminase and dehydrogenase *in vivo* will depend on the intracellular Ca^{2+} concentration, decarboxylation probably occurs more slowly than transamination in intact muscles as well. As shown in Fig. 3, diaphragm

and soleus incubated with [1- 14 C]leucine released amounts of 4-methyl-2-oxopentanoate comparable with the rate of 14 CO $_2$ production. The results in Table 10 also suggest that in incubated muscles it is the dehydrogenase and not prior or subsequent steps that determine the rate of leucine degradation. Accordingly, starvation stimulates the decarboxylation of leucine to the same extent as that of the whole molecule. In addition the increased decarboxylation occurs concomitantly with a decreased release of 4-methyl-2-oxopentanoate, the substrate of the dehydrogenase. By means of the cross-over theorem, it can be inferred that regulation has occurred at the dehydrogenase step. The conclusion that the decarboxylation of 4-methyl-2-oxopentanoate is the rate-limiting step in leucine oxidation by whole muscle is also consistent with the results of others (Buse *et al.*, 1975; Shinnick & Harper, 1976) on rat diaphragm. These findings and our results with muscle homogenates from animals deprived of food (Odessey, 1973) do not support a report by Adibi *et al.* (1975) that leucine oxidation increases in starvation through enhancement of transaminase activity.

These findings emphasize the difference between liver and muscle with respect to branched-chain amino acid metabolism. Unlike in skeletal muscle, in liver transaminase activity appears to be the rate-limiting step in the degradation of the branched-chain amino acids (Dawson & Hird, 1967; MacFarlane & Von Holt, 1969; Shinnick & Harper, 1976). The hepatic concentration of leucine transaminase activity is 10–20-fold lower than it is in skeletal muscle (Young, 1970; Shinnick & Harper 1976). The addition of transaminase stimulates leucine oxidation by liver homogenates, but not by skeletal muscle (Dawson & Hird, 1967; Shinnick & Harper, 1976) (Table 1). These findings are also consistent with physiological evidence that the liver plays a relatively minor role in the degradation of ingested leucine, isoleucine and valine. After a protein meal, the branched-chain amino acids account for more than 60% of the amino acids released into the circulation, although they contribute only about 20% of the amino acids ingested (Wahren *et al.*, 1976).

Many of the properties of branched-chain oxo acid dehydrogenase demonstrated here and by others (Wohlheuter & Harper, 1970; Goedde & Keller, 1967) resemble those of other mitochondrial 2-oxo acid dehydrogenases such as pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase. All these enzymes require NAD $^+$, thiamin pyrophosphate and CoA for optimal activity and are inhibited by arsenite (Tables 3 and 7). In addition, Mg $^{2+}$ and Ca $^{2+}$ stimulate both branched-chain oxo acid dehydrogenase and pyruvate dehydrogenase (Table 7) (Chiang & Sactor, 1975; Linn *et al.*, 1969; Denton *et al.*, 1972). It has also been found that the phosphorylation of pyruvate

dehydrogenase by ATP markedly inhibits catalytic activity (Chiang & Sactor, 1975; Linn *et al.*, 1969). It is interesting to speculate that the branched-chain oxo acid dehydrogenase may also be regulated by phosphorylation, since the present experiments have demonstrated that ATP also inhibits this dehydrogenase (Table 8). Accordingly, none of the non-metabolizable ATP analogues tested had any inhibitory effect (Table 8), including the imido analogue, which is sterically very similar to ATP and substitutes for ATP in many binding reactions (Yount *et al.*, 1971). Perhaps the inability of the analogues to inhibit branched-chain oxo acid dehydrogenase results from their failure to permit phosphorylation of the dehydrogenase. This latter process may even involve the same enzyme that phosphorylates pyruvate dehydrogenase.

The existence of the phosphorylation–dephosphorylation cycle for the dehydrogenase would also be consistent with the observed ionic effects on activity. By analogy with pyruvate dehydrogenase, stimulation by Mg $^{2+}$ and Ca $^{2+}$ could result from an activation of the phosphatase (Linn *et al.*, 1969; Denton *et al.*, 1972; Seiss & Wieland, 1972; Severson *et al.*, 1974) or an inhibition of the kinase (Cooper *et al.*, 1974). Also, K $^+$ may stimulate the dehydrogenase by causing an inhibition of the kinase (Roche & Reed, 1974). Similarly, inhibition of branched-chain oxo acid dehydrogenase by NADH and isovaleryl-CoA may be due to effects on enzyme phosphorylation (Pettit *et al.*, 1975; Hansford, 1976). Other explanations of the data are possible, and work to decide between these hypotheses deserves investigation.

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References

- Adibi, S. A. (1976) *Metabolism* **25**, 1287–1302
- Adibi, S. A., Peterson, S. A. & Krzysik, B. A. (1975) *Am. J. Physiol.* **228**, 432–435
- Aki, K., Ogawa, K., Shirai, A. & Ichihara, A. (1967) *J. Biochem. (Tokyo)* **62**, 610–617
- Aki, K., Ogawa, K. & Ichihara, A. (1968) *Biochim. Biophys. Acta* **159**, 276–284
- Arese, P., Kirsten, R. & Kirsten, E. (1965) *Biochem. Z.* **341**, 523–533
- Arrigoni, O. & Singer, T. P. (1962) *Nature (London)* **193**, 1256–1258
- Buse, M. G. & Reid, S.*S. (1976) *J. Clin. Invest.* **56**, 1250–1261
- Buse, M. G., Biggers, F., Drier, C. & Buse, J. F. (1973) *J. Biol. Chem.* **248**, 697–706

- Buse, M. G., Jursinic, S. & Reid, S. S. (1975) *Biochem. J.* **148**, 363–374
- Chance, B., Holmes, W., Higgins, J. J. & Connelly, C. M. (1958) *Nature (London)* **182**, 1190–1191
- Chang, T. W. & Goldberg, A. L. (1978a) *J. Biol. Chem.* **253**, 3677–3684
- Chang, T. W. & Goldberg, A. L. (1978b) *J. Biol. Chem.* **253**, 3685–3695
- Chang, T. W. & Goldberg, A. L. (1978c) *J. Biol. Chem.* **253**, 3696–3701
- Chappell, J. B. (1968) *Br. Med. Bull.* **24**, 150–157
- Chiang, P. K. & Sactor, B. (1975) *J. Biol. Chem.* **250**, 3399–3408
- Cleland, W. W. (1967) *Adv. Enzymol.* **29**, 1–32
- Connelly, J. L., Danner, D. J. & Bowden, J. A. (1968) *J. Biol. Chem.* **243**, 1198–1203
- Cooper, R. H., Randle, P. J. & Denton, R. M. (1974) *Biochem. J.* **143**, 625–641
- Dancis, J., Hutzler, J. & Levitz, M. (1960) *Biochim. Biophys. Acta* **43**, 342–343
- Dancis, J., Hutzler, J. & Levitz, M. (1961) *Biochim. Biophys. Acta* **52**, 60–64
- Danner, D. J., Lemmon, S. K. & Elsas, L. J., II (1978) *Biochem. Med.* **19**, 27–38
- Dawson, A. J. & Hird, F. J. R. (1967) *Arch. Biochem. Biophys.* **122**, 426–433
- Denton, R. M., Randle, P. J. & Martin, B. R. (1972) *Biochem. J.* **128**, 161–163
- Ebashi, S. & Endo, M. (1968) *Prog. Biophys. Mol. Biol.* **18**, 123–183
- Felig, P. (1975) *Annu. Rev. Biochem.* **44**, 933–954
- Fink, K., Cline, R. E. & Fink, R. M. (1963) *Anal. Chem.* **35**, 389–398
- Fulks, R., Li, J. B. & Goldberg, A. L. (1975) *J. Biol. Chem.* **250**, 290–298
- Goedde, H. W. & Keller, W. (1967) in *Amino Acid Metabolism and Genetic Variation*, pp. 191–218, McGraw-Hill, New York
- Goldberg, A. L. & Chang, T. W. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 2301–2307
- Goldberg, A. L. & Odessey, R. (1972) *Am. J. Physiol.* **223**, 1384–1391
- Gornall, A. G., Bardawill, D. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Green, D. E. & Ziegler, D. M. (1963) *Methods Enzymol.* **6**, 416–424
- Hansford, R. G. (1976) *J. Biol. Chem.* **251**, 5483–5489
- Ichihara, A. & Koyama, E. (1966) *J. Biochem. (Tokyo)* **59**, 160–169
- Ichihara, A., Takahashi, H., Aki, K. & Shirai, A. (1967) *Biochem. Biophys. Res. Commun.* **26**, 674–678
- Johnson, W. A. & Connelly, J. L. (1968) *J. Biol. Chem.* **243**, 3526–3531
- Linn, T. C., Pettit, F. R. & Reed, L. J. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **62**, 234–241
- MacFarlane, I. G. & Von Holt, C. (1969) *Biochem. J.* **111**, 563–571
- Meister, A. (1951) *J. Biol. Chem.* **190**, 269–276
- Miller, L. L. (1961) *Amino Acid Pools*, pp. 708–721, Elsevier, Amsterdam
- Notari, R. E. & Munson, J. W. (1969) *J. Pharm. Sci.* **58**, 1060–1064
- Odessey, R. (1973) Ph.D. Thesis, Harvard University
- Odessey, R. & Goldberg, A. L. (1972) *Am. J. Physiol.* **223**, 1376–1383
- Odessey, R., Khairallah, E. A. & Goldberg, A. L. (1974) *J. Biol. Chem.* **249**, 7623–7629
- Pettit, F. H., Pelley, J. W. & Reed, L. J. (1975) *Biochem. Biophys. Res. Commun.* **65**, 575–582
- Portzehl, H., Caldwell, P. C. & Ruegg, J. C. (1964) *Biochim. Biophys. Acta* **79**, 581–591
- Roche, T. E. & Reed, L. J. (1974) *Biochem. Biophys. Res. Commun.* **59**, 1341–1348
- Rolleston, F. S. (1972) *Curr. Top. Cell. Regul.* **5**, 47–75
- Ruderman, N. B. (1975) *Annu. Rev. Med.* **26**, 245–258
- Seiss, E. A. & Wieland, O. H. (1972) *Eur. J. Biochem.* **26**, 96–105
- Severson, D. L., Denton, R. M., Pask, H. T. & Randle, P. J. (1974) *Biochem. J.* **140**, 225–237
- Shinnick, F. L. & Harper, A. E. (1976) *Biochim. Biophys. Acta* **437**, 477–486
- Sketcher, R. D., Fern, E. B. & James, W. P. T. (1974) *Br. J. Nutr.* **31**, 333–342
- Solberg, H. E. & Bremer, J. (1970) *Biochim. Biophys. Acta* **222**, 372–380
- Wahren, J., Felig, P., Havel, R. J., Jordefeldt, L., Pernow, B. & Saltin, B. (1973) *N. Engl. J. Med.* **288**, 774–777
- Wahren, J., Felig, P. & Hagenfelt, J. (1976) *J. Clin. Invest.* **57**, 987–999
- Wieland, T. & Fischer, E. (1949) *Naturwissenschaften* **36**, 219
- Wohlheuter, R. M. & Harper, A. E. (1970) *J. Biol. Chem.* **245**, 2391–2401
- Young, V. R. (1970) in *Mammalian Protein Metabolism* (Munro, H. N., ed), vol. 4, pp. 586–674, Academic Press, New York
- Yount, R. G., Babcock, D., Ballantyne, W. & Ojala, D. (1971) *Biochemistry* **10**, 2484–2489
- Zak, R., Etlinger, J. & Fischman, D. A. (1970) *Excerpta Med. Found. Int. Congr. Ser. no. 240: Research in Muscle Development and the Muscle Spindle*, pp. 163–135