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Partial Purification and Properties of Branched-Chain 2-Oxo Acid Dehydrogenase of Ox Liver

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1. A branched-chain 2-oxo acid dehydrogenase was partially purified from ox liver mitochondria. 2. The preparation oxidized 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutyrate and D- and L-3-methyl-2-oxopentanoate. The apparent K_m values for the oxo acids and for thiamin pyrophosphate, CoA, NAD^+ and Mg^{2+} were determined. 3. The oxidation of each oxo acid was inhibited by isovaleryl (3-methylbutyryl)-CoA (competitive with CoA) and by NADH (competitive with NAD^+); K_i values were determined. 4. The preparation showed substrate inhibition with each 2-oxo acid. The oxidative decarboxylation of 4-methyl-2-oxo[1-^{14}C]pentanoate was inhibited by 3-methyl-2-oxobutyrate and DL-3-methyl-2-oxopentanoate, but not by pyruvate. The $V_{max.}$ with 3-methyl-2-oxobutyrate as variable substrate was not increased by the presence of each of the other 2-oxo acids. 5. Ox heart pyruvate dehydrogenase did not oxidize these branched-chain 2-oxo acids and it was not inhibited by isovaleryl-CoA. The branched-chain 2-oxo acid dehydrogenase activity (unlike that of pyruvate dehydrogenase) was not inhibited by acetyl-CoA. 6. It is concluded that the branched-chain 2-oxo acid dehydrogenase activity is distinct from that of pyruvate dehydrogenase, and that a single complex may oxidize all three branched-chain 2-oxo acids.

There is little information available about the properties and specificity of enzyme(s) catalysing the oxidative decarboxylation of the branched-chain 2-oxo acids formed by deamination of valine, leucine and isoleucine. Connelly *et al.* (1968) and Bowden & Connelly (1968) described the partial purification and some properties of ox liver enzyme(s). Their evidence suggested the presence of two enzymes, which may catalyse reactions analogous to those of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes. The K_m values for 2-oxo acids were high (in the mM range) and they described inhibition by isovalerate (3-methylbutyrate). Wohlhueter & Harper (1970) have studied the decarboxylation of ^{14}C-labelled branched-chain 2-oxo acids in extracts of rat liver. The apparent K_m for 4-methyl-2-oxopentanoate was 0.2mM and their results suggested that a single enzyme may catalyse decarboxylation of all three branched-chain 2-oxo acids. Apart from this, lack of information about the relevant enzyme(s) contrasts with current interest in regulation of branched-chain amino acid metabolism and in the deficiency of branched-chain 2-oxo acid dehydrogenase activity in maple-syrup-urine disease.

We describe here kinetic properties and end-product inhibition of branched-chain 2-oxo acid dehydrogenase activity partially purified from ox liver. We have been unable to purify the activity further. The main factors are low activity in tissues (relative to that of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase, which may be analogous enzyme complexes) and poor recovery (which is comparable with that achieved with pig heart pyruvate dehydrogenase complex in this laboratory). The problems (as with pyruvate dehydrogenase complex) were low yield of mitochondria in bulk preparations and loss of activity after removal of mitochondrial membranes. However, by using two features that have been successful in preparing pyruvate dehydrogenase complex (use of digitonin to disrupt lysosomes before freezing and thawing of mitochondria, and centrifugation of active material on to a sucrose cushion) preparations have been obtained that could be reliably assayed by spectrophotometric methods based on NAD^+ reduction.

Experimental

Materials

The sodium salts of branched-chain 2-oxo acids 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutyrate and DL-3-methyl-2-oxopentanoate were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Solutions were assayed with branched-chain 2-oxo acid dehydrogenase complex (see under 'Methods'); the yields, as a fraction of the amount weighed, were: 0.75 (4-methyl-2-oxopentanoate); 0.91 (3-methyl-2-oxobutyrate); 0.99 (DL-3-

methyl-2-oxopentanoate). Other reagents and enzymes were from Boehringer Corp. (London) Ltd., London W.5, U.K., or from Sigma, except for the following. Sephadex G-25 was from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. PCl_3, rotenone, DL-leucine and digitonin were from BDH Chemicals, Poole, Dorset, U.K. L-Carnitine chloride was from Koch–Light Laboratories, Colnbrook, Bucks., U.K. DL-[1-^{14}C]Leucine (50μCi/μmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. It was converted into 4-methyl-2-oxo[1-^{14}C]pentanoate enzymically (see under 'Methods'). Isovaleryl (3-methylbutyryl)-CoA was synthesized starting from isovaleric acid (see under 'Methods').

Methods

Preparation of isovaleryl-CoA. Isovaleryl chloride was prepared from isovaleric acid with PCl_3, and the fraction distilling at 114.5–115.5°C collected. Isovaleryl chloride was treated with an equimolar quantity of anhydrous sodium isovalerate to yield isovaleryl anhydride, which was collected by distillation at 212°C. Isovaleryl-CoA was synthesized from isovaleryl anhydride by the method described for the synthesis of acetyl-CoA by Simon & Shemin (1953), but with a 1.5-fold excess of anhydride. The conversion of CoA into isovaleryl-CoA was 86%. Isovaleryl-CoA was assayed as CoA released from isovaleryl-CoA by L-carnitine and carnitine acetyltransferase (Chase & Tubbs, 1965). The assay buffer contained 1mM-L-carnitine and 4 units of carnitine acetyltransferase. The preparation of isovaleryl-CoA was free of CoA.

Preparation of 4-methyl-2-oxo[1-^{14}C]pentanoate. DL-[1-^{14}C]Leucine (50μCi) was added with carrier to 1ml of 0.1M-Tris/HCl, pH7.5, to yield a final concentration of 5mM-DL-leucine. Conversion was effected by incubation at 25°C for 2.75h with 0.7 unit of L-amino acid oxidase, 5 units of D-amino acid oxidase and 20000 units of catalase. The product was separated from enzymes on a column (12cm×0.9cm) of Sephadex G-25, developed with 0.1M-Tris/HCl, pH7.5. The radioactive fractions were pooled and 4-methyl-2-oxopentanoate was assayed spectrophotometrically, as the 2,4-dinitrophenylhydrazone, at 552nm against standard solutions. Conversion of leucine was in excess of 80%.

Preparation of ox liver branched-chain 2-oxo acid dehydrogenase complex. Fresh ox liver (1.75kg) was collected on ice and transported to the laboratory (20min). All operations were performed at 2°C. Liver was cut into thin slices and dispersed in a Waring blender into approx. 2 litres of 30mM-potassium phosphate/3mM-EDTA, pH7.5 (usually 3×5s bursts). The homogenate was centrifuged at 1500rev./min for 10min in an M.S.E. 6L centrifuge. The supernatant was filtered through cheesecloth and then centrifuged at 18000*g* for 40min in an M.S.E. 18 centrifuge (6×300ml head). After removal of the supernatant, each crude mitochondrial pellet was suspended in 50ml of phosphate/EDTA buffer containing 80mg of digitonin and left at 2°C for 2min to disrupt lysosomes. After dilution to 250ml with the phosphate/EDTA buffer, the mitochondrial pellets were re-collected by centrifugation (18000*g*; 40min). They were then washed once with 250ml of phosphate/EDTA buffer and re-centrifuged (18000*g*; 40min). The pellets were then taken up in phosphate/EDTA buffer to approx. 1.4 litres and shell-frozen and thawed three times in liquid N_2 and at 30°C. The resulting mixture was then centrifuged for 2h at 45000*g* in an M.S.E. 75 centrifuge (6×300ml head). The supernatant (approx. 1.25 litres) was adjusted to 55% saturation with solid $(NH_4)_2SO_4$ (315g/litre) and, after stirring for 10min, the pellets were collected at 380000*g*-min. The pellets were resuspended in 120–150ml of phosphate/EDTA buffer containing 1mM-2-mercaptoethanol in a Teflon/glass Potter–Elvehjem homogenizer and dialysed overnight against 3×2 litres of the same buffer. The contents of the sac were adjusted to pH6.1 with 10% (v/v) acetic acid and clarified by centrifugation for 5min at 38000*g*. The supernatant was adjusted to pH7.2 with KOH, shell-frozen and stored at −10°C for 4–5 days. The solution was then thawed, and the partially purified branched-chain 2-oxo acid dehydrogenase complex concentrated by centrifuging for 45min at 150000*g* [9ml layered on to 0.5ml of 60% (w/v) sucrose in phosphate/EDTA buffer containing 3mM-dithiothreitol], and the top layer removed. The crude branched-chain 2-oxo acid dehydrogenase complex, which collected at the interface, was dissolved in the sucrose layer by sucking up and down in a Pasteur pipette. The solution of enzyme complex was stored at −10°C in 0.5ml or 1ml samples.

The degree of purification achieved was only 25-fold compared with the supernatant after shell-freezing and thawing. The specific activity was only 1munit/mg of protein. [For protein estimation see Gornall *et al.* (1949).] The preparations were stable for at least 2 months at −10°C. The yield of branched-chain 2-oxo acid dehydrogenase complex was approx. 2 units in 10ml. The preparations contained pyruvate dehydrogenase (approx. 4 units in 10ml), but were devoid of 2-oxoglutarate dehydrogenase.

Assay of branched-chain 2-oxo acid dehydrogenase complex. Spectrophotometric assay was based on NAD^+ reduction followed at 340nm. The assay buffer was 30mM-potassium phosphate, pH7.5, and the reaction was initiated (in 0.5ml volume) with 2-oxo acid. Other additions were as given in the text, Tables or Figures. One of the three preparations used showed NADH oxidase activity, which was suppressed with either rotenone (16μg/ml) or 2-heptyl-4-hydroxyquinoline *N*-oxide (100μg/ml). Rates of

NAD^+ reduction were proportional to added enzyme over the range used (2–8 munits/cuvette).

Radioactivity assay was based on $^{14}CO_2$ production from 4-methyl-2-oxo[1-^{14}C]pentanoate. In this method incubations were made in glass liquid-scintillation vials sealed with a serum stopper. The assay buffer was 30mM-potassium phosphate, pH 7.5, with other additions as given in the text, Tables or Figures, in a total volume of 0.5ml. The reaction was initiated by addition of enzyme. Incubations were made with shaking (45 cycles/min) at 30°C for the time given and terminated by injection of 100μl of 50mM-glycine/HCl, pH 3.0. $^{14}CO_2$ liberated over 90min was collected into 0.2ml of methanolic Hyamine hydroxide (10%, w/v) (in a small tube incorporated into the incubation vessel). Radioactivity in Hyamine was assayed by liquid-scintillation spectrometry by using a toluene-based scintillator (Severson *et al.*, 1974). Quench corrections were applied by means of an external standard.

Assay of 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutyrate and DL-*3-methyl-2-oxopentanoate.* These were assayed spectrophotometrically with branched-chain 2-oxo acid dehydrogenase complex by allowing NAD^+ reduction to go to completion. This was achieved (in 30mM-potassium phosphate, pH 7.5) with 1mM-NAD^+, 0.4mM-CoA, 0.4mM-thiamin pyrophosphate, 2mM-$MgSO_4$, 1mM-L-carnitine, 0.5 unit of carnitine acetyltransferase and 2 munits of branched-chain 2-oxo acid dehydrogenase complex. The reaction took no more than 5min to complete.

Expression of results. A unit of enzyme activity is the amount of enzyme catalysing the formation of 1μmol of product (NADH) in 1min at 30°C. In studies of enzyme kinetics K_m and $V_{max.}$ were computed by the method of Jones (1970). This method weights values at high substrate concentrations and yields unbiased values of K_m and $V_{max.}$ by selecting a fit that gives least residual sum of squares in linear-regression analysis of the Lineweaver–Burk equation. Enzyme–inhibition data were analysed by fitting to the equation for mixed non-competitive inhibition, and comparing the significance of fit (F-test) with that obtained by using the equations for competitive, simple linear non-competitive and uncompetitive inhibition (see Cleland, 1963*a*,*b*).

Results and Discussion

It was found, by spectrophotometric assay, that the branched-chain 2-oxo acid dehydrogenase activity of these preparations required CoA, thiamin pyrophosphate and Mg^{2+} for NAD^+ reduction. In determining apparent K_m values for a reaction involving five substrates or cofactors (the above four plus 2-oxo acid), four have been fixed at concentrations not less than 10 times their apparent K_m values.

Kinetics of oxidation of 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutyrate and DL-*3-methyl-2-oxopentanoate by branched-chain 2-oxo acid dehydrogenase complex*

The apparent K_m values for 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutyrate and DL-3-methyl-2-oxopentanoate were determined in the range 0–200μM (Table 1). All three observed K_m values

Table 1. *Substrate kinetics for branched-chain 2-oxo acid dehydrogenase complex of ox liver*

For details of enzyme preparation and spectrophotometric assay see the Experimental section. The variable concentrations in determination of apparent K_m are shown in column 1. The non-variable concentrations were, as applicable: thiamin pyrophosphate, 0.4mM; NAD^+, 1mM; $MgSO_4$, 2mM; 2-oxo acid (as specified in parentheses), 0.15mM. Results are means±S.E.M.; the numbers in parentheses in column 2 are the numbers of individual rate measurements. The relative values for $V_{max.}$ are based on five rate measurements over a range of 10–100μM-2-oxo acid.

Substrate (range of concns.)	Apparent K_m (μM)	$V_{max.}$ (nmol/min)
4-Methyl-2-oxopentanoate (1–200μM)	8.7 ± 1.25 (51)	1.83±0.09
3-Methyl-2-oxobutyrate (1–140μM)	15.6 ± 1.76 (25)	2.07±0.04
DL-3-Methyl-2-oxopentanoate (1–140μM)	17.2 ± 4.04 (23)	1.18±0.07
CoA (1–800μM) (4-methyl-2-oxopentanoate)	7.1 ± 0.64 (55)	—
	2.5 ± 0.17 (13)*	—
CoA (37.5–400μM) (3-methyl-2-oxobutyrate)	9.0 ± 2.3 (8)	—
CoA (3.1–400μM) (DL-3-methyl-2-oxopentanoate)	7.0 ± 0.67 (11)	—
NAD^+ (10μM–5mM) (4-methyl-2-oxopentanoate)	109 ±10.76 (29)	—
NAD^+ (50μM–1mM) (3-methyl-2-oxobutyrate)	126 ± 5.04 (20)	—
NAD^+ (50μM–1mM) (DL-3-methyl-2-oxopentanoate)	101 ± 7.24 (22)	—
Thiamin pyrophosphate (0.4–80μM) (4-methyl-2-oxopentanoate)	0.35± 0.08 (23)§	—
Mg^{2+} (0.04–60μM) (4-methyl-2-oxopentanoate)	4.2 ± 1.1 (8)†	—

* Measured in the presence of 1mM-L-carnitine and 0.5 unit of carnitine acetyltransferase (≡approx. 17 munits of carnitine isovaleryltransferase activity).

† In MgEDTA buffers (1mM-EDTA): Mg^{2+} concentrations were computed from the following dissociation constants: MgEDTA 4.26μm, $MgSO_4$ or $MgHPO_4$ 5.37mM.

§ Free Mg^{2+} calculated at 281μM.

were between 8 μM and 20 μM in ascending order: 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutyrate and DL-3-methyl-2-oxopentanoate. The value determined for DL-3-methyl-2-oxopentanoate is complex, since both the D and L forms were oxidized by these enzyme preparations (shown by 99.6% conversion as assayed by NAD^+ reduction). All three 2-oxo acids showed substrate inhibition at and above 500 μM (Fig. 1). The relative $V_{max.}$ values for the three 2-oxo acids, also determined in the range 0–200 μM, were 1.13:1.0:0.64 for 3-methyl-2-oxobutyrate, 4-methyl-2-oxopentanoate and DL-3-methyl-2-oxopentanoate respectively.

Apparent K_m values for CoA and NAD^+ and inhibition by isovaleryl-CoA and NADH. The apparent K_m values for CoA with any one of the three 2-oxo acids were not significantly different (Table 1). The K_m for CoA was lowered by the addition of 0.5 unit of carnitine acetyltransferase and carnitine to the assay medium, which acts to remove the end product isovaleryl-CoA. The K_m of isovaleryl-CoA (for preparation see under 'Methods') for carnitine acetyltransferase (determined by the method of Chase & Tubbs, 1965) was 14.5±2.9 μM and the $V_{max.}$ for the carnitine isovaleryltransferase activity relative to carnitine acetyltransferase activity was 34:1000.

The inhibition of branched-chain 2-oxo acid dehydrogenase complex by isovaleryl-CoA (Fig. 2) was competitive with respect to CoA irrespective of which of the three 2-oxo acids was used as substrate. The K_i values shown in Table 2 were determined with a program that does not weight values obtained at higher substrate concentrations (CoA). When calculated in this way the K_i for isovaleryl-CoA with DL-3-methyl-2-oxopentanoate as substrate was significantly higher than the K_i obtained with the other two 2-oxo acids (Table 2). The K_i values have also been calculated by using the program for K_m (which weights values at higher substrate concentration; see the Experimental section) and the formula for competitive inhibition. With this calculation the values were 11.5 μM (4-methyl-2-oxopentanoate), 11.2 μM (3-methyl-2-oxobutyrate) and 8.9 μM (DL-3-methyl-2-oxopentanoate). These values did not differ significantly.

The apparent K_m for NAD^+ was independent of the 2-oxo acid used as substrate (Table 1). The K_m for NAD^+ has not been determined under conditions where NADH does not accumulate.

The inhibition of branched-chain 2-oxo acid dehydrogenase complex by NADH is shown in Fig. 3. The K_i values obtained with each of the three 2-oxo acids as one of the fixed substrates are shown in Table 2. There was no significant difference between the determined K_i values, and in each case NADH was competitive with NAD^+.

Apparent K_m for thiamin pyrophosphate and Mg^{2+}. The apparent K_m for thiamin pyrophosphate (Table 1) was independent of the concentration of 4-methyl-2-oxopentanoate over the range 10–500 μM (results not shown). This contrasts with the pyruvate dehydro-

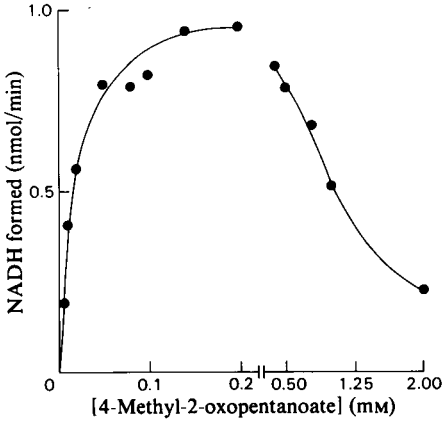

Fig. 1. *Substrate inhibition of branched-chain 2-oxo acid dehydrogenase complex by high concentrations of 4-methyl-2-oxopentanoate*

Branched-chain 2-oxo acid dehydrogenase complex was purified from ox liver and assayed spectrophotometrically (see under 'Methods'). The concentrations of the other substrates were: thiamin pyrophosphate, 0.4 mM; CoA, 0.4 mM; NAD^+, 1 mM; $MgSO_4$, 2 mM. Assays with this preparation were made in the presence of rotenone (16 μg/ml) to suppress NADH oxidase activity.

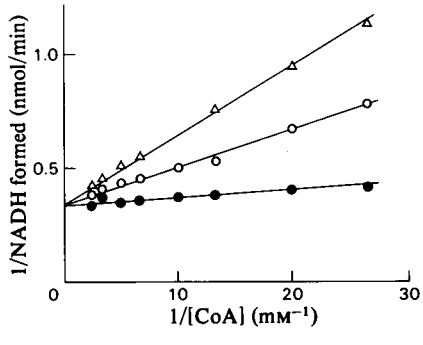

Fig. 2. *Inhibition of branched-chain 2-oxo acid dehydrogenase complex by isovaleryl-CoA*

Branched-chain 2-oxo acid dehydrogenase complex was purified from ox liver and assayed spectrophotometrically (see under 'Methods'). Concentrations were: thiamin pyrophosphate, 0.4 mM; NAD^+, 1 mM; $MgSO_4$, 2 mM; 3-methyl-2-oxobutyrate, 0.15 mM; isovaleryl-CoA, zero (●), 50 μM (○), 100 μM (△).

Table 2. *Inhibition by isovaleryl-CoA and NADH of branched-chain 2-oxo acid dehydrogenase of ox liver*

See the Experimental section for details of enzyme preparation, spectrophotometric assay and isovaleryl-CoA preparation. Where applicable the fixed concentrations were: thiamin pyrophosphate, 0.4mM; CoA, 0.4mM; NAD^+, 1mM; $MgSO_4$, 2mM. For NADH inhibition, the ranges of [NAD^+] were: 2.5μM–1mM for 4-methyl-2-oxopentanoate; 50μM–1mM for the other two branched-chain 2-oxo acids. The ranges of [CoA] used in the isovaleryl-CoA inhibition were 2.5–400μM, 37.5–400μM and 3.7–400μM for 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutyrate and DL-3-methyl-2-oxopentanoate respectively. The first column shows the oxo acid used (in each case 0.15mM), and in parentheses the number of rate determinations at each inhibitor concentration. The second column shows the inhibitor and the concentrations (μM) used. K_i values are means ± S.E.M.

2-Oxo acid	Inhibitor	K_i (μM)
4-Methyl-2-oxopentanoate (8, 8, 8, 4)	NADH (0, 25, 50, 250)	51.3 ± 5.9
3-Methyl-2-oxobutyrate (6, 6, 6)	NADH (0, 25, 50)	41.4 ± 7.3
3-Methyl-2-oxopentanoate (12, 6, 6)	NADH (0, 25, 50)	33.4 ± 6.3
4-Methyl-2-oxopentanoate (16, 12, 12)	Isovaleryl-CoA (0, 50, 100)	7.5 ± 0.7
3-Methyl-2-oxobutyrate (8, 8, 7)	Isovaleryl-CoA (0, 50, 100)	8.3 ± 1.4
3-Methyl-2-oxopentanoate (12, 12, 10)	Isovaleryl-CoA (0, 50, 100)	17.9 ± 2.7

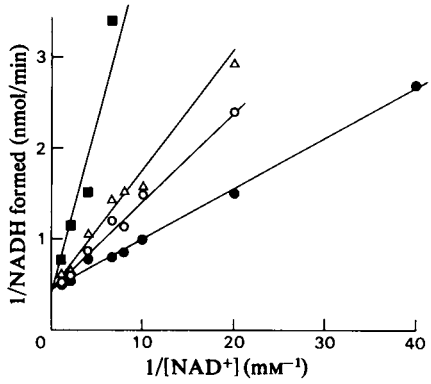

Fig. 3. *Inhibition of branched-chain 2-oxo acid dehydrogenase complex by NADH*

See under 'Methods' for details of branched-chain 2-oxo acid dehydrogenase preparation and spectrophotometric assay. Concentrations were: thiamin pyrophosphate, 0.4mM; CoA, 0.4mM; $MgSO_4$, 2mM; 4-methyl-2-oxopentanoate, 0.15mM; NADH, zero (●), 25μM (○), 59μM (△), 250μM (■).

genase complex, where the apparent K_m for thiamin pyrophosphate decreases with increasing pyruvate concentration (Walsh *et al.*, 1976). The apparent K_m for Mg^{2+} (measured with MgEDTA buffers) was 4.2μM (see Table 1). The K_m for thiamin pyrophosphate was dependent on the Mg^{2+} concentration, being higher at low Mg^{2+} concentrations (results not shown). This might suggest that thiamin pyrophosphate binding requires Mg^{2+}, as has been demonstrated with pyruvate dehydrogenase (Walsh *et al.*, 1976).

Branched-chain 2-oxo acid dehydrogenase and pyruvate dehydrogenase activities

All preparations of branched-chain 2-oxo acid dehydrogenase complex contained pyruvate dehydrogenase complex activity, but not 2-oxoglutarate dehydrogenase activity. The $V_{max.}$ of the pyruvate dehydrogenase complex in these preparations was less than twice that of the branched-chain 2-oxo acid dehydrogenase activity. However, there is substantial evidence to suggest that the branched-chain 2-oxo acid dehydrogenase activity does not reside in the pyruvate dehydrogenase complex. The oxidation of 52μM-4-methyl-2-oxo[1-^{14}C]pentanoate by branched-chain 2-oxo acid dehydrogenase complex was not inhibited by the presence of 1.25mM-pyruvate (Fig. 4; see under 'Methods' for radiochemical assay).

Pyruvate dehydrogenase complex purified from ox heart by the method of Linn *et al.* (1972) did not oxidize any of the three branched-chain 2-oxo acids (assayed spectrophotometrically through NADH formation with 1mM-NAD^+, 0.4mM-CoA, 0.4mM-thiamin pyrophosphate, 2mM-$MgSO_4$ and 0.15mM-branched-chain 2-oxo acid). The limit of detection in these experiments was 2% of the activity with pyruvate. Moreover, the branched-chain 2-oxo acids (at 150μM) did not inhibit the oxidation of pyruvate (at 100μM) by ox heart pyruvate dehydrogenase complex (results not shown). The ox heart pyruvate dehydrogenase complex was not inhibited by isovaleryl-CoA, at 20μM- and 200μM-CoA and at 250μM-isovaleryl-CoA (results not shown). It was, however, inhibited by acetyl-CoA (K_m for CoA, 13.2μM; K_i for acetyl-CoA, 36.5μM; see also Tsai *et al.*, 1973). The branched-chain 2-oxo acid dehydrogenase activity was inhibited by isovaleryl-CoA (see preceding section), but not by acetyl-CoA (CoA concentrations 5–60μM, acetyl-CoA concentration 114μM; results not shown).

Is there a single branched-chain 2-oxo acid dehydrogenase complex?

The present study has shown that the branched-chain 2-oxo acid dehydrogenase activity in a

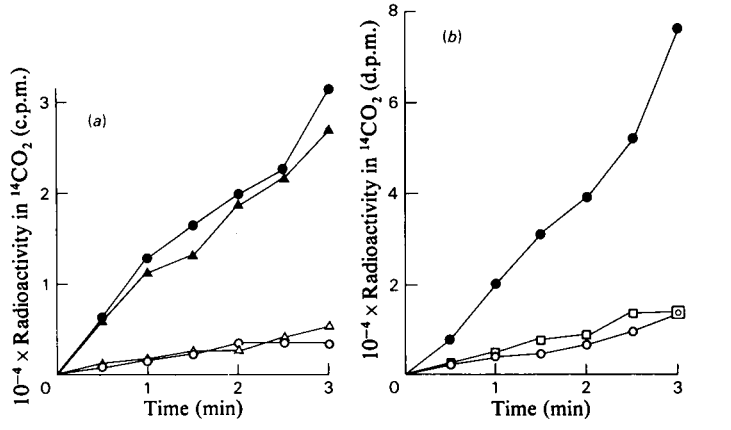

Fig. 4. *Decarboxylation of 4-methyl-2-oxo[1-^{14}C]pentanoate by branched-chain 2-oxo acid dehydrogenase complex*
For details of preparation of branched-chain 2-oxo acid dehydrogenase complex and radioactivity assay see under 'Methods'. In (*a*) concentrations were: thiamin pyrophosphate, 0.4mM; $MgSO_4$, 2mM; CoA, 0.4mM; NAD^+, 1mM; 4-methyl-2-oxo[1-^{14}C]pentanoate, 57μM (1.25μCi/μmol); ●, no other addition; ▲, 1.5mM-sodium pyruvate; ○, 1.5mM-3-methyl-2-oxobutyrate; △, 1.5mM-DL-3-methyl-2-oxopentanoate. In (*b*) concentrations were: thiamin pyrophosphate, 0.4mM; $MgSO_4$, 2mM; CoA, 0.4mM; NAD^+, 1mM; 4-methyl-2-oxo[1-^{14}C]pentanoate, 35μM (2.3μCi/μmol); ●, no other addition; □, 100μM-3-methyl-2-oxobutyrate; ○, 100μM-DL-3-methyl-2-oxopentanoate. In both (*a*) and (*b*) two observations were made at each point. Blank corrections were applied from incubations without enzyme.

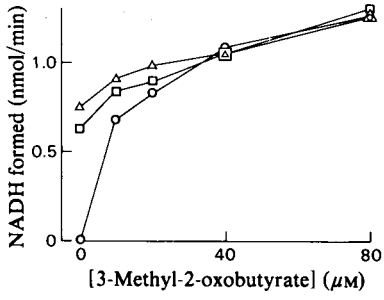

Fig. 5. *Effect of 4-methyl-2-oxopentanoate and* DL-*3-methyl-2-oxopentanoate on NADH production from 3-methyl-2-oxobutyrate*
See under 'Methods' for details of preparation of branched-chain 2-oxo acid dehydrogenase complex and spectrophotometric assay. Other concentrations were: thiamin pyrophosphate, 0.4mM; CoA, 0.4mM; $MgSO_4$, 2mM; NAD^+, 1mM; ○, no other addition; □, 40μM-DL-3-methyl-2-oxopentanoate; △, 40μM-4-ethyl-2-oxopentanoate.

preparation from ox liver sediments in 20% (w/v) sucrose at 8.1×10^6*g*-min. This indicates that the activity is of high molecular weight. In this respect and in the nature of the reaction catalysed, the requirements for substrates and cofactors, and competitive inhibition by isovaleryl-CoA (versus CoA) and by NADH (versus NAD^+), it is analogous to pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes. The question as to whether this activity resides in a high-molecular-weight complex containing decarboxylase, dihydrolipoyl acyltransferase and dihydrolipoyl dehydrogenase components can only be resolved by purification and analysis. We shall assume for the purposes of further discussion that the activity is due to such a complex.

The following evidence may indicate that a single complex can catalyse the oxidative decarboxylation of 4-methyl-2-oxopentanoate, 3-methyl-2-oxobutyrate and D- and L-3-methyl-2-oxopentanoates. Fig. 4 shows that the formation of $^{14}CO_2$ from 4-methyl-2-oxo[1-^{14}C]pentanoate is inhibited by 3-methyl-2-oxobutyrate and DL-3-methyl-2-oxopentanoate (*a*) at concentrations that produce substrate inhibition and (*b*) at concentrations below those showing substrate inhibition (see Fig. 1). Under the conditions used in Fig. 4(*b*) the relative velocities (means ± S.E.M. from linear-regression analyses) were 100 (4-methyl-2-oxopentanoate alone), 16.4±1.8 (with DL-3-methyl-2-oxopentanoate) and 19.5±1.9 (with 3-methyl-2-oxobutyrate).

Fig. 5 shows that rates of NADH production with 3-methyl-2-oxobutyrate alone at 10, 20, 40 and 80μM (selected because it has the highest $V_{max.}$) were not increased by the further addition of 40μM-4-methyl-2-oxopentanoate or 40μM-3-methyl-2-oxopentanoate. If more than one dehydrogenase activity were involved in the oxidation of the three 2-oxo acids one might

expect to see evidence of additive rates. No such evidence was obtained (Fig. 5). As mentioned above, the oxidation of all three branched-chain 2-oxo acids was inhibited by isovaleryl-CoA (competitive with CoA) and the K_i values were comparable. The observation that the D and L isomers of 3-methyl-2-oxopentanoate are both oxidized also suggests the presence of a dehydrogenase complex of broad specificity.

Connelly *et al.* (1968) and Bowden & Connelly (1968) have described the partial purification of branched-chain 2-oxo acid dehydrogenase activities from ox liver. They have concluded that there may be two separate dehydrogenase complexes, one catalysing the oxidation of 4-methyl-2-oxopentanoate and DL-3-methyl-2-oxopentanoate and the other catalysing the oxidation of 3-methyl-2-oxobutyrate. The preparation that we have used is obviously different in its kinetic properties and apparently in its specificity. The K_m values for 2-oxo acids given by Connelly *et al.* (1968) were in the mM range. Our enzyme preparation gave K_m values two orders of magnitude less, and moreover showed marked substrate inhibition by 2-oxo acids in the mM range. Our observations regarding substrate specificity and inhibition at high substrate concentration agree with the results of studies on the decarboxylation of branched-chain 2-oxo acids by extracts of rat liver (Wohlhueter & Harper, 1970).

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