Biochem. J. (1984) **220**, 273–281
Printed in Great Britain

The activity state of the branched-chain 2-oxo acid dehydrogenase complex in rat tissues

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(*Received 5 December 1983/Accepted 6 February 1984*)

An assay is described to define the proportion of the branched-chain 2-oxo acid dehydrogenase complex that is present in the active state in rat tissues. Activities are measured in homogenates in two ways: actual activities, present in tissues, by blocking both the kinase and phosphatase of the enzyme complex during homogenization, preincubation, and incubation with 1-^{14}C-labelled branched-chain 2-oxo acid, and total activities by blocking only the kinase during the 5min pre-incubation (necessary for activation). The kinase is blocked by 5mM-ADP and absence of Mg^{2+} and the phosphatase by the simultaneous presence of 50mM-NaF. About 6% of the enzyme is active in skeletal muscle of fed rats, 7% in heart, 20% in diaphragm, 47% in kidney, 60% in brain and 98% in liver. An entirely different assay, which measures activities in crude tissue extracts before and after treatment with a broad-specificity protein phosphatase, gave similar results for heart, liver and kidney. Advantages of our assay with homogenates are the presence of intact mitochondria, the simplicity, the short duration and the high sensitivity. The actual activities measured indicate that the degradation of branched-chain 2-oxo acids predominantly occurs in liver and kidney and is limited in skeletal muscle in the fed state.

Most conclusions about the capability of tissues to degrade branched-chain amino acids and 2-oxo acids so far have been based on activity measurements in tissue homogenates and in intact tissue preparations (e.g. Odessey & Goldberg, 1972; Buse *et al.*, 1975; Shinnick & Harper, 1976; Goldberg & Chang, 1978; Odessey & Goldberg, 1979; Veerkamp & Wagenmakers, 1981; Wagenmakers & Veerkamp, 1982). The results from these studies have given rise to the view that transamination occurs predominantly in extrahepatic tissues and that oxidative decarboxylation is both hepatic and extrahepatic. Of the extrahepatic tissues, skeletal muscle is considered to be the major site of branched-chain amino acid degradation because of its high transaminase and considerable oxidative-decarboxylation activity (e.g. Shinnick & Harper, 1976; Veerkamp & Wagenmakers, 1981; Wagenmakers & Veerkamp, 1982) and because of its large mass (about 40% of total body weight).

It is not evident, however, whether the measured activities represent the actual activity present in the tissues or whether they are changed during preparation and incubation of tissues and homogenates. This is important, since a large body of evidence has accumulated in recent years that the branched-chain 2-oxo acid dehydrogenase complex is subject to covalent modification *in vitro*, with inactivation by phosphorylation and activation by dephosphorylation (reviewed by Randle, 1983). In the present study we have adapted an assay described previously (Van Hinsbergh *et al.*, 1978; Veerkamp *et al.*, 1980) in such a way that both actual and total activities can be measured in homogenates of rat skeletal muscle, heart, liver, kidney and brain. The results obtained with this assay are compared with those obtained with an assay which measures activities in crude tissue extracts before and after treatment with a broad-specificity protein phosphatase (Harris *et al.*, 1982*a*,*b*; Gillim *et al.*, 1983).

Materials and methods

DL-2-Chloro-4-methylpentanoate was a gift from Dr. Ronald Simpson, Sandoz Inc., East Hanover, NJ, U.S.A.; lipoamide dehydrogenase (EC 1.6.4.3) from pig heart, Co A (lithium salt) and thiamin pyrophosphate were from Sigma, St. Louis, MO, U.S.A.; phosphorylase *a*, ADP and

NAD^+ (grade 1) were from Boehringer, Mannheim, Germany; calf serum was from Gibco Europe, Paisley, Scotland, U.K.; Tos-Lys-CH_2Cl (7-amino-1-chloro-3-L-tosylamidoheptan-2-one) was from Fluka A.G., Buchs, Switzerland. Sources of other chemicals and radiochemicals used have been reported previously (Wagenmakers & Veerkamp, 1982). Male albino Wistar rats weighing 110–120g were used, except for the experiments of Table 4 (180–220g animals). The rats were fed on a commercial stock diet (RMH-TM, obtained from Hope Farms BV, Woerden, The Netherlands) and water *ad libitum*. The rats were used in the fed state and killed by cervical dislocation.

Preparation of homogenates

Quadriceps muscle, diaphragm, heart, liver, kidney(s) and brain were removed and immediately cooled in ice-cold buffer, consisting of 0.25M-sucrose, 2mM-EDTA and 10mM-Tris/HCl (pH7.4). Further procedures were performed at 0°C. Tissues were washed, blotted, weighed, and dissected with scissors, and homogenates [10% (w/v) for quadriceps muscle and brain; 5% (w/v) for the other tissues] were prepared in the same buffer by homogenization by hand with a Teflon–glass Potter–Elvehjem homogenizer, with three pestles of different diameters (0.35, 0.12 and 0.05mm clearance). With soft tissues the first pestle was omitted. The good quality of mitochondria isolated from these homogenates (Van Hinsbergh *et al.*, 1979; Veerkamp *et al.*, 1980) indicates that the homogenates contain intact mitochondria.

Basic assay for the total activity in homogenates

This assay is performed as described by Van Hinsbergh *et al.* (1978) and Veerkamp *et al.* (1980), with some modifications. Oxidative-decarboxylation rates were determined in triplicate. A volume (100μl) of tissue homogenate was added to 300μl of a medium containing 16.7mM-KH_2PO_4, 25.0mM-$NaHCO_3$, 100mM-Tris/HCl (pH7.4), 41.7mM-sucrose, 25.0mM-KCl, 8.33mM-$MgCl_2$, 1.67mM-EDTA, 1.67mM-2-oxoglutarate, 8.33mM-ADP and 3.33mM-L-carnitine. After preincubation for 5min at 37°C, 100μl of 0.5mM 1-^{14}C-labelled branched-chain 2-oxo acid was added. After incubation for 15min at 37°C, reactions were terminated and $^{14}CO_2$ was collected and measured as described previously (Veerkamp *et al.*, 1980). Oxidative-decarboxylation rates were calculated from the specific radioactivity in the medium (1500–3000d.p.m./nmol) and were expressed in nmol of ^{14}C-labelled compound metabolized/min per g of tissue. A blank correction was subtracted (incubation without homogenate). Rates were constant for at least 15min. Addition of 1mM-thiamin pyrophosphate, 1mM-NAD^+ or 1mM-CoA, separately or in combination, did not affect the measured oxidative-decarboxylation rates. This indicates that the mitochondria remained intact during the entire procedure. When the mitochondria in the homogenates were broken by freezing and thawing, the activity decreased considerably and was markedly dependent on these cofactors (Van Hinsbergh *et al.*, 1979; Veerkamp *et al.*, 1980). The 2-oxo acid concentration of 0.1mM is below the apparent K_m in rat muscle mitochondria (Van Hinsbergh *et al.*, 1980). Oxidative-decarboxylation rates consequently do not represent maximal rates.

Assay for the activity state in homogenates

This assay is divided into two sub-assays, one to measure the actual activity present in tissues and one to measure the total activity under the same reaction conditions. Tissue samples were washed and homogenates prepared in a buffer consisting of 0.25M-sucrose, 5mM-EDTA, 10mM-Tris/HCl (pH7.4), 5mM-ADP and 50mM-NaF (for actual activity) or 0.25M-sucrose, 2mM-EDTA and 10mM-Tris/HCl (pH7.4) (for total activity). The tissue sample in which the actual activity was assayed was always homogenized first as quickly as possible. Homogenization was completed 1–2min after the rats were killed. The second tissue sample, for the total-activity assay, was kept in ice-cold washing buffer during that time. The preincubation, after addition of 100μl of homogenates to 300μl of the assay media, was for 5min at 37°C. During the preincubation the concentrations of additions were the same as in the basic assay except for $MgCl_2$ (omitted) and EDTA (6.25mM). Furthermore, 62.5mM-NaF was present during the preincubation in the sub-assay for the actual activity. After the preincubation, 100μl of 0.5mM-1-^{14}C-labelled 2-oxo acid (actual activity) or 100μl of 0.5mM-2-oxo acid/250mM-NaF (total activity) was added, to obtain the same conditions in both sub-assays during the incubations with radioactive substrate. Incubations at 37°C were for 10min (quadriceps muscle), 5min (diaphragm and brain) or 2min (heart, liver and kidney), unless otherwise stated. The activity state (%) is obtained from the ratio of the oxidative-decarboxylation rates found with the sub-assays for the actual and the total activity.

Assay for the activity state in tissue extracts

Heart, liver and kidneys from 180–200g rats were removed and freeze-clamped within 30s at liquid-N_2 temperature. Extracts were prepared by the procedures of Parker & Randle (1980) and Gillim *et al.* (1983), with some minor modifications. Liver and heart tissue were powdered under liquid N_2 with a pestle and mortar. After warming

to about −10°C, the powder was extracted into an ice-cold buffer consisting of 30mM-KH_2PO_4 (pH7.5), 3mM-EDTA, 5mM-dithiothreitol, 0.5mM-4-methyl-2-oxopentanoate, 1mM-Tos-Lys-CH_2Cl and 5% (v/v) Triton X-100 (about 5ml/g of tissue) by vortex-mixing and freezing (liquid N_2) and thawing (30°C) three times, followed by homogenization with a Teflon–glass Potter–Elvehjem homogenizer as described above. Kidneys were extracted in the same medium, except for the addition of 3% (v/v) calf serum to the extraction buffer. The extracts were centrifuged at 33000*g* for 5min at 4°C. The supernatants were collected and centrifuged at 135000*g* for 120min at 4°C. The pellets originating from heart and liver extracts were suspended in 50mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (pH7.0), 0.5mM-dithiothreitol and 0.1% Triton X-100 (1–2ml/g of original tissue) with a Teflon–glass Potter–Elvehjem homogenizer. The pellet from kidney extracts was suspended in the same buffer containing 1mM-Tos-Lys-CH_2Cl and 3% calf serum.

To activate the branched-chain 2-oxo acid dehydrogenase complex by dephosphorylation, 200μl of extract was treated at 37°C with 80μl of a broad-specificity protein phosphatase, as described by Harris *et al.* (1982*a*,*b*) and Gillim *et al.* (1983). The phosphatase preparation was isolated from rat liver by a simplified procedure, which is a combination of both procedures described by Brandt *et al.* (1974, 1975). Frozen rat livers (100g) were homogenized with a Waring Blendor in 350ml of ice-cold medium consisting of 0.1M-NaCl in IED buffer [50mM-imidazole (pH7.45), 5mM-EDTA and 0.5mM-dithiothreitol]. Further operations were carried out at 4°C, unless otherwise stated. The homogenate was centrifuged at 10000*g* for 20min. To the supernatant 200ml of saturated $(NH_4)_2SO_4$ solution was added. The precipitate was collected by centrifugation (10000*g*, 20min) and resuspended in 100ml of IED buffer. Then 95% (v/v) ethanol at room temperature (500ml) was added. The precipitate was immediately collected by centrifugation (5000*g*, 10min) and extracted with 100ml of IED buffer. After centrifugation (16000*g*, 15min), the supernatant was dialysed overnight against the same buffer. To the dialysed extract saturated $(NH_4)_2SO_4$ solution was added to give 40% saturation. After 30min, the precipitate was removed by centrifugation (10000*g*, 15min) and discarded. The supernatant was adjusted to 75% saturation by the addition of saturated $(NH_4)_2SO_4$ solution. After 30min, the precipitate was collected by centrifugation as above. The pellet was dissolved in 5ml of IED buffer and dialysed overnight against two changes of the same buffer. The dialysed extract was then adjusted to 60% (v/v) glycerol and concentrated to about 5ml with an Amicon PM 10 filter. The activity of this preparation was estimated with phosphorylase *a* as substrate by the method described by Brandt *et al.* (1975). Since the activity falls gradually on storage at −70°C, we only used fresh preparations in our experiments.

Branched-chain 2-oxo acid dehydrogenase complex activities were measured in duplicate in tissue extracts before and after treatment with the phosphatase preparation by following NAD^+ reduction at 340nm at 30°C. The assay buffer (final volume 2.0ml) consisted of 30mM-KH_2PO_4 (pH7.5), 2mM-$MgCl_2$, 0.4mM-thiamin pyrophosphate, 0.4mM-CoA, 1mM-NAD^+, 1mM-dithiothreitol, 0.1% Triton X-100 and 0.2mM-4-methyl-2-oxopentanoate. To assay activities in extracts from liver and kidney, 20 units of lipoamide dehydrogenase were included. Assays were started by the addition of 25–100μl of tissue extract. Rates were corrected for the rate of change of A_{340} in the absence of 4-methyl-2-oxopentanoate (measured in duplicate). After a short stabilization period, rates were proportional to volume of extract and were constant for 2min or more. Activities are expressed in nmol of NADH produced/min per g of tissue.

Phosphatase treatments were performed for 15 and 30min. Maximal activation was assumed when the longer treatment gave no higher activity. This was always the case with a fresh phosphatase preparation. Although calf serum and Tos-Lys-CH_2Cl were added to inhibit proteolysis in kidney extracts, branched-chain 2-oxo acid dehydrogenase activity decreased on incubation at 37°C. In extracts incubated for 15min (without phosphatase) the activity was 66±7% ($n=3$) of that in unincubated extracts. For that reason the activities measured in the phosphatase-treated samples were corrected for the loss of activity in a parallel incubation of kidney extracts without phosphatase (Gillim *et al.*, 1983).

Statistics

Results are shown as means±S.D., with the numbers of experiments (*n*) in parentheses. Student's paired and unpaired *t* tests were used for the statistical analyses.

Results

Although incubation of mitochondria from rat heart and skeletal muscle and perfusion of rat hearts without substrate and with branched-chain 2-oxo acids were reported to result in a rapid activation of the branched-chain 2-oxo acid dehydrogenase complex (reviewed by Randle,

1983), we could not detect this phenomenon with the assay used in our laboratory. This basic assay measures the $^{14}CO_2$ production from ^{14}C-labelled branched-chain amino acids and 2-oxo acids in intact mitochondria and in tissue homogenates (Van Hinsbergh *et al.*, 1978; Veerkamp *et al.*, 1980). The reason for the inability to observe activation may be that it contains a preincubation step of 5min (originally meant for thermo-equilibration). Furthermore, the assay medium contains 5mM-ADP, which inhibited the kinase of the branched-chain 2-oxo acid dehydrogenase complex (Lau *et al.*, 1982). Aftring *et al.* (1982), moreover, showed with liver mitochondria that extra-mitochondrial ADP (1mM) more than doubled the intra-mitochondrial ADP concentration. For all of these reasons the branched-chain 2-oxo acid dehydrogenase complex may be fully activated during the preincubation. This was confirmed by the absence of an effect of another potent inhibitor of the kinase, 2-chloro-4-methylpentanoate (Harris *et al.*, 1982*a*). When 0.5mM of this compound was included during preincubation and incubation, together with ADP, no change was found in the activity measured in homogenates of heart, quadriceps muscle and liver (two paired experiments each).

These indications that our basic assay gave total activities challenged us to try to adapt this assay in such a way that the actual activity could be measured, too. Therefore it was necessary to block the kinase and phosphatase reactions. Possible tools with which to block the kinase are, besides addition of ADP, omission of Mg^{2+} from the assay medium in combination with an increase in the EDTA concentration to bind tissue Mg^{2+}, since the branched-chain 2-oxo acid dehydrogenase kinase reaction possibly requires Mg^{2+} as a cofactor (Parker & Randle, 1978*c*; Lau *et al.*, 1982). The phosphatase can be blocked with NaF in mitochondria (Odessey, 1980) and in the isolated enzyme complex (Fatania *et al.*, 1983) and by the absence of Mg^{2+}, since it also requires Mg^{2+} as a cofactor (Fatania *et al.*, 1983).

The reaction conditions that may be applicable to inhibit the kinase activity have been tested with quadriceps-muscle homogenates (Table 1). The basic assay gave a higher $^{14}CO_2$ production in the absence of ADP than in its presence. The reason for this increase is not evident. It was not found with 3-methyl-2-oxobutanoate as substrate ($87 \pm 7\%$; $n = 4$). With liver and heart homogenates lower activities were found in the absence of ADP both with 4-methyl-2-oxopentanoate (64 ± 19 and $41 \pm 2\%$ respectively; $n = 3$) and with 3-methyl-2-oxobutanoate (75 ± 6 and $55 \pm 9\%$ respectively; $n = 3$) as substrate. Since the presence of 0.5mM-DL-2-chloro-4-methylpentanoate during preincubation and incubation gave no increase in the presence or in the absence of ADP (quadriceps muscle, heart and liver; two experiments each), full activation during preincubation was probably reached in both cases. The absence of Mg^{2+} gave a small increase in the presence of ADP and had no effect in its absence (Table 1). Furthermore, in the presence of ADP no effect of the absence of Mg^{2+} was found on the oxidation of 4-methyl-2-oxopentanoate by liver and heart homogenates (104 ± 10 and $100 \pm 5\%$ respectively; $n = 3$) and on the oxidation of 3-methyl-2-oxobutanoate by quadriceps muscle homogenates ($99 \pm 4\%$, $n = 4$). These results indicate that the 5min preincubation also gave full activation in the absence of Mg^{2+}.

The presence of kinase activity will be most evident when the branched-chain 2-oxo acid dehydrogenase complex is fully active and when at the same time the phosphatase is inhibited. For that reason 50mM-NaF was added in some experiments after the preincubation (Table 1). Its addition decreased $^{14}CO_2$ production under all

Table 1. *Production of $^{14}CO_2$ from 4-methyl-2-oxo[1-^{14}C]pentanoate by quadriceps muscle homogenates in the presence and absence of ADP and Mg^{2+}*

Except for the indicated changes, assays were performed as for the basic assay. When $MgCl_2$ was omitted, the EDTA concentration of the medium was simultaneously increased to 8.33mM (to bind tissue Mg^{2+}). DL-2-Chloro-4-methylpentanoate was added in a concentration of 0.5mM. After 5min preincubation 50mM-NaF was added in some of the experiments as well as radioactive 2-oxo acid (0.1mM). Values (means ± S.D. for four paired experiments) are given as % of the $^{14}CO_2$ production given by the basic assay during an 15min incubation (488 ± 20nmol/g of muscle). [a,b] Significantly different at $P < 0.001$ and < 0.01 respectively. Abbreviation: N.D., not determined.

Presence of NaF (after preincubation)	Presence of Mg^{2+}	Presence of 2-chloro-4-methylpentanoate	+ADP	−ADP
−	+	−	100	147 ± 11^b
−	−	−	111 ± 3^b	148 ± 10^b
+	+	−	48 ± 4^a	31 ± 3^a
+	+	+	N.D.	46 ± 4^a
+	−	−	80 ± 3^b	66 ± 5^a
+	−	+	80 ± 3^b	N.D.

conditions. The decrease was largest when Mg^{2+} was present and ADP was omitted. Since the presence of 0.5mM-DL-2-chloro-4-methylpentanoate during preincubation and incubation increased the $^{14}CO_2$ production in that case by $42 \pm 12\%$ ($n = 4$), the decrease in the presence of NaF was probably due to kinase activity, not compensated by phosphatase activity. The decrease in the presence of NaF was smallest when ADP was present and Mg^{2+} was omitted. Since 2-chloro-4-methylpentanoate had no effect in that case, kinase activity was probably adequately blocked by ADP and omission of Mg^{2+}.

Under these conditions the optimal concentration of NaF to block the phosphatase activity was subsequently determined. When 5mM- or 25mM-NaF was present during preincubation and incubation, the activity increased during preincubation and the first minutes of incubation (results not shown). For that reason a NaF concentration of 50mM was used. This concentration of NaF, 5mM-ADP and extra EDTA (up to 5mM) were also added to the homogenization buffer, to block kinase and phosphatase activity as soon as possible after the tissues had been isolated. Furthermore, tissues were homogenized as quickly as possible, to arrest interconversion, although no significant changes were observed when the tissues were kept deliberately in ice-cold homogenization buffer for a few minutes before the homogenization was started. Under the applied conditions, the actual activity in quadriceps-muscle homogenates did not change on preincubation (Fig. 1) and the time-dependence of the $^{14}CO_2$ production from 4-methyl-2-oxo[1-^{14}C]pentanoate was linear from zero time until 10–15min of incubation (Fig. 2*a*). The basic assay for the total activity was adapted in such a way that the same conditions were applied as for the assay of the actual activity during the incubation with radioactive substrate (see the Materials and methods section). The main modification was that NaF was added after the preincubation. The activity measured with this assay rose on preincubation (Fig. 1) and reached a maximal value after 5min of preincubation. The ability to achieve full activation in the absence of Mg^{2+} is remarkable, since Fatania *et al.* (1983) reported that the phosphatase reaction is dependent on Mg^{2+}. Under the conditions used, the intramitochondrial Mg^{2+} concentration was apparently kept high enough to maintain a phosphatase activity which gave full enzyme activity within 5min of preincubation with kinase activity blocked by ADP and the low Mg^{2+} concentration. Furthermore, during the incubation rates were constant up to 10min (Fig. 2*a*).

The conditions developed with quadriceps muscle were also applicable to assay the activity state of the enzyme in diaphragm, heart, liver, kidney and brain. The time-dependencies of $^{14}CO_2$ production are shown in Fig. 2. Especially for heart, the actual activity rose still at incubation times of more than 5min. An increase of the NaF concentration to 75mM lengthened the linear part of the time curve, without an effect on the initial rate (results not shown). Total activities measured with the modified assay fell for most tissues at incubation times of more than 5min, whereas those measured with the basic assay were constant for 15min. This difference may relate to inhibition of extramitochondrial ATPases by EDTA and NaF in the modified assay. The increase of ATP

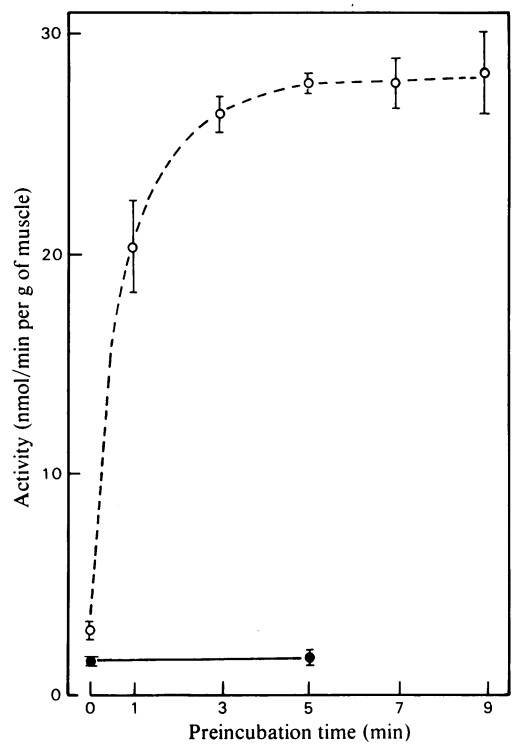

Fig. 1. *Effect of time of preincubation on actual and total activities in quadriceps-muscle homogenates*

Activities were measured with 0.1mM-4-methyl-2-oxo[1-^{14}C]pentanoate as substrate. Assays without preincubation were started with the addition of homogenate to a prewarmed assay medium containing all additions, including radioactive substrate. The assay for the actual activity (●) contains all components to block both kinase and phosphatase activity during homogenization, preincubation and incubation. In the assay for the total activity (○), the phosphatase is blocked only after the preincubation by the addition of 50mM-NaF. Values are means ± S.D. for at least three experiments.

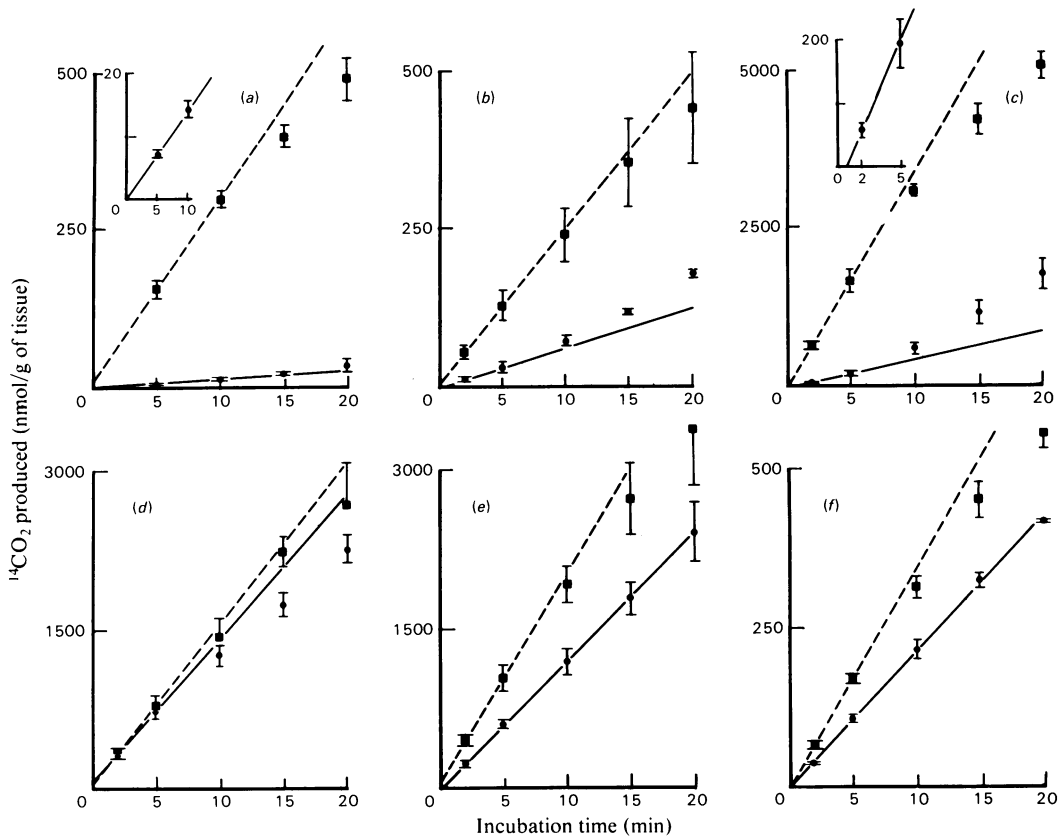

Fig. 2. *Time-dependence of $^{14}CO_2$ production for the actual and total activity assays*
The oxidation of 0.1 mM-4-methyl-2-oxo[1-^{14}C]pentanoate was measured in homogenates of quadriceps muscle (*a*), diaphragm (*b*), heart (*c*), liver (*d*), kidney (*e*) and brain (*f*). Straight lines were drawn through the points indicating the amount of $^{14}CO_2$ produced at the two shortest assay times to illustrate initial linearity from zero time, for both actual (●——●) and total (■----■) activity assays. Values are means ± S.D. for three to four experiments.

may consequently cause phosphorylation (inactivation) at longer incubation times. For these reasons we chose to maintain exactly the same assay conditions for all tissues, except for the assay time. On the basis of the results shown in Fig. 2 we chose assay periods of 10 min for quadriceps muscle, of 5 min for diaphragm and brain and of 2 min for heart, liver and kidney for both sub-assays.

The assay would be simpler if the preparation of one homogenate would suffice. Actual activities (in nmol/min per g) rose, however, when NaF, ADP and extra EDTA were omitted from the homogenization buffer for quadriceps muscle [1.6 ± 0.3 (n = 12) to 2.9 ± 0.4 (n = 3)], heart [20 ± 3 (n = 6) to 43 (n = 2)] and kidney [109 ± 7 (n = 5) to 158 (n = 2)]. When the homogenates were prepared in the indicated way, however, both actual and total activity did not change when the homogenates were kept on ice for 30 min (all tissues).

The activities measured with the basic assay and with the two modified assays and the activity states are given in Table 2. The total activity measured with the basic assay was in most cases somewhat higher than that measured with the modified assay. This difference was probably caused by an effect of the changed reaction conditions on the total activity and not by an inability to achieve full activation within the 5 min preincubation. The ability to achieve full activation has been discussed above, on the basis of the results given in Table 1, and was indicated by the results of Fig. 1.

The activity state is a property of the branched-chain 2-oxo acid dehydrogenase complex. Since the enzyme complex catalyses the oxidative decarboxylation of the branched-chain 2-oxo acids from leucine, valine and isoleucine (Danner *et al.*, 1978; Parker & Randle, 1978*a*,*b*; Pettit *et al.*, 1978), we expected to measure the same activity state independently of the branched-chain 2-oxo acid used as substrate. This indeed appeared to hold for

the enzyme complex of quadriceps muscle, heart and liver. No statistically significant difference was found between the activity states measured with 4-methyl-2-oxopentanoate (Table 2) and 3-methyl-2-oxobutanoate (Table 3) as substrate.

While we were developing our assay, Gillim *et al.* (1983) published another method to measure the activity state of the branched-chain 2-oxo acid dehydrogenase complex. In their assay the activity of the enzyme complex was assayed in crude extracts from rat heart, liver and kidney before and after treatment with a broad-specificity protein phosphatase. This enzyme appeared to be able to dephosphorylate the branched-chain 2-oxo acid dehydrogenase complex and to produce full enzyme activity (Harris *et al.*, 1982*a*,*b*). They found activity states of 48, 94 and 77% in heart, liver and kidney, respectively, of fed normal 200–300g rats. Apart from the liver, their results do not agree with the values in Tables 2 and 3. Our results are, however, in accordance with previous estimations of the activity state of rat heart *in vivo*, or perfused *in vitro* with glucose, which amounted to 5–15% (Parker & Randle, 1978*c*, 1980; Waymack *et al.*, 1980; Harris *et al.*, 1982*a*,*b*). In 200g rats we also measured an activity state of $7.8 \pm 0.9\%$ ($n = 4$) with our assay. The weight of the rats consequently cannot be the basis for the observed discrepancy. To elucidate this discrepancy, we repeated part of the experiments of Gillim *et al.* (1983). The results of these experiments (Table 4) agreed quite well with the results of Tables 2 and 3 for heart, liver

Table 2. *Activities and activity state of the branched-chain 2-oxo acid dehydrogenase complex in rat tissues measured with 4-methyl-2-oxopentanoate*

Total activities were measured in homogenates with the basic assay (A) or with the assay for the activity state (B) under the same conditions as the actual activity. As substrate 0.1 mM-4-methyl-2-oxo[1-^{14}C]pentanoate was used. The activity state (100 × ratio actual activity/total activity; measured with assay B) was obtained in paired measurements. Values are means ± S.D. for the numbers of experiments in parentheses. [a,b,c] Significantly different from the total activity measured with assay A at $P < 0.001$, < 0.01 and < 0.05 respectively.

	Activity (nmol/min per g of tissue)			
	Total		Actual	Activity state
Tissue	A	B	B	(%)
Quadriceps muscle	32 ± 3.6 (18)	29 ± 4.1 (10)[c]	1.6 ± 0.3 (12)	6.0 ± 1.4 (6)
Diaphragm	46 ± 12 (10)	38 ± 9.3 (5)	7.8 ± 2.3 (5)	20 ± 2.5 (5)
Heart	330 ± 42 (7)	279 ± 23 (6)[c]	20 ± 3.4 (6)	7.1 ± 1.0 (6)
Liver	178 ± 28 (10)	171 ± 18 (5)	167 ± 24 (5)	98 ± 12 (5)
Kidney	300 ± 15 (5)	234 ± 15 (5)[a]	109 ± 7 (5)	47 ± 1.6 (5)
Brain	39 ± 4.0 (5)	32 ± 2.4 (6)[b]	19 ± 2.5 (6)	60 ± 5.2 (6)

Table 3. *Activities and activity state of the branched-chain 2-oxo acid dehydrogenase complex in rat tissues measured with 3-methyl-2-oxobutanoate*

As substrate 0.1 mM-3-methyl-2-oxo[1-^{14}C]butanoate was used. Other details are given in Table 2.

	Activity (nmol/min per g of tissue)			
	Total		Actual	Activity state
Tissue	A	B	B	(%)
Quadriceps muscle	82 ± 18 (12)	57 ± 12 (7)[c]	2.2 ± 0.7 (6)	4.3 ± 2.0 (6)
Heart	430 ± 32 (5)	411 ± 20 (3)	30 ± 2.1 (3)	7.3 ± 0.6 (3)
Liver	253 ± 51 (6)	279 ± 33 (5)	275 ± 64 (3)	99 ± 19 (5)

Table 4. *Activity state of the branched-chain 2-oxo acid dehydrogenase complex measured in crude tissue extracts*

Activities were measured, before and after treatment with a broad-specificity protein phosphatase, as described in the Materials and methods section. Values are means ± S.D. for the numbers of extracts given in parentheses.

	Activity (nmol/min per g of tissue)		
Tissue	Control	Phosphatase-treated	Activity state (%)
Heart	22 ± 6.6 (6)	221 ± 16 (3)	12 ± 2.6 (3)
Liver	354 ± 16 (4)	387 ± 32 (3)	93 ± 4.9 (3)
Kidney	90 ± 19 (5)	197 ± 10 (3)	49 ± 8.8 (3)

and kidney. The actual activity of skeletal muscle was too low to allow a reliable spectrophotometric assay on crude extracts. The enzyme activities that we measured before and after phosphatase treatment were considerably lower than those found by Gillim *et al.* (1983). The actual enzyme activity and the activity state that we found in heart extracts agreed, however, quite well with those measured by others (Parker & Randle, 1980; Waymack *et al.*, 1980).

Discussion

The assay described above to determine the activity state of the branched-chain 2-oxo acid dehydrogenase complex in tissue homogenates has several advantages over the assay in tissue extracts. The enzyme activity is assayed in intact mitochondria, with the enzyme complex located at its original site on the mitochondrial inner membrane. The laborious part of the assay (not including $^{14}CO_2$ collection and measurement of radioactivity) only lasts 15–30min and consists of several relatively simple operations. The assay with tissue extracts, including the isolation of the phosphatase preparation, takes several days. Since the phosphatase preparation loses its activity on storage, it can be used for only a few days. The operations necessary to prepare the extracts are complex. The loss of activity of kidney extracts on incubation at 37°C is a serious disadvantage. The activity measured in heart extracts (without phosphatase treatment) is at the lower level of detectability. Larger quantities of extract cause disturbances of the spectrophotometric measurements (precipitation of protein). For that reason, that assay is not sensitive enough to assay the actual activity and the activity state in skeletal muscle and other tissues that contain low activities. With our assay with homogenates it is possible to measure activities one-tenth of the actual activity of rat skeletal muscle with about 100mg of tissue.

On the basis of earlier activity measurements in homogenates, the view has arisen that a considerable part of the degradation of branched-chain amino acids and 2-oxo acids occurs in skeletal muscle (see the introduction). The actual activities measured in this study indicate, however, that the degradation of branched-chain 2-oxo acids predominantly occurs in liver and kidney and is limited in skeletal muscle in fed rats. The transfer *in vivo* of 2-oxo acids produced in skeletal muscle to the liver (Livesey & Lund, 1980) is in agreement with this conclusion. Furthermore, in liver and kidney an activator protein was found (Fatania *et al.*, 1982), which may be involved in directing branched-chain 2-oxo acid degradation towards these organs (Randle, 1983).

The total activity in rat brain is not high, but the actual activity is considerably higher than that of skeletal muscle and as high as that of heart. For that reason it will be interesting to study the significance of branched-chain amino acids and 2-oxo acids as metabolic fuels for this organ, especially during periods of diminished glucose availability.

The rapidity and ease of our assay with homogenates make it very suitable to study the significance of covalent modification in regulating the branched-chain 2-oxo dehydrogenase complex under various nutritional and hormonal conditions. An attempt to do this has been made by Gillim *et al.* (1983). On the basis of the data from the present study, it is evident, however, that we doubt the correctness of their results and conclusions. Our assay should be able to define the proportion of the enzyme in the active state in tissues from starved and exercised rats and in skeletal muscle after incubations *in vitro* with various metabolites.

We thank Bellona Driessen for typing of the manuscript.

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