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Regulation of the branched-chain 2-oxo acid dehydrogenase complex in hepatocytes isolated from rats fed on a low-protein diet

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Hepatocytes isolated from rats fed on a chow diet or a low-protein (8%) diet were used to study the effects of various factors on flux through the branched-chain 2-oxo acid dehydrogenase complex. The activity of this complex was also determined in cell-free extracts of the hepatocytes. Hepatocytes isolated from chow-fed rats had greater flux rates (decarboxylation rates of 3-methyl-2-oxobutanoate and 4-methyl-2-oxopentanoate) than did hepatocytes isolated from rats fed on the low-protein diet. Oxidizable substrates tended to inhibit flux through the branched-chain 2-oxo acid dehydrogenase, but inhibition was greater with hepatocytes isolated from rats fed on the low-protein diet. 2-Chloro-4-methylpentanoate (inhibitor of branched-chain 2-oxo acid dehydrogenase kinase), dichloroacetate (inhibitor of both pyruvate dehydrogenase kinase and branched-chain 2-oxo acid dehydrogenase kinase) and dibutyryl cyclic AMP (inhibitor of glycolysis) were effective stimulators of branched-chain oxo acid decarboxylation with hepatocytes from rats fed on a low-protein diet, but had little effect with hepatocytes from rats fed on chow diet. Activity measurements indicated that the branched-chain 2-oxo acid dehydrogenase complex was mainly (96%) in the active (dephosphorylated) state in hepatocytes from chow-fed rats, but only partially (50%) in the active state in hepatocytes from rats fed on a low-protein diet. Oxidizable substrates markedly decreased the activity state of the enzyme in hepatocytes from rats fed on a low-protein diet, but had much less effect in hepatocytes from chow-fed rats. 2-Chloro-4-methylpentanoate and dichloroacetate increased the activity state of the enzyme in hepatocytes from rats fed on a low-protein diet, but had no effect on the activity state of the enzyme in hepatocytes from chow-fed rats. The results indicate that protein starvation greatly increases the sensitivity of the hepatic branched-chain 2-oxo acid dehydrogenase complex to regulation by covalent modification.

INTRODUCTION

The interest in the regulation of the catabolism of the branched-chain amino acids stems in part from the possibility that these amino acids (especially leucine) may play important regulatory roles in protein synthesis (Goldberg & Tischler, 1981), protein degradation (Morgan *et al.*, 1981) and insulin release (Panten *et al.*, 1972; Hutton *et al.*, 1981). In addition, striking changes in plasma concentration of these amino acids occur in various physiological (starvation) and pathological conditions (diabetes, obesity, cancer) that are poorly understood (Walser, 1984). Previous work from our laboratory has established that feeding a low-protein diet to rats results in inactivation of the branched-chain 2-oxo acid dehydrogenase complex (Gillim *et al.*, 1983). The activity state of the enzyme (i.e. percentage of the enzyme in the active dephosphorylated form) was determined in the latter study by measuring the activity of the liver enzyme in crude extracts of freeze-clamped tissue before and after treatment of the extract with a broad-specificity phosphoprotein phosphatase (Harris *et al.*, 1982*b*). The purpose of the present study was to compare the effects of various factors on branched-chain oxo acid decarboxylation rates as well as branched-chain 2-oxo acid dehydrogenase activity states in hepatocytes prepared from rats in which the liver enzyme is predominantly in the active state, i.e. chow-fed rats, and in hepatocytes prepared from rats in which the liver enzyme is predominantly in the inactive state, i.e. low-protein-fed rats. It was found that the activity state of the enzyme reflected the nutritional state of the animal from which the hepatocytes were prepared. Furthermore, the flux through branched-chain 2-oxo acid dehydrogenase and its activity/phosphorylation state were more easily altered in hepatocytes prepared from low-protein-fed rats than in hepatocytes prepared from chow-fed rats.

MATERIALS AND METHODS

Isolation and incubation of hepatocytes

Hepatocytes were isolated from male Wistar rats (200–230 g) given free access to chow diet [Purina Rodent Laboratory Chow 5001; minimum 23% protein, minimum 4.5% fat, maximum 6.0% fibre (all w/w)] or a low-protein diet (ICN Nutritional Biochemicals, Cleveland, OH, U.S.A.; 8% (w/w) casein, 0.3% (w/w) methionine, 26% (w/w) corn starch, 52% (w/w) sucrose, 10% (w/w) vegetable oil, 4% (w/w) salts, plus appropriate vitamins) for 2–3 weeks by the procedure of Berry & Friend (1969), with modifications described previously (Harris *et al.*, 1985). Food consumption was not measured, but rats on

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the chow diet grew about twice as fast as rats on the low-protein diet.

Hepatocytes (30–40 mg wet wt) were incubated in 2 ml of Krebs–Henseleit (1932) buffer supplemented with 2.5% (w/v) bovine serum albumin (bovine albumin powder CRG-7; Armour Pharmaceutical Co.; dialysed) under an atmosphere of O_2/CO_2 (19:1) in 25 ml Erlenmeyer flasks sealed with rubber serum caps fitted with hanging centre wells. Incubations were carried out in a shaking water bath maintained at 37 °C. Oleate and chloro-acids were added to the incubation flasks as sodium salts.

Assays of metabolites

Incubations conducted for the measurement of metabolites were terminated by the addition of 0.1 ml of 50% (w/v) $HClO_4$. Metabolite assays were performed on KOH-neutralized $HClO_4$ extracts by the methods of Hohorst *et al.* (1959) for pyruvate and lactate, Williamson *et al.* (1962) for acetoacetate and 3-hydroxybutyrate, Slein (1965) for glucose, Gutmann & Bergmeyer (1974) for urea, and McCune *et al.* (1981) for glycogen. Acid-soluble thiol groups (mainly glutathione) present in $HClO_4$ extracts of hepatocytes were measured by the reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) (Riddles *et al.*, 1979) in 0.4 M-sodium phosphate (pH 8.0)/1 mM-EDTA and 0.1 mM-5,5′-dithiobis-(2-nitrobenzoic acid). Fat was extracted by the procedure described by Radin (1981) and determined gravimetrically.

Determination of flux through the branched-chain 2-oxo acid dehydrogenase with intact hepatocytes

Hepatocytes were incubated for 15 min as described above, with the additions shown. 1-^{14}C-labelled oxo acid (100 c.p.m./nmol) was then added to a final concentration of 0.2 mM and the incubation continued for an additional 15 min. Nearly linear flux rates were obtained over this relatively short incubation period. The reaction was terminated by the addition of 0.3 ml of phenethylamine/methanol (1:1, v/v) to the centre well and 1.0 ml of 5 M-H_2SO_4 to the reaction mixture. Incubations were continued for 1 h at room temperature to collect $^{14}CO_2$ in the hanging centre wells. The wells were removed and counted for radioactivity in 10 ml of xylene-based scintillation fluid supplemented with 0.5 ml of methanol and 0.5 ml of phenethylamine.

Determination of branched-chain 2-oxo acid dehydrogenase complex activity in extracts of isolated hepatocytes

Hepatocytes were incubated for 20 min as described above, except that the incubation volume and quantity of hepatocytes was doubled. Samples (1.5 ml) were removed in duplicate from each flask and the hepatocytes sedimented by centrifugation for 30 s in an Eppendorf centrifuge. The pellets were quickly frozen in liquid N_2 and stored at −70 °C. Cell-free extracts were prepared by homogenizing the hepatocytes with a Polytron homogenizer (lowest possible setting for 10 s) in an ice-cold solution containing 50 mM-Hepes, pH 7.5, 3 mM-EDTA, 5 mM-dithiothreitol, 0.1 mM-2-chloro-4-methylpentanoate, 1 mM-*N*-α-tosyl-L-lysylchloromethane and 5% (v/v) Triton X-100. In some experiments a broad-specificity phosphoprotein phosphatase was added to the hepatocyte extracts to activate the complex completely by dephosphorylation (Harris *et al.*, 1982*b*). Branched-chain 2-oxo acid dehydrogenase activity of the extracts was assayed in a solution containing 30 mM-potassium phosphate, pH 7.5, 2 mM-$MgCl_2$, 0.4 mM-thiamin pyrophosphate, 0.4 mM-CoA, 1 mM-dithiothreitol, 1 mM-NAD^+, 0.1% Triton X-100 and 3.75 units of pig heart dihydrolipoamide reductase (EC 1.8.1.4)/ml. Assay volume was 2 ml in 25 ml Erlenmeyer flasks sealed with rubber serum caps fitted with hanging centre wells. The flasks were preincubated for 5 min at 30 °C before initiation of the reaction by the addition of 0.2 mM-3-methyl-2-oxo[1-^{14}C]butanoate (250 c.p.m./nmol). The reaction was terminated 5 min later and $^{14}CO_2$ was collected as described above for flux measurements. Linearity with respect to time and quantity of tissue extract was established.

Statistical analyses

Statistical significance was determined by using Student's unpaired *t* test for the effect of diet and Student's paired *t* test for the effect of addition to hepatocyte incubations.

Sources of materials

Radioactive compounds were obtained from Amersham Corp., Arlington Heights, IL, U.S.A. Collagenase was obtained from Cooperbiomedical, Malvern, PA, U.S.A.; broad-specificity phosphoprotein phosphatase was isolated from rabbit liver by the procedure given previously (Paxton & Harris, 1984*a*); other enzymes and biochemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A. DL-2-Chloro-4-methylpentanoate was given by Dr. Ronald Simpson of Sandoz, East Hanover, NJ, U.S.A.

RESULTS

Characterization of hepatocytes isolated from rats fed on chow or low-protein diets

Trypan Blue exclusion, ATP content and lactate dehydrogenase release rate of hepatocytes prepared from rats fed on the chow diet and from rats fed on the low-protein diet (8% protein plus 0.3% methionine) indicated that hepatocytes of comparable and good quality (> 92% viability) were prepared from both groups of animals (Harris *et al.*, 1985). The contents of acid-soluble thiol compounds were similar in the two types of hepatocytes (4.8 ± 0.4 and 5.0 ± 0.4 μmol/g wet wt. respectively; means ± S.E.M. for four hepatocyte preparations), suggesting comparable contents of glutathione. This measurement of acid-soluble thiol compound is noted because considerable difficulty was experienced in the preparation of hepatocytes with good viability from rats fed on a low-protein (8%) diet without supplemental methionine. Low glutathione contents (< 1.5 μmol/g wet wt.) probably contributed to the poor viability, since glutathione protects cells against oxidative stress (Orrenius *et al.*, 1983).

The glycogen content of hepatocytes prepared from rats fed on a low-protein diet was markedly higher than in those from chow-fed rats (Table 1). This greater glycogen reserve probably accounts, at least in part, for the greater release of glucose from these hepatocytes during incubation with and without dibutyryl cyclic AMP (Table 1). The cytosolic redox state, indicated by the lactate/pyruvate ratio, was not different in the two types of hepatocytes. However, hepatocytes from rats fed on a

Table 1. Some metabolic properties of hepatocytes prepared from rats fed on chow and low-protein (8%) diets

Procedures for the treatment of the rats and the isolation of hepatocytes are given in the text. Incubations were for 30 min without additions except dibutyryl cyclic AMP (0.05 mM), oleate (2 mM), alanine (10 mM) and ornithine (1 mM) as indicated. Results are means ± S.E.M. for four hepatocyte preparations. * $P < 0.01$ for effect of diet; † $P < 0.01$ for effect of indicated addition to incubation.

Measurement	Hepatocytes prepared from rats fed on: Chow diet	Low-protein diet
Glycogen (μmol of glucose/g wet wt.)	200 ± 20	537 ± 19*
Fat (% of wet wt.)	6.3 ± 0.2	8.8 ± 0.4*
Glucose release (μmol/min per g wet wt.)	1.08 ± 0.03	1.87 ± 0.15*
Glucose release with dibutyryl cyclic AMP (μmol/min per g wet wt.)	2.86 ± 0.24†	4.11 ± 0.39*†
Lactate + pyruvate accumulation (μmol/min per g wet wt.)	32 ± 7	60 ± 5*
Lactate/pyruvate ratio	3.9 ± 0.3	3.6 ± 0.4
Lactate + pyruvate accumulation with dibutyryl cyclic AMP	2.9 ± 0.9†	14.9 ± 0.4*†
Ketone-body production (μmol/min per g wet wt.)	< 0.1	< 0.1
Ketone-body production with oleate (μmol/min per g wet wt.)	1.2 ± 0.2	0.8 ± 0.1
Urea synthesis with alanine, oleate and ornithine (μmol/min per g wet wt.)	0.60 ± 0.10	0.07 ± 0.01*

low-protein diet accumulated much more lactate and pyruvate, perhaps again reflecting the greater content of glycogen. Although the fat content of the hepatocytes from rats fed on a low-protein diet was greater, ketone-body production rate could not be accurately measured without added substrate with hepatocytes prepared from either group of rats. The addition of oleate greatly enhanced ketone-body production, with a trend for greater ketogenesis with the hepatocytes from chow-fed rats. The rate of urea synthesis by the two preparations of cells reflected their physiological state, i.e. hepatocytes prepared from rats fed on a low-protein diet had much less capacity to synthesize urea (Table 1), as described by others (Briggs & Freedland, 1977).

Flux measurements through the branched-chain 2-oxo acid dehydrogenase with hepatocytes prepared from rats fed on chow and low-protein diets

Flux rates as measured by the decarboxylation of 3-methyl-2-oxobutanoate and 4-methyl-2-oxopentanoate (Table 2) were much lower in hepatocytes from rats fed on the low-protein diet. The former substrate was decarboxylated at a faster rate than the latter, as also observed by Corkey *et al.* (1982), and as would be predicted from the known substrate preference of purified branched-chain 2-oxo acid dehydrogenases (Pettit *et al.*, 1978; Paxton & Harris, 1982). 2-Chloro-4-methylpentanoate, a potent inhibitor of branched-chain 2-oxo acid

Table 2. Effect of various substrates and 2-chloro-4-methylpentanoate on decarboxylation of 3-methyl-2-oxobutanoate and 4-methyl-2-oxopentanoate by hepatocytes isolated from chow-fed and low-protein-diet-fed rats

Hepatocytes were preincubated for 15 min with the additions given below. Either 3-methyl-2-oxo[1-^{14}C]butanoate (0.2 mM) or 4-methyl-2-oxopentanoate (0.2 mM) was added and the incubation continued for another 15 min. $^{14}CO_2$ was collected by the procedure described in the text. Values are means ± S.E.M. for three hepatocyte preparations. * $P < 0.02$ for the effect of diet; † $P < 0.05$ for the effect of 2-chloro-4-methylpentanoate versus corresponding condition without this compound; ‡ $P < 0.05$ for effect of given addition versus control (none) incubation.

Addition (mM)	Rate of 3-methyl-2-oxobutanoate decarboxylation (nmol/min per g wet wt.) by hepatocytes prepared from rats fed on: Chow diet	Low-protein diet	Rate of 4-methyl-2-oxopentanoate decarboxylation (nmol/min per g wet wt.) by hepatocytes prepared from rats fed on: Chow diet	Low-protein diet
None	304 ± 13	100 ± 6*	193 ± 14	84 ± 7*
2-Chloro-4-methylpentanoate (0.1)	307 ± 13	199 ± 7*†	206 ± 16†	118 ± 12*†
Glucose (20)	309 ± 16	103 ± 9*	199 ± 13	76 ± 5*
Glucose + 2-chloro-4-methylpentanoate	313 ± 14	200 ± 7*†‡	213 ± 12†‡	122 ± 12*†‡
Lactate (10) + pyruvate (2.5)	156 ± 6‡	10 ± 3*‡	123 ± 8‡	12 ± 2*‡
Lactate + pyruvate + 2-chloro-4-methylpentanoate	146 ± 8‡	60 ± 9*†‡	125 ± 8‡	40 ± 8*†‡
DL-3-Hydroxybutyrate (20)	309 ± 20	40 ± 9*‡	226 ± 17‡	83 ± 10*
DL-3-Hydroxybutyrate + 2-chloro-4-methylpentanoate	309 ± 22	192 ± 13*†‡	242 ± 19†‡	119 ± 15*†‡
Oleate (2)	237 ± 36	7 ± 1*‡	191 ± 25	19 ± 2*†‡
Oleate + 2-chloro-4-methylpentanoate	239 ± 35	62 ± 11*†‡	198 ± 25	29 ± 4*†‡

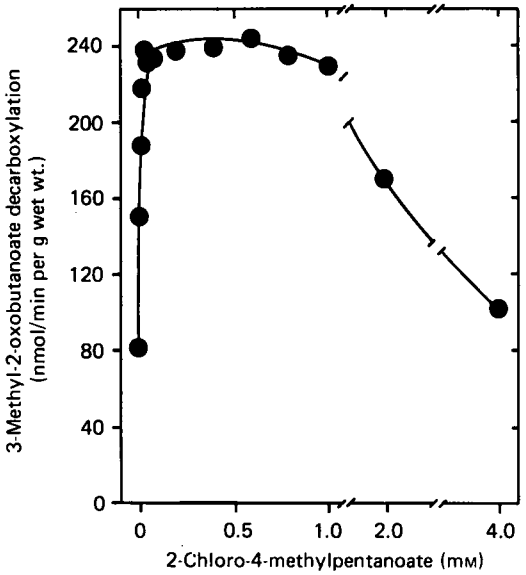

Fig. 1. Effect of 2-chloro-4-methylpentanoate on the decarboxylation of 3-methyl-2-oxobutanoate by hepatocytes isolated from a rat fed on a low-protein diet

Conditions are given in Table 2. Values shown are the results obtained with one hepatocyte preparation. Similar results were obtained in two separate experiments.

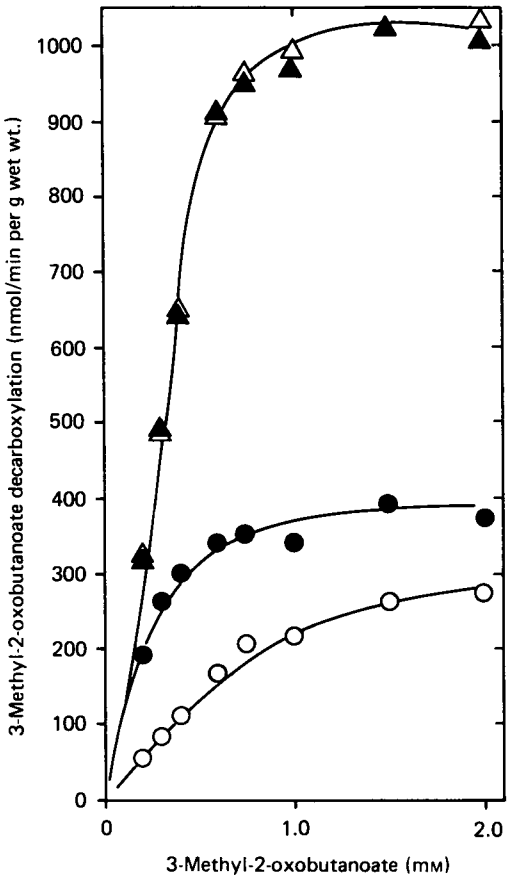

Fig. 2. Effect of 3-methyl-2-oxobutanoate concentration on its decarboxylation by isolated hepatocytes in the absence and presence of 2-chloro-4-methylpentanoate

Conditions are given in Table 2. 3-Methyl-2-oxobutanoate concentration was varied as indicated. Hepatocytes isolated from rats fed on the chow diet (△, ▲) and low-protein diet (○, ●) were incubated in the absence (△, ○) and presence (▲, ●) of 2-chloro-4-methylpentanoate (0.1 mM). Similar results were obtained in a separate experiment.

dehydrogenase kinase (Harris *et al.*, 1982*a*), was found to increase flux greatly with hepatocytes prepared from rats fed on the low-protein diet (Table 2 and Fig. 1). This was observed with either 3-methyl-2-oxobutanoate or 4-methyl-2-oxopentanoate (Table 2) as substrate. In contrast, with hepatocytes prepared from chow-fed rats there was only a slight activating effect of 2-chloro-4-methylpentanoate on 4-methyl-2-oxopentanoate decarboxylation, and no effect at all on 3-methyl-2-oxobutanoate decarboxylation. In several experiments carried out as shown in Fig. 1, half-maximal activation of flux in hepatocytes isolated from rats fed on low-protein diet occurred in the range of 10–20 μM-2-chloro-4-methylpentanoate. Very high concentrations of 2-chloro-4-methylpentanoate (> 1 mM) resulted in inhibition of flux, as would be expected, since this compound acts as a weak competitive inhibitor of the dehydrogenase (Harris *et al.*, 1982*a*). Although 2-chloro-4-methylpentanoate was effective with hepatocytes from rats fed on low-protein diets over a broad range of 3-methyl-2-oxobutanoate concentrations, no effect was found at any concentration with hepatocytes from chow-fed rats (Fig. 2). The effect of 2-chloro-4-methylpentanoate on hepatocytes from low-protein-diet-fed rats was more dramatic with 3-methyl-2-oxobutanoate than with 4-methyl-2-oxopentanoate (Table 2). Exogenous glucose (20 mM) had little effect with either hepatocyte preparation, regardless of the 2-oxo acid substrate used or the presence of 2-chloro-4-methylpentanoate. In contrast, lactate plus pyruvate inhibited flux in both types of hepatocytes, although inhibition was greater in hepatocytes from rats fed on low-protein diets. Furthermore, 2-chloro-4-methylpentanoate was effective in decreasing the inhibition of flux in the presence of lactate plus pyruvate only in the latter hepatocytes. 3-Hydroxybutyrate inhibited flux with hepatocytes prepared from rats fed on a low-protein diet, as measured by the decarboxylation of 3-methyl-2-oxobutanoate but not of 4-methyl-2-oxopentanoate (Table 2). 3-Hydroxybutyrate was without inhibitory effects with hepatocytes prepared from chow-fed rats and even had a slight stimulatory effect on the decarboxylation of 4-methyl-2-oxopentanoate (Table 2). Once more 2-chloro-4-methylpentanoate had its most marked stimulatory effect in the presence of 3-hydroxybutyrate with hepatocytes prepared from rats fed on the low-protein diet. Oleate inhibited flux as measured with both substrates in hepatocytes prepared from rats fed on the low-protein diet, but was without significant effect with hepatocytes prepared from chow-fed rats (Table 2). Again, 2-chloro-4-methylpentanoate was effective at stimulating flux in the presence of oleate only with hepatocytes prepared from low-protein-diet-fed rats.

Effect of dibutyryl cyclic AMP on flux through the branched-chain 2-oxo acid dehydrogenase

Dibutyryl cyclic AMP stimulated decarboxylation of 3-methyl-2-oxobutanoate, in both the absence and the

Table 3. Effect of dibutyryl cyclic AMP on the decarboxylation of 3-methyl-2-oxobutanoate and 4-methyl-2-oxopentanoate by hepatocytes prepared from chow-fed and low-protein-diet-fed rats

Conditions were as described in Table 2. * $P < 0.05$ for effect of diet; † $P < 0.05$ for effect of dibutyryl cyclic AMP versus corresponding condition without this compound.

1-^{14}C-labelled substrate	Addition (mM)	Rate of 2-oxo acid decarboxylation (nmol/min per g wet wt.) by hepatocytes prepared from rats fed on: Chow diet	Low-protein diet
3-Methyl-2-oxobutanoate	None	304±13	100±6*
	Dibutyryl cyclic AMP (0.05)	316±23	148±13*†
	Oleate (2)	237±36	7±1*
	Oleate+dibutyryl cyclic AMP	223±55	41±8*†
	Lactate (10)+pyruvate (2.5)	156±6	10±3*
	Lactate+pyruvate+dibutyryl cyclic AMP	153±6	12±5*
4-Methyl-2-oxopentanoate	None	193±14	84±7*
	Dibutyryl cyclic AMP	201±15	90±10*
	Oleate	191±25	19±2*
	Oleate+dibutyryl cyclic AMP	212±18	42±7*†
	Lactate+pyruvate	123±8	12±2*
	Lactate+pyruvate+dibutyryl cyclic AMP	127±11	13±4*

presence of oleate, by hepatocytes prepared from rats fed on the low-protein diet, but not the chow diet (Table 3). Decarboxylation of 4-methyl-2-oxopentanoate was only stimulated in the presence of oleate. Lactate and pyruvate completely prevented any effect of dibutyryl cyclic AMP with both oxo acids. The stimulatory effects of dibutyryl cyclic AMP were substantially less than those caused by 2-chloro-4-methylpentanoate (compare Table 2 with Table 3), and its effects were also greatly influenced by the time of preincubation of the hepatocytes before measurement of flux (Fig. 3). Although 2-chloro-4-methylpentanoate was effective without preincubation, dibutyryl cyclic AMP only became effective as the preincubation time progressed and appeared to block a time-dependent loss of flux through the branched-chain 2-oxo acid dehydrogenase (Fig. 3).

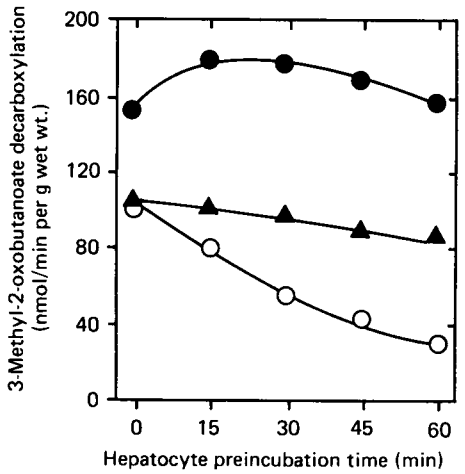

Fig. 3. Effect of preincubation time on stimulation by 2-chloro-4-methylpentanoate and dibutyryl cyclic AMP of 3-methyl-2-oxobutanoate decarboxylation by hepatocytes prepared from rats fed on a low-protein diet

Conditions are given in Table 2, except that preincubation time before measuring flux through the branched-chain 2-oxo acid dehydrogenase complex was varied from 0 to 60 min as indicated in the Figure. Incubation time with 0.2 mM-3-methyl-2-oxo[1-^{14}C]butanoate was 15 min. ○, no additions; ●, +0.1 mM-2-chloro-4-methylpentanoate; ▲, +0.05 mM-dibutyryl cyclic AMP. Similar results were attained in a separate experiment.

Effect of dichloroacetate on flux through the branched-chain 2-oxo acid dehydrogenase

Dichloroacetate, a potent inhibitor of pyruvate dehydrogenase kinase (Whitehouse *et al.*, 1974) and a less potent inhibitor of branched-chain 2-oxo acid dehydrogenase kinase (Paxton & Harris, 1982, 1984*b*), produced effects similar to those of 2-chloro-4-methylpentanoate on the decarboxylation of 3-methyl-2-oxobutanoate (Table 4). No significant effects of dichloroacetate were observed with hepatocytes from rats fed on the chow diet in either the absence or the presence of oxidizable substrates (Table 4). Stimulation of 3-methyl-2-oxobutanoate decarboxylation required about 0.8 mM-dichloroacetate for half-maximal effect (Fig. 4). Dichloroacetate partially blocked stimulation of flux by 2-chloro-4-methylpentanoate (Table 4), most likely because pyruvate decarboxylation would be stimulated by dichloroacetate but not by 2-chloro-4-methylpentanoate.

Activity and activity state of the branched-chain 2-oxo acid dehydrogenase complex in isolated hepatocytes

As expected from the flux measurements given above for the two types of hepatocytes, the activity of the branched-chain 2-oxo acid dehydrogenase complex was much greater in cell-free extracts of hepatocytes prepared from chow-fed rats (Table 5). On the other hand, the

Table 4. Effect of dichloroacetate on the decarboxylation of 3-methyl-2-oxobutanoate by hepatocytes prepared from chow-fed and low-protein-diet-fed rats

Conditions are described in Table 2. * $P < 0.01$ for effect of diet; † $P < 0.05$ for the effect of dichloroacetate versus the corresponding condition without this compound.

Addition (mM)	Rate of 3-methyl-2-oxobutanoate decarboxylation (nmol/min per g wet wt.) by hepatocytes prepared from rats fed on:	
	Chow diet	Low-protein diet
None	304±13	100±6*
Dichloroacetate (2.5)	289±26	166±8*†
Oleate (2)	237±36	7±1*
Oleate+dichloroacetate	211±32	48±7*†
2-Chloro-4-methylpentanoate (0.1)	307±13	199±7*
2-Chloro-4-methylpentanoate+dichloroacetate	285±23	176±8*†
Lactate (10)+pyruvate (2.5)	156±6	10±3*
Lactate+pyruvate+dichloroacetate	153±11	35±7*†

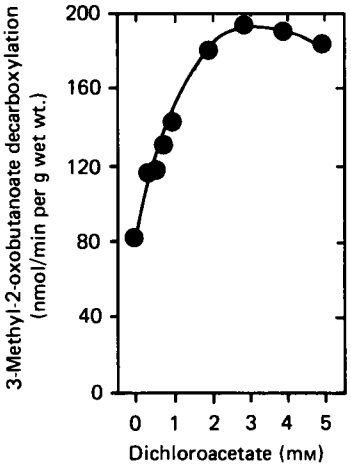

Fig. 4. Effect of dichloroacetate on the decarboxylation of 3-methyl-2-oxobutanoate by hepatocytes prepared from a rat fed on a low-protein diet

Conditions are given in Table 2. Values shown are the results obtained with one hepatocyte preparation. Similar results were obtained in a separate experiment.

activity of the complex varied much more with various incubation conditions in cell-free extracts of hepatocytes prepared from low-protein-diet-fed rats (Table 5). It was found that incubation of hepatocytes with no additions other than 2-chloro-4-methylpentanoate completely activated the complex, i.e. converted the enzyme into the 100%-active/dephosphorylated form. This was established by the finding that no additional activation of the complex could be achieved by incubating extracts of these hepatocytes with broad-specificity phosphoprotein phosphatase (results not shown). Thus the activity state (% of active form) of the enzyme for each incubation condition (given in parentheses in Table 5) could be calculated relative to the activity of the enzyme in hepatocytes incubated only with 2-chloro-4-methylpentanoate.

Some 50% of the enzyme of hepatocytes from low-protein-diet-fed rats incubated without additions was in the active state (Table 5). Oxidizable substrates (lactate plus pyruvate, 3-hydroxybutyrate and oleate) caused a marked inactivation of the enzyme and a corresponding decrease in the activity state. 2-Chloro-4-methylpentanoate opposed these effects, but to varying degrees, and did not completely prevent the inhibitory effects of any of the substrates. Additional evidence that these changes in activity state reflect the phosphorylation state of the complex is given in Fig. 5. The very low enzyme activity of extracts of 3-hydroxybutyrate-incubated hepatocytes was increased markedly by the action of the broad-specificity phosphoprotein phosphatase, indicating that only about 10% of the enzyme was in the active dephosphorylated state. The phosphatase had much less effect with extracts of hepatocytes previously incubated with 3-hydroxybutyrate plus 2-chloro-4-methylpentanoate (Fig. 5), presumably because most (74%) of the enzyme was already in the active/dephosphorylated state.

2-Chloro-4-methylpentanoate was without significant effect on the activity of the enzyme in extracts of hepatocytes prepared from chow-fed rats (Table 5). This result, plus the finding that the broad-specificity phosphoprotein phosphatase could not cause an increase in activity (results not shown), suggest that the enzyme of these hepatocytes is completely active/dephosphorylated. Incubation of chow-fed-rat hepatocytes (Table 5) with oleate as well as with lactate plus pyruvate (but not with 3-hydroxybutyrate) produced some apparent inactivation of the enzyme (Table 5). It should be noted, however, that these effects were marginal and completely insensitive to reversal or prevention by 2-chloro-4-methylpentanoate. It is not clear, therefore, that these effects of oxidizable substrates were due to inactivation of the complex by phosphorylation.

Dibutyryl cyclic AMP had no significant effect on the activity state of the enzyme, regardless of the hepatocyte preparation and/or incubation condition (Table 5). Dichloroacetate also had no significant effects in the absence of oxidizable substrate, but increased the activity state of the enzyme significantly with low-protein-diet-fed rat hepatocytes incubated with lactate plus pyruvate, 3-hydroxybutyrate or oleate (Table 5). As observed with 2-chloro-4-methylpentanoate, no significant effects of

Table 5. Activity of branched-chain 2-oxo acid dehydrogenase complex in extracts of hepatocytes prepared from chow-fed and low-protein-diet-fed rats

Hepatocytes prepared from three chow-fed rats and four low-protein-diet-fed rats were incubated for 20 min under conditions described in the text with the additions indicated below. Extracts of the hepatocytes were prepared and assayed for branched-chain 2-oxo acid dehydrogenase as described in the text. Means ± S.E.M. are given; the numbers in parentheses correspond to the percentage of the value obtained with hepatocytes incubated with 2-chloro-4-methylpentanoate. * $P < 0.05$ for the effect of addition versus control (none) incubation; † $P < 0.05$ for effect of 2-chloro-4-methylpentanoate versus corresponding condition without this compound; ‡ $P < 0.05$ for effect of dichloroacetate versus corresponding condition without this compound.

Addition (mM)	Activity of branched-chain 2-oxo acid dehydrogenase complex (nmol/min per g wet wt.) of hepatocytes prepared from rats fed on:	
	Chow diet	Low-protein diet
None	787 ± 73 (96)	152 ± 32 (50)
2-Chloro-4-methylpentanoate (0.1)	816 ± 71 (100)	302 ± 23*† (100)
Lactate (10) + pyruvate (2.5)	648 ± 47* (79)	51 ± 10* (17)
Lactate + pyruvate + 2-chloro-4-methylpentanoate	591 ± 48* (72)	145 ± 31‡ (48)
DL-3-Hydroxybutyrate (20)	693 ± 95 (85)	26 ± 7* (9)
DL-3-Hydtroxybutyrate + 2-chloro-4-methylpentanoate	669 ± 36 (82)	210 ± 19† (67)
Oleate (2)	643 ± 58* (79)	24 ± 6* (8)
Oleate + 2-chloro-4-methylpentanoate	650 ± 54 (80)	100 ± 29† (33)
Dibutyryl cyclic AMP (0.05)	778 ± 91 (95)	184 ± 30 (61)
Dibutyryl cyclic AMP + lactate + pyruvate	608 ± 42* (75)	42 ± 8* (14)
Dibutyryl cyclic AMP + DL-3-hydroxybutyrate	762 ± 88 (93)	41 ± 14* (14)
Dibutyryl cyclic AMP + oleate	632 ± 60 (77)	24 ± 6* (8)
Dichloroacetate (5)	675 ± 81 (83)	190 ± 22 (63)
Dichloroacetate + 2-chloro-4-methylpentanoate	671 ± 58 (82)	257 ± 19* (85)
Dichloroacetate + lactate + pyruvate	622 ± 71* (76)	98 ± 20*‡ (32)
Dichloroacetate + DL-3-hydroxybutyrate	690 ± 69 (85)	186 ± 22‡ (62)
Dichloroacetate + oleate	630 ± 61 (77)	86 ± 24‡ (28)

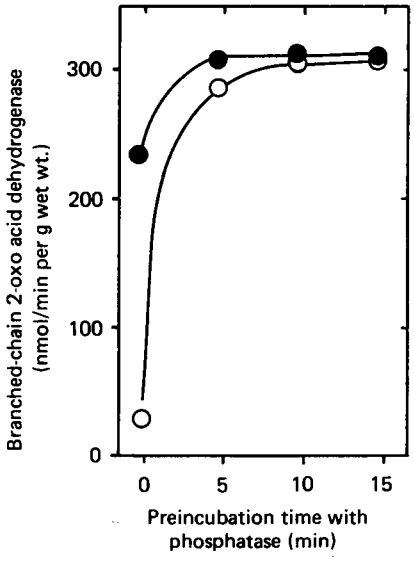

Fig. 5. Activation by broad-specificity phosphoprotein phosphatase of branched-chain 2-oxo acid dehydrogenase complex in cell-free extracts

Preparation of cell-free extracts of hepatocytes is described in the text. Equal volumes of extract and phosphatase [suspended in a solution containing 50 mM-imidazole/HCl, pH 7.5, 0.5 mM-EDTA, 0.5 mM-dithiothreitol and 40% (v/v) glycerol] were incubated at 30 °C. Samples were removed at the indicated times and assayed for branched-chain 2-oxo acid dehydrogenase activity by the procedure given in the test. ○, Hepatocytes incubated for 20 min with 20 mM-DL-3-hydroxybutyrate before extraction; ●, hepatocytes incubated for 20 min with 20 mM-DL-3-hydroxybutyrate plus 0.1 mM-2-chloro-4-methylpentanoate before extraction. Similar results were obtained in a separate experiment.

dichloroacetate were observed with chow-fed-rat hepatocytes.

DISCUSSION

Now that covalent modification has been clearly established for the branched-chain 2-oxo acid dehydrogenase complex of several mammalian tissues (Fatania *et al.*, 1981; Odessey, 1982; Paxton & Harris, 1982), the physiological significance of this mode of regulation of the enzyme is receiving considerable attention. Obviously much remains to be learned with respect to the factors involved in setting the phosphorylation/activity state of the complex; however, some insight has been gained from studies of the characteristics of the kinase and phosphatase responsible for inactivation and activation of the complex respectively (Lau *et al.*, 1982; Paxton & Harris, 1984*a*; Damuni *et al.*, 1984). It appears certain that these enzymes are different from the kinase and phosphatase responsible for regulation of the pyruvate dehydrogenase complex. Indeed, the relative activity states of the pyruvate dehydrogenase complex and the branched-chain

2-oxo acid dehydrogenase complex are usually very different and apparently independently regulated in any given tissue. In normal fed rats, for example, hepatic pyruvate dehydrogenase is primarily in the inactive, phosphorylated, state (Wieland *et al.*, 1971), whereas hepatic branched-chain 2-oxo acid dehydrogenase is primarily in the active, dephosphorylated, state (Gillim *et al.*, 1983). Starvation of such rats for 48 h further decreases the activity state of the hepatic pyruvate dehydrogenase complex (Wieland *et al.*, 1971), but has no effect on the activity state of the hepatic branched-chain 2-oxo acid dehydrogenase complex (Gillim *et al.*, 1983; Wagenmakers *et al.*, 1984; but see also Patston *et al.*, 1984). In contrast, restricting protein intake rather than energy has exactly the opposite effect, i.e. no effect on the activity state of the hepatic pyruvate dehydrogenase complex (R. A. Harris & G. Goodwin, unpublished work), but markedly inactivates the hepatic branched-chain 2-oxo acid dehydrogenase complex (Gillim *et al.*, 1983; Patston *et al.*, 1984). Such studies indicate that different factors regulate the respective kinases and phosphatases responsible for covalent modification of these complexes and, furthermore, that this mode of regulation of branched-chain 2-oxo acid dehydrogenase is as important to the control of branched-chain amino acid metabolism as the analogous regulation of the pyruvate dehydrogenase complex is to glucose metabolism.

Hepatocytes isolated from low-protein-diet-fed rats have much less capacity to oxidize branched-chain 2-oxo acids than have hepatocytes isolated from chow-fed rats (Brand & Hauschildt, 1984; Harris *et al.*, 1985). Rates of branched-chain 2-oxo acid decarboxylation found in the present study are within the range reported in previous studies with isolated hepatocytes (Livesey, 1983; Corkey *et al.*, 1982) or the perfused rat liver (Buxton *et al.*, 1982). The decreased capacity of low-protein-diet-fed rat hepatocytes to decarboxylate branched-chain 2-oxo acids can be explained by the finding that liver from such rats has less total enzyme plus a greater percentage of the complex in the phosphorylated/inactive state (Gillim *et al.*, 1983).

Activation of branched-chain 2-oxo acid oxidation by dichloroacetate was observed in the present study, but only with hepatocytes isolated from low-protein-diet-fed rats. This effect can be explained, at least in part, by conversion of the branched-chain 2-oxo acid dehydrogenase complex into the less phosphorylated, more active, form. Although dichloroacetate is better known as an inhibitor of pyruvate dehydrogenase kinase (Whitehouse *et al.*, 1974), it has been shown to activate the branched-chain 2-oxo acid dehydrogenase complex of the perfused rat heart (Sans *et al.*, 1980; Paxton & Harris, 1984*b*) and to inhibit the kinase associated with the branched-chain 2-oxo acid dehydrogenase purified from rabbit liver (Paxton & Harris, 1982, 1984*b*).

Dibutyryl cyclic AMP was also found to stimulate the decarboxylation of branched-chain 2-oxo acids in low-protein-diet-fed rat hepatocytes, but, again, not with hepatocytes from chow-fed rats. Unlike the findings with dichloroacetate, no evidence was found for activation of the complex by conversion into its more active, less phosphorylated, state. Since dibutyryl cyclic AMP inhibits glycolysis, and is therefore very effective in decreasing generation of lactate and pyruvate in isolated hepatocytes (Harris, 1975), and since exogenous lactate plus pyruvate inhibit flux through the branched-chain 2-oxo acid dehydrogenase complex (Sans *et al.*, 1980; Patel *et al.*, 1981), it seems likely that prevention of the inhibition of flux through branched-chain 2-oxo acid dehydrogenase by lactate plus pyruvate explains the effects of dibutyryl cyclic AMP. This proposed mechanism is favoured by the observed time-dependency of the dibutyryl cyclic AMP stimulation as well as the prevention of this stimulation by exogenous lactate plus pyruvate. However, this mechanism ignores the observation that lactate plus pyruvate (at least at high concentrations) cause inactivation of the branched-chain 2-oxo acid dehydrogenase complex in low-protein-diet-fed rat hepatocytes. This may mean that multiple mechanisms are involved, varying in importance with concentration of lactate plus pyruvate. Interestingly, Palmer *et al.* (1983) found dibutyryl cyclic AMP inhibition rather than stimulation of branched-chain 2-oxo acid dehydrogenase flux in rat diaphragm muscle. That observation is not inconsistent with the present work, however, since dibutyryl cyclic AMP stimulates rather than inhibits glycolysis in diaphragm muscle (Palmer *et al.*, 1983).

2-Chloro-4-methylpentanoate, a potent and specific inhibitor of branched-chain 2-oxo acid dehydrogenase kinase (Harris *et al.*, 1982*a*), proved the most useful tool of the compounds investigated in the present study. The activating effect of this compound on the decarboxylation of branched-chain 2-oxo acids suggested: (*a*) a rate-limiting role for the branched-chain 2-oxo acid dehydrogenase complex in the decarboxylation of branched-chain 2-oxo acids by hepatocytes prepared from low-protein-diet-fed rats and (*b*) the branched-chain 2-oxo acid dehydrogenase must be partially in the phosphorylated, inactive, state in the same hepatocytes. The latter point was confirmed by measuring the activity and activity state of the complex in cell-free extracts of hepatocytes incubated with and without 2-chloro-4-methylpentanoate. It was found with low-protein-diet-fed rat hepatocytes that 2-chloro-4-methylpentanoate increased both flux and activity state of the enzyme under a wide variety of incubation conditions. In contrast, no corresponding increase in flux or activity state was produced by this compound with hepatocytes isolated from chow-fed rats. These results indicate, therefore, that the activity state of the complex in isolated hepatocytes is qualitatively similar to the activity state of the enzyme and it existed in the intact tissue. This 'preservation' of activity state is surprising, considering the time and steps involved in hepatocyte preparation, and suggests that conditions within the mitochondrial matrix space of isolated hepatocytes may not be grossly altered from the situation *in vivo*.

Williamson *et al.* (1979) concluded, from quantitative analysis of the CoA esters produced during branched-chain 2-oxo acid catabolism, that the rate-limiting step of branched-chain 2-oxo acid oxidation by isolated hepatocytes is distal to the branched-chain 2-oxo acid dehydrogenase complex. The addition of oleate, known to inhibit branched-chain 2-oxo acid oxidation in a number of model liver systems, caused the branched-chain 2-oxo acid dehydrogenase complex to become rate-limiting in the isolated hepatocyte system studied by Williamson *et al.* (1979) and Corkey *et al.* (1982). Although no attempt was made to establish rigorously the rate-limiting step with the hepatocytes used in the present study, the activating effect of 2-chloro-4-methylpenta-

noate and the greater inhibitory effects of oxidizable substrates would suggest that the low activity of branched-chain 2-oxo acid dehydrogenase caused this enzyme to become rate-limiting in hepatocytes prepared from low-protein-diet fed rats. Although beyond the scope of the present study, acyl-CoA esters should accumulate in hepatocytes from chow-fed rats but not from low-protein-diet-fed rats when incubated with branched-chain 2-oxo acids. Indeed, preliminary studies suggest significant effects of 4-methyl-2-oxopentanoate (1 mM) on glycolytic flux in hepatocytes prepared from chow-fed rats but not from low-protein-diet-fed rats (R. A. Harris, unpublished work). Since CoA esters originating from branched-chain 2-oxo acids have dramatic effects on metabolic processes (Walajtys-Rode *et al.*, 1979), these observations are consistent with the accumulation of such compounds in hepatocytes from chow-fed rats, but not those from low-protein-diet-fed rats.

Oxidizable substrates, used in this study at concentrations higher than normally found under physiological conditions, caused a marked inhibition of flux through the branched-chain 2-oxo acid dehydrogenase as well as decreased activity state of the complex in hepatocytes prepared from low-protein-diet-fed rats. In general, the effects of oxidizable substrates on flux with hepatocytes from chow-fed rats were much less marked, and convincing evidence was not found for inactivation of the complex by covalent modification. It is not clear at this time why inactivation of the complex was readily affected in low-protein-diet-fed hepatocytes, whereas the enzyme was apparently resistant in chow-fed-rat hepatocytes. A difference in concentration of effectors of the kinase and phosphatase within the mitochondria of these hepatocytes is one possible explanation. Branched-chain 2-oxo acids are inhibitors of the kinase (Paxton & Harris, 1984*a*), and a decrease in their concentration is probably important for inactivation of the complex during protein starvation (Gillim *et al.*, 1983). The other substrates as well as the products of the reaction catalysed by the complex have not been observed to regulate the kinase in a manner analogous to that seen with pyruvate dehydrogenase (Paxton & Harris, 1984*a*). For example, pyruvate dehydrogenase kinase is subject to inhibition by substrates of the complex (pyruvate, NAD^+ and CoA) and to activation by its products (acetyl-CoA and NADH) (for a review, see Randle, 1981). Lau *et al.* (1982) reported a slight stimulatory effect of high acyl-CoA/CoA ratios on the kinase, but this was not observed by Paxton & Harris (1984*a*). Likewise, Odessey (1980) found that NADH promoted apparent phosphorylation of branched-chain 2-oxo acid dehydrogenase in crude mitochondrial extracts, but, again this has not been observed with a purified enzyme preparation (Paxton & Harris, 1984*a*). However, initial attempts to show allosteric regulation of pyruvate dehydrogenase kinase also yielded conflicting results, suggesting that optimal conditions may need to be established for detecting such effects with the branched-chain 2-oxo acid dehydrogenase kinase.

The branched-chain 2-oxo acid dehydrogenase phosphatase has also not been studied in great detail. Only one purified preparation of the enzyme has been reported, with the most interesting feature being that no bivalent cations were required for activity (Damuni *et al.*, 1984), in marked contrast with the requirement of the pyruvate dehydrogenase phosphatase for Mg^{2+} and Ca^{2+} (for a review, see Randle, 1981). It should be noted, however, that Fatania *et al.* (1983) found Mg^{2+} to be essential and that Paxton & Harris (1984*c*) found Mg^{2+} to be stimulatory in cruder enzyme preparations. Furthermore, Patel & Olson (1982) reported both Ca^{2+} and Mg^{2+} to be necessary for activation of the complex in intact mitochondria. Clearly, much remains to be learned about regulation of the phosphatase.

Although it is clear that hepatic branched-chain 2-oxo acid dehydrogenase from several mammalian species and tissues is subject to inactivation by phosphorylation, there is suggestive evidence that phosphorylation may be slower with the liver enzyme than with the enzymes of other tissues (Odessey, 1982; Paxton & Harris, 1982). This may represent an artifact of loss or inactivation of the kinase during extraction/isolation of the hepatic complex. Nevertheless, different tissues may contain different ratios of kinase to complex and/or kinase to phosphatase, and these ratios may be markedly different in chow-fed-rat liver and in low-protein-diet-fed-rat liver. A substantial increase in the amount of pyruvate dehydrogenase kinase appears to be an important factor in starvation-induced inactivation of the pyruvate dehydrogenase complex (Kerbey *et al.*, 1984). It is also possible that the 'activator protein', described by Fatania *et al.* (1982) and shown to correspond to E_1 of the complex by Yeaman *et al.* (1984), plays some role in 'buffering' covalent modification of branched-chain 2-oxo acid dehydrogenase in liver of well-fed rats. It becomes of considerable importance, therefore, to determine if 'activator protein' is an artifact of extraction, as suggested by Yeaman *et al.* (1984), or of physiological significance, as argued by Espinal *et al.* (1985). Regardless of the exact mechanism involved in the apparent resistance of the hepatic complex of well-fed rats to regulation by covalent modification, the present work, along with activity-state measurements *in vivo* (Gillim *et al.*, 1983), clearly establish that this type of regulation plays an important role in modulation of branched-chain 2-oxo acid oxidation in liver of low-protein-diet-fed rats. Whether this mode of regulation is physiologically important in liver in other nutritional and hormonal states remains to be established.

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