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Metabolism of Branched-Chain Amino Acids by Rat Skeletal Muscle: Regulation of Branched-Chain Alpha-Keto Acid Dehydrogenase

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METABOLISM OF BRANCHED-CHAIN AMINO ACIDS BY RAT SKELETAL MUSCLE:

REGULATION OF BRANCHED-CHAIN α-KETO ACID DEHYDROGENASE

by

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ROBERT PAUL AFTRING. Metabolism of Branched-Chain Amino Acids by Rat Skeletal Muscle: Regulation of Branched-Chain α-Keto Acid Dehydrogenase. (Under the direction of Maria G. Buse.)

Catabolism of the essential branched-chain amino acids (BCAA) by skeletal muscle of the rat was investigated. In diaphragm muscles incubated in vitro oxidation and transamination of leucine or valine were accelerated by diabetes or starvation overnight. In the presence of pyruvate, transamination of BCAA by diaphragms was increased; oxidation of BCAA by diaphragms from diabetic or starved rats was increased by pyruvate but was inhibited in diaphragms of normal, fed rats. The effects on transamination were verified by direct measurements of the α-ketoisocaproate (KIC) product of leucine transamination. The effect of pyruvate on KIC oxidation by diaphragms was similar to the effect on leucine oxidation. Branched-chain α-keto acid dehydrogenase (BCDH) catalyzes the committed step in BCAA catabolism, its activity is regulated by a reversible phosphorylation-dephosporylation cycle. A method to measure active (initial) and total (active + inactive) BCDH in a soluble preparation from muscle was developed. Initial BCDH activity in fed, postabsorptive rats was about 1 nmol/min/g muscle. Total activity was ~35 nmol/min/g muscle; thus ~2% of BCDH was in the active state. Intravenous infusion of leucine rapidly increased BCDH activity in a dose-dependent fashion; activity subsequently declined in parallel with plasma leucine concentration. Valine had no effect on BCDH activity while isoleucine was effective only at high doses. BCDH activity increased after 25% or 50% but not 9% protein meals in rats fed an adequate protein diet. Feeding a 50%

protein diet chronically increased postabsorptive BCDH. Dose-dependent response of BCDH to leucine was blunted in rats fed 9% protein chronically. In insulinopenic diabetic rats plasma BCAA and muscle BCDH activity were increased. Insulin therapy or adrenalectomy of diabetic rats decreased plasma BCAA but BCDH activity remained elevated. Starvation increased BCDH activity significantly only after four days. BCDH activation in response to increased leucine was blunted in starved or diabetic rats. Conclusions: In normal fed rats, muscle BCDH activity increases in response to hyperleucinemia from meal consumption or infusion. The response to hyperleucinemia is attenuated by diabetes, starvation or consumption of a low protein diet. In diabetes increased muscle BCAA oxidation results from increased BCAA transamination and increased BCDH activity.

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Lastly I would be remiss if I did not acknowledge the continuing support of my parents through the many travails of my graduate education. They always had words of encouragement regardless of how stormy the times and were remarkably tolerant of my continuing student status. I would like to dedicate this dissertation to them for without their

support and encouragement from childhood on through my many years of college and post-baccalaureate education I would not be what I am today.

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List of Abbreviations

BCAA; branched-chain amino acids

BCKA; branched-chain α-keto acids

BCDH; branched-chain α-keto acid dehydrogenase complex

BCAT; branched-chain amino acid aminotransferase

HPLC; high-performance liquid chromatography

KIC; α-ketoisocaproate

PEG; polyethylene glycol

Chapter I

General Introduction

The branched-chain amino acids (BCAA*; L-leucine, L-isoleucine and L-valine) are among the nine essential amino acids in man and the rat (156,158). As a group the BCAA account for a large proportion, about 45% (157,158), of the total essential amino acid requirement in man. The substantial requirement for these amino acids is reflected in their abundance in proteins; they account for 35% of the essential and 14% of the total amino acids in skeletal muscle (159), the largest repository of metabolically active protein (44). Among the essential amino acids BCAA (and leucine in particular) are unique in several respects: 1) they are metabolized extensively by peripheral tissues other than the liver (77,126); 2) their circulating concentrations change markedly in states such as altered protein intake, starvation, hepatic failure and insulinopenic diabetes (172); and 3) there is evidence that leucine can regulate protein turnover other than in its role as a precursor for protein synthesis (29,127).

Catabolism of the BCAA.

The BCAA can be completely degraded by mammals and the catabolic pathways for the amino acids were established by a number of investigators (reviewed in 125). The essential features of their catabolic pathways are shown in Figure 1. The initial two catabolic steps are

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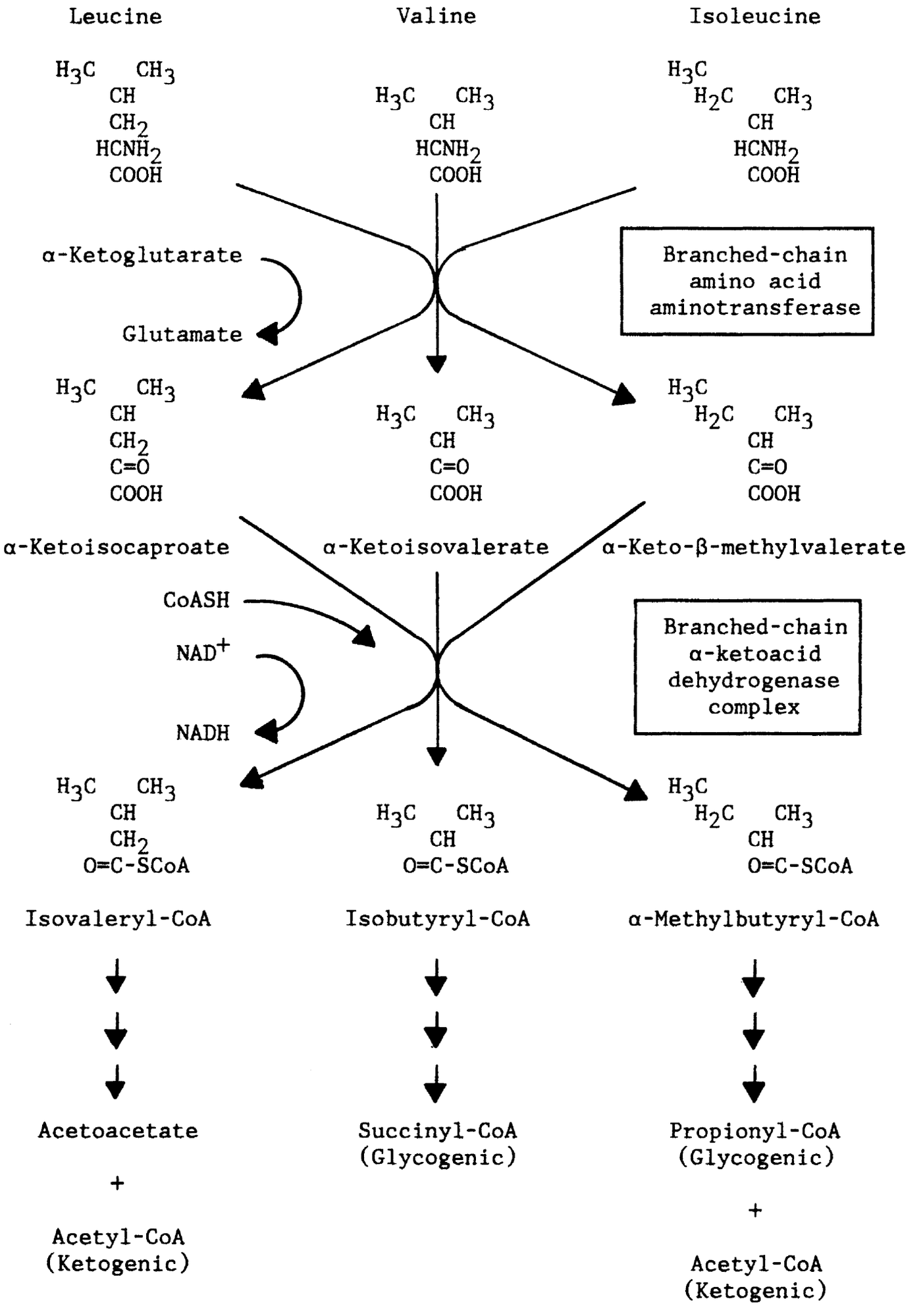
* Abbreviations used in this chapter are: BCAA, branched-chain amino acids; BCKA, branched-chain α-keto acids; BCDH, branched-chain α-keto acid dehydrogenase; BCAT, branched-chain amino acid aminotransferase; KIC, α-ketoisocaproate.

Figure 1. Catabolic pathways of the branched-chain amino acids.



common to all three BCAA. The first step (I), involves transamination of the amino acid to its corresponding branched-chain α-keto acid (BCKA) by branched-chain amino acid aminotransferase (BCAT); i.e. leucine to α-ketoisocaproate (KIC), valine to α-ketoisovalerate, and isoleucine to α-keto-β-methylvalerate. The second (II), and committed step (thus rendering these amino acids essential), is irreversible oxidative decarboxylation of BCKA catalyzed by branched-chain α-keto acid dehydrogenase complex (BCDH). Further catabolism of the three branched-chain acyl-CoA products of BCDH action on the three BCKA diverges with respect to the enzymes and the specific catabolic products but in general the pathways are similar to β-oxidation of fatty acids.

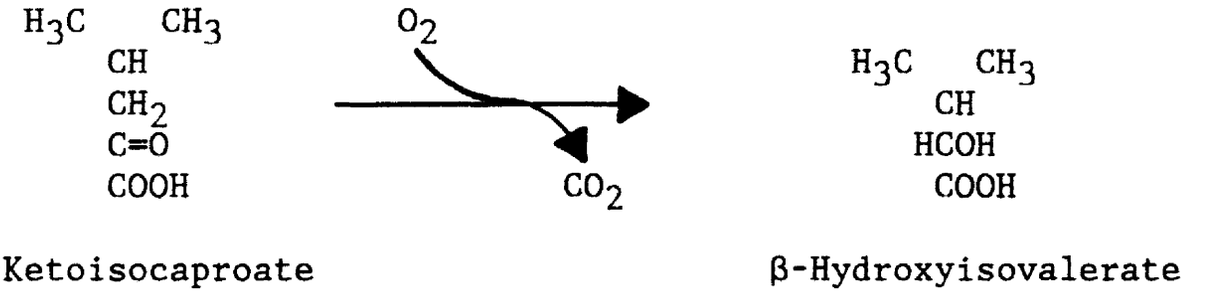
There are several noteworthy features of these catabolic pathways. The ultimate products of leucine oxidation are acetyl-CoA and aceto-acetyl-CoA, leucine catabolism therefore yields only ketogenic products and cannot contribute to gluconeogenesis. 3-Hydroxy-3-methylglutaryl-CoA is an intermediate in leucine catabolism, thus its carbon skeleton can act as a direct precursor for cholesterol synthesis without oxidation to acetyl-CoA. Valine is ultimately catabolized to succinyl-CoA, and therefore contributes only a gluconeogenic precursor. Catabolism of isoleucine yields propionyl-CoA and acetyl-CoA thereby providing precursors for both ketogenesis and gluconeogenesis.

The existence of additional catabolic pathways for leucine deserve mention. A cytosolic enzyme which catalyzes O_2-dependent oxidative decarboxylation of KIC has been identified in liver (161). This enzyme is an Fe-dependent dioxygenase (162,163) which catalyzes KIC oxidation

to β-hydroxyisovalerate (Figure 2). The significance of this catabolic sequence in disposal of leucine is unknown but the product of this reaction appears to be an ideal precursor for sterol synthesis, requiring only activation to its CoA derivative to enter the sterol synthetic pathway; experiments using rat liver homogenates designed to demonstrate incorporation of α-ketoisocaproate carbons into cholesterol under conditions favoring utilization of this pathway were however unsuccessful (R.P. Aftring, M. Owens, and M.G. Buse, unpublished observations).

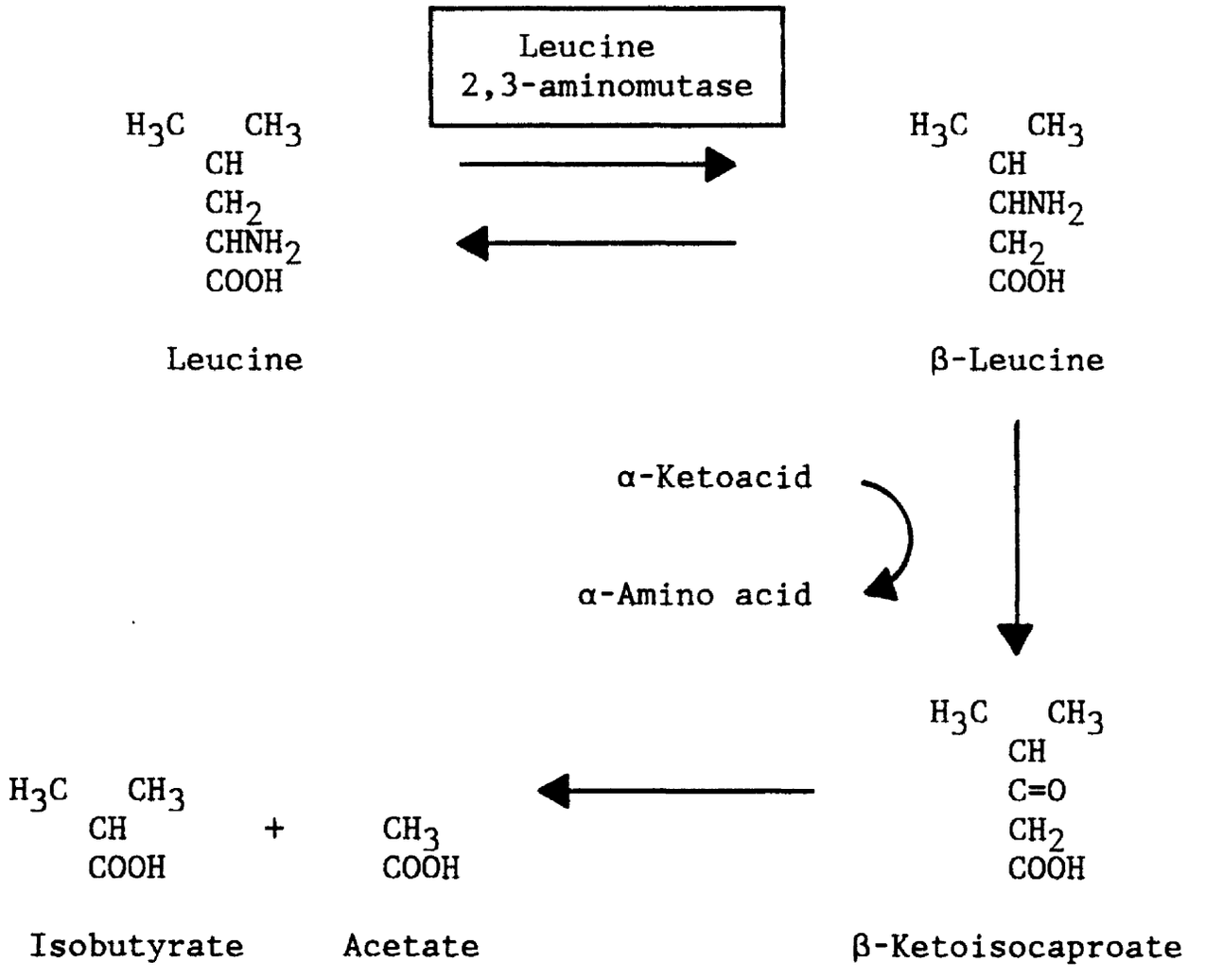
Liver of mammals also synthesizes a cobalamin-requiring enzyme (leucine 2,3-aminomutase) which catalyzes the conversion of leucine to β-leucine (Figure 3; ref. 147). The resulting β-leucine can be transaminated to β-ketoisocaproate (an inherently unstable β-keto acid) which in a bacterial system was further catabolized to isobutyrate and acetate. In a group of patients with pernicious anemia serum leucine concentration was decreased while serum β-leucine concentration was increased. This observation suggested the possibility that the β-leucine pathway operated in the direction of synthesis (148). Furthermore, β-leucine was synthesized from valine, isobutyryl-CoA or branched-chain fatty acids in vitro in a rat liver system (148). The importance of this metabolic pathway is unknown, but it suggests that although leucine is essential in the diet (i.e. required for growth and positive N balance), mammals may have limited capacity to synthesize this amino acid.

Figure 2. Catabolism of α-ketoisocaproate by a cytosolic oxygenase.



α-Ketoisocaproate β-Hydroxyisovalerate

Figure 3. Metabolism of leucine by leucine 2,3-aminomutase.



Leucine β-Leucine

Isobutyrate Acetate β-Ketoisocaproate

Branched-chain amino acid aminotransferases.

BCAT activity in tissues is comprised of at least three and perhaps more isoenzymes with somewhat different physical properties and substrate specificities (reviewed in 95,96). As a group they catalyze a pyridoxal-5'-phosphate dependent transamination reaction similar to a large number of others (e.g. aspartate aminotransferase, alanine aminotransferase). The reaction catalyzed is the transfer of the amino group from the branched-chain amino acid to an α-keto acid with production of the corresponding BCKA and amino acid (from the α-keto acid, see Figure 1). In general α-ketoglutarate is thought to be the preferred amino group acceptor in vivo for this group of enzymes but the branched-chain α-keto acids are also efficient amino group acceptors (13,174).

The BCAT enzymes are present in all mammalian tissues examined. The highest activities were found in stomach, pancreas, lactating mammary gland, salivary gland and heart, intermediate activities in kidney, brain, and muscle and low activities in liver, spleen and lung (95,99). Three isoenzymes of BCAT have been described in the rat. Isoenzyme I was ubiquitous in tissues and actively transaminates all three BCAA; isoenzyme III, also active with all BCAA, was found only in brain, placenta and ovary (99). Isoenzyme II, present only in liver, was active with leucine and methionine and may exist as distinct cytosolic and mitochondrial forms (97). In human tissues isoenzyme III was found in many tissues but isoenzyme I was the predominant BCAT; isoenzyme II has not been identified in human tissues (72).

Branched-chain α-keto acid dehydrogenase complex.

The BCDH complex is analogous to the pyruvate and α-ketoglutarate dehydrogenase complexes, requiring NAD^+ and CoASH as cofactors for the oxidation of BCKA producing NADH and branched-chain acyl CoA (one carbon shorter than the BCKA) as end products (45,150,199, see Figure 1). BCDH is a large multienzyme complex, located on the inner aspect of the inner mitochondrial membrane (119,180). Active BCDH consists of three enzymatic components (38,85,144,146): 1) a decarboxylase moiety (E1) composed of nonidentical α and β subunits with thiamine pyrophosphate as a prosthetic group; 2) a transacylase moiety (E2) with lipoic acid as a prosthetic group; and 3) a flavin-containing lipoamide dehydrogenase moiety (E3) which is probably identical to that found in the pyruvate and α-ketoglutarate dehydrogenase complexes (85,199).

BCDH activity is subject to several types of regulation. The complex is subject to feedback regulation by NADH, its branched-chain acyl CoA products and perhaps catabolites of leucine distal to isovaleryl-CoA (reviewed in 150). It was recently established that BCDH is regulated by a phosphorylation-dephosphorylation cycle similar to that described for pyruvate dehydrogenase (150,152). BCDH is inactivated by the action of a specific kinase which catalyzes ATP-dependent phosphorylation of serine residue(s) of the α-subunit of the decarboxylase moiety (37,58,108,133). Purified BCDH kinase activity is inhibited by BCKA (KIC >> α-keto-β-methylvalerate > α-ketoisovalerate; ref. 142). The non-physiological compound, α-chloroisocaproate, is a relatively specific and highly potent inhibitor of BCDH kinase, in vivo and in vitro (82). Purified inactive BCDH can be reactivated in vitro

by dephosphorylation of phosphoserine by the action of a non-specific phosphoprotein phosphatase, cytosolic phosphoprotein phosphatase II (69). There is evidence for a specific phosphatase for BCDH, purified from bovine kidney mitochondria, which may be responsible for reactivation of the complex in vivo (40). BCDH phosphatase activity is unaffected by divalent cations and is inhibited by several di- and triphosphonucleosides in the absence of Mg^{2+} (40,152). Phosphatase activity is stimulated by protamine, poly(L-lysine), and histone H3 and inhibited by heparin, CoASH and several acyl-CoA products of BCAA catabolism (40,152). Recently, a protein inhibitor of BCDH phosphatase was purified from bovine kidney mitochondria. The protein inhibitor is distinct from protein phosphatase inhibitors -1 and -2 and appears to be specific for BCDH phosphatase (39,41). Mg^{2+} and spermine reverse the inhibitory effect of this protein on BCDH phosphatase (39,41). The system of a phosphoprotein phosphatase with a protein inhibitor is reminiscent of the cytosolic protein phosphatases I and II with their respective protein inhibitors I and II (reviewed in 36). Whether the BCDH phosphatase inhibitor is present in other tissues and if it has a role in the regulation of BCAA catabolism are unknown.

Protein factors which can alter BCDH activity have been found in several tissues and plasma (137,141). A cytoplasmic protein factor which activates BCDH in liver mitochondria has been prepared from skeletal muscle and is also found in plasma, but not in smooth muscle, kidney or liver (141). This skeletal muscle factor also activates α-ketoglutarate dehydrogenase but inhibits pyruvate dehydrogenase in rat liver mitochondria. A protein factor from kidney and liver

mitochondria activates phosphorylated BCDH, the effect does not involve dephosphorylation of the complex (57,198). This latter factor (termed BCDH activator protein) has been purified and was identified as free E1 (decarboxylase) or an isoenzyme of E1, which presumably increases BCDH activity by replacing inactive (i.e. phosphorylated) E1 in the BCDH reaction (56,198). It is noteworthy that phosphorylated E1 has lower affinity for the complex than does the non-phosphorylated enzyme (38). The role of these protein effectors of BCDH activity in regulation of BCDH activity in vivo remains unknown.

The discovery of interconvertible forms of BCDH presents a problem in the determination of BCDH activity in tissues, and casts doubt on the results and interpretation of early studies of BCDH activity. Studies of BCDH activity which consider the active and inactive forms find that BCDH is nonuniformly distributed between tissues (69,137, 183). Liver, kidney and heart have large total quantities of BCDH (69,137,183). In livers of fed rats nearly all of the BCDH was in the active state, in kidney the complex was 50-70% active, and in heart the activity state ranged between 7% and 50% in studies from different laboratories (69,137,183). Total BCDH activity in skeletal muscle was lower than in other tissues examined (137,183), although direct comparisons of the measured activities are difficult due to differences in assay methods. Patston et al. (137) estimated the BCDH was less than 20% active in detergent extracts of muscle mitochondria but the actual activity was below the limit of detection of their assay. In muscle homogenates Wagenmakers et al. (183) and Kasperek et al. (100) found about 5-6% of BCDH was in the active state in fed rats.

Interorgan cooperativity in BCAA catabolism.

A number of reports have established the importance of peripheral (non-splanchnic) tissues in BCAA catabolism. Studies of amino acid oxidation by eviscerated rat carcasses showed that of the essential amino acids only the BCAA were oxidized in the peripheral tissues and provided early evidence that BCAA are oxidized primarily by extrahepatic tissues, while other essential amino acids are oxidized by the liver (126). The importance of the peripheral tissues in BCAA oxidation was confirmed in studies of hepatectomized dogs (122) and rats (86), in which the concentrations of most amino acids increased steadily while those of the BCAA remained unchanged or declined. A wide variety of extrahepatic tissues have the ability to oxidize BCAA, including skeletal muscle (25,132), kidney (46,47), brain (21,35), peripheral nerve (28), aorta (28), adipose tissue (59), and heart (25). Determinations of the clearance of amino acids in humans after a protein meal (49,54) or amino acid infusion (2,49,54,67) demonstrated that skeletal muscle is a prominent site for BCAA disposal (disposing of 65-70% of infused leucine, ref. 67).

Although BCAT and BCDH are present in all tissues examined, their distributions in various tissues are markedly different (i.e. liver had low BCAT activity but high BCDH activity, muscle had high BCAT activity but low BCDH activity, and kidney had relatively high activities of BCAT and BCDH). The observation that perfused rat hindlimbs release substantial quantitities of BCKA when perfused with BCAA (90,91) led to the suggestion that there is interorgan cooperativity in the catabolism of BCAA (reviewed in 77). In view of the low activity of hepatic BCAT

it is thought that most BCAA entering the splanchnic circulation after a protein meal pass through the liver unaltered. Most BCAA from oral ingestion therefore enter the systemic circulation for distribution to peripheral tissues. In the periphery BCAA are taken up for protein synthesis and catabolism. In muscle some fraction of the BCAA which are transaminated to BCKA are oxidized by BCDH and the remaining BCKA enter the venous system and are distributed to other tissues for oxidation or reamination. Since liver has high BCDH activity it is thought to be an important site for the clearance of BCKA in the circulation and indeed hepatic-portal venous BCKA gradients in the rat show that liver clears virtually all BCKA supplied to it (112).

The conceptual model of interorgan cooperation for BCAA metabolism is well supported in the rat, but its applicability to humans has been questioned (172). Studies of leucine metabolism by human muscle in vivo established that leucine is oxidized by muscle but consistently found considerably less export of the transamination product KIC (2,49) by human muscle than has been found for rat muscle (90,91). The difference in the systems was suggested to be due to different distributions of BCDH in human and rat tissues. Khatra et al. (102) reported that 60% of human total body BCDH was present in muscle while only 15% of the rat enzyme was present in muscle. However, these studies were done without knowledge of the phosphorylation-dephosphorylation cycle which regulates BCDH activity (as discussed above). Furthermore the BCDH activities obtained by these authors in rat tissues are much lower than recent estimates of these activities (94,151); 4-6 fold lower in liver and nearly 2 orders of magnitude lower in skeletal muscle. At

present, in the absence of measurements of BCDH activity in human tissues using more accurate methods it is difficult to assess the similarity of BCAA metabolism in man and rat, but there appears to be no compelling reason to dismiss data from the rat as inapplicable to humans.

Alterations in BCAA metabolism by starvation, disease, and injury.

Dramatic changes in plasma BCAA concentrations are observed under several conditions. In starvation, diabetes mellitus, and after injury the circulating concentrations of BCAA are elevated (4,18,51,139,154). In contrast, plasma BCAA are markedly depressed in hepatic failure, while the concentrations of aromatic amino acids are increased (62). These alterations of plasma BCAA concentrations presumably reflect changes in supply and/or removal. Despite increased plasma BCAA there is evidence for increased BCAA oxidation by skeletal muscle in starvation, diabetes, and trauma (4,9,51). These results seem paradoxical in view of the fact that in short-term starvation, and probably in trauma as well, the source of increased BCAA is thought to be skeletal muscle (34,44). There is some dispute over whether the increased BCAA oxidation observed in vitro in incubated muscles represents the in vivo response to starvation or diabetes. Studies of leucine oxidation in vivo in rats starved for three days however clearly demonstrated that whole body oxidation of leucine was increased (182). In vivo whole body oxidation of leucine was also increased in patients with type I diabetes after insulin withdrawal (154,179) and in starved obese patients (4). Despite increased BCAA oxidation, the leucine clearance

rate in diabetic patients (51) or in normal humans fasted for 3 (169) or 4 (51) days was decreased.

Recent studies from Randle and coworkers (reviewed in 151) found that BCDH activity in kidney and liver from rats was decreased by alloxan-diabetes and 2 days starvation. BCDH activity in heart was unaffected by starvation but increased in diabetes. Earlier studies found no effect of starvation or diabetes on kidney or liver BCDH activity (69,184) but did not take into account changes in the BCDH activator protein which is present in liver and kidney. It was postulated that the decrease in whole body BCDH activity (considering only changes in kidney, liver and heart BCDH activity) in diabetes could explain the increased circulating BCAA in diabetes (151). This explanation may be inadequate though since no consideration was given to possible changes in BCDH activity in tissues such as skeletal muscle or brain or to the observations of increased whole body oxidation of BCAA in insulinopenic diabetes (154,179). Indeed, Brosnan and coworkers have demonstrated increased BCAA clearance by the brain in diabetic rats (20), and determined that the activity state of BCDH in brain is increased by diabetes (21). Further study of protein degradation and the regulation of BCAA disposal, especially in skeletal muscle are required to fully elucidate the factors which contribute to increased BCAA in starvation, diabetes and trauma.

The depressions in plasma BCAA observed in hepatic failure are most likely due to accelerated oxidation by peripheral tissues, as suggested by studies in hepatectomized animals (86,122). Skeletal muscle is a likely site for increased BCAA disposal. Indeed, increased clearance

of BCAA by the leg was observed in patients with decompensated liver cirrhosis (83). The activity of skeletal muscle BCDH in liver failure has not been determined and no factors identified which may cause increased BCAA clearance in hepatic failure.

Effect of diet and meal consumption on BCAA metabolism.

Plasma BCAA increased in parallel with the protein concentration of the diet in rats maintained on diets with protein content varying between 5% and 75% casein; other plasma amino acids remained constant or declined (10,145). These results suggest that BCAA oxidation does not increase proportionately with their dietary intake. This is in contrast to other essential amino acids whose plasma concentrations are essentially constant despite increased intake (145). The adaptive advantage of this response to increased dietary intake of BCAA is unclear since increased amino acid concentrations in general can be toxic. This is certainly so for the BCAA as evidenced by Maple Syrup urine disease, a congenital disease with greatly decreased BCDH activity (42) which results in large increases in plasma BCAA and BCKA and is associated with mental retardation and diminished growth (43). Nevertheless whole body leucine oxidation increased in rats fed diets with increasing protein content (109), and in humans leucine oxidation was increased following a protein meal (87). Curiously, when animals are fed diets containing adequate protein but excesses of leucine, growth rates are diminished and circulating concentrations of valine and isoleucine are depressed (reviewed in 77). The site(s) of increased BCAA oxidation after a protein meal or during increased protein intake is not known, nor is the cause of depressed valine and isoleu-

cine concentrations in the presence of elevated leucine. It is noteworthy however that BCDH activity in liver and kidney was greater in rats fed a normal diet (20% protein) than those fed a 9% protein diet (81,151).

BCAA as regulators of protein turnover.

In incubated diaphragm muscles from rats, leucine inhibited protein degradation and promoted protein synthesis (29,66,127). Similar effects on protein turnover were demonstrated in perfused rat hearts (128); the effect on protein synthesis was thought to involve initiation of translation (128). In fasted rats in vivo, a mixture of BCAA given intraperitoneally after administration of insulin stimulated formation of more polysomes in the psoas muscle than were found after insulin alone (23). In skeletal muscles incubated in vitro, the effects of BCAA on protein synthesis were attributed to a specific, concentration-dependent effect of leucine (31,66,127). The inhibitory effect of BCAA on protein degradation however required metabolism of leucine to α-ketoisocaproate (127,176); proteolysis was inhibited by high concentrations of 3-methylvalerate in one study (127). It remains unclear if the positive effects of BCAA on protein turnover obtain in vivo. In isolated, perfused rat hindlimbs high concentrations of leucine increased protein synthesis in immature rat preparations but not in preparations from adult rats (111). Bolus injection of 0.1 mmol of leucine in vivo had no effect on skeletal muscle protein synthesis by fed, starved or protein-deprived rats (123), while infusion of a mixture of the three BCAA in starved rats stimulated protein synthesis in certain muscles (32). The mechanism(s) of the effects of leucine

(or its metabolites) on protein turnover remain undefined. However, a recent study suggests that glycoprotein synthesis may be involved in the stimulatory effect of leucine on protein synthesis since inhibitors of glycoprotein synthesis abolished the effect of a mixture of BCAA but not of insulin on protein synthesis in diaphragm muscles incubated in vitro (149).

Therapeutic uses of BCAA.

The reported anabolic effects of BCAA coupled with evidence for increased oxidation of BCAA in clinical states such as injury and sepsis (9,51,160) have generated considerable interest in the use of alimentation formulas supplemented with increased rations of BCAA in clinical nutrition. It is thought that increased supply of BCAA may be beneficial in improving nitrogen balance in selected patients, both by supplying additional substrate for oxidation and through their anabolic effects (reviewed in 189). The use of BCAA supplementation in injured or surgical patients is being encouraged despite the incomplete understanding of BCAA metabolism and its regulation and inconclusive and contradictory results in clinical studies.

BCAA catabolism by skeletal muscle.

The BCAA are among a limited number of amino acids which can be oxidized by skeletal muscle (70,115); muscle has substantial capacity for the degradation of BCAA as demonstrated with isolated muscle preparations and perfused rat hindlimbs (reviewed in 77). Furthermore, the apparent oxidation of BCAA by muscle is accelerated in protein catabolic states such as diabetes (26,139), starvation (24,139), trauma (9), and perhaps by exercise (74,110,194,196) as well. The large pool

of inactive BCDH in muscle suggests that increases in the activity state of BCDH may account for accelerated BCAA catabolism observed in the conditions discussed above. Kasperek et al. found that the activity state of muscle BCDH increased markedly during exercise and declined toward the initial value in the postexercise period (100). However, Wagenmakers et al. found only a minimal change in the activity state of muscle BCDH during exercise or starvation (183). Different assays were used in these studies and both are less than ideal for the determination of the activity state of BCDH.

At present there is no method in use which fulfills the "ideal" requirements of sampling the muscle in a manner to "trap" BCDH in its in vivo activity state and from a single muscle sample render BCDH soluble in a form allowing assay of both the in vivo activity and with appropriate manipulation the total (sum of inactive and active) BCDH activity. In the method of Wagenmakers and coworkers (183) BCDH activity was measured in two homogenates of muscle prepared in an iso-osmotic buffer with addition of fluoride and α-chloroisocaproate to the sample used for in vivo activity determination but no effort was made to determine if a transport barrier existed for the BCKA substrate or if there was a difference in the two samples. In the sample containing fluoride (for measurement of "in vivo" activity) the tonicity is altered and the mitochondria are likely to be uncoupled. Hutson has shown that BCKA are transported by a specific transporter whose activity is dependent on the mitochondrial pH gradient (92) and that the rate of BCKA oxidation by muscle mitochondria was dependent on the isolation procedure and media employed (94). The method of Kasperek

and coworkers (100) also utilizes two homogenates, a homogenate for determination of initial activity was prepared with fresh muscle while the homogenate for total BCDH activity was prepared with frozen muscle. The BCDH assays were carried out in the presence of detergent (eliminating the possiblity of a permeability barrier) over a 20 min period. As observed by Hutson (94) assays performed over this time frame can underestimate the true activity, even though the assay appears to be linearly dependent on time.

BCDH activity is generally thought to be rate-limiting for BCAA degradation, since BCAT activity is much greater in skeletal muscle than BCDH activity and BCKA are released by muscle. Nonetheless it is possible that under some conditions flux through BCDH may be limited by BCKA production by BCAT. The BCAT reaction is freely reversible thus its activity in the direction of BCAA --> BCKA is determined by the ratio of products to reactants. Studies of purified BCAT isozyme I, the isozyme present in muscle (99), found that α-ketoglutarate and BCKA are the preferred amino group acceptors in the BCAA --> BCKA reaction (174). In view of the the concentrations of the available substrates and their affinities for the BCAT enzyme, it was suggested that α-ketoglutarate is the amino group acceptor for 95% of BCAA transamination in skeletal muscle (131). However in diabetes the glutamate/α-ketoglutarate ratio was increased in diaphragm muscles (30), which could slow tansamination of BCAA due to a relative deficiency of amino group acceptor. This raises the possibility that in muscles from diabetic animals flux through BCDH may be restrained by the rate of transamination.

Despite the potential importance of skeletal muscle in the whole body metabolism of BCAA there is little information on the activity state of BCDH, which may be the rate-limiting enzyme for muscle BCAA catabolism, especially under conditions of altered BCAA metabolism. Furthermore since muscle is likely the principal source of circulating BCKA to be metabolized by other tissues, information on factors regulating flux through BCAT is important to understanding the degree of interorgan cooperativity in BCAA metabolism. The experiments detailed in the following chapters will address: 1) the role of transamination in increased BCAA oxidation by muscle in diabetes and fasting; 2) the activity state of BCDH in skeletal muscle; and 3) alterations of BCDH activity in skeletal muscle under physiolosgical and pathological conditions.

Chapter II

Catabolism of Branched-Chain Amino Acids by Diaphragm Muscles of Fasted and Diabetic Rats

The contents of this chapter have been previously published in similar form in Metabolism 34: 702-711, 1985.

Summary

In vitro catabolism of branched-chain amino acids, leucine and valine, was investigated using diaphragm muscles from normal, streptozotocin-diabetic and overnight fasted rats. Oxidation and transamination of [1-^{14}C]branched-chain amino acids were both stimulated to a similar extent by diabetes or fasting, when diaphragms were incubated with glucose. Transamination of leucine and valine was increased when diaphragms were incubated with pyruvate; stimulation of transamination was greatest in diaphragms from diabetic rats. Leucine and valine oxidation by control diaphragms was inhibited by pyruvate while it was unchanged or slightly stimulated in diaphragms from fasted or diabetic rats. Thus diaphragms from diabetic rats oxidized 2-3 fold more branched-chain amino acids than controls when they were incubated with pyruvate. The specific radioactivity of extracellular α-ketoisocaproate (KIC*; the product of leucine transamination) produced by diaphragms incubated with [1-^{14}C]leucine was similar for all groups (fed, fasted or diabetic) in the presence or absence of pyruvate. Oxidation of [1-^{14}C]α-KIC by diaphragms from fasted or diabetic rats, incubated with glucose, was the same or less than KIC oxidation by control diaphragms. Incubation with pyruvate inhibited KIC oxidation by control diaphragms to a significantly greater degree than that by diaphragms from diabetic or fasted rats. These data suggest: 1) Flux through branched-chain amino acid transaminase is limited by the availability of amino group acceptors in diaphragms from normal and

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* Abbreviations used in this chapter are: KIC, α-ketoisocaproate; HPLC, high-performance liquid chromatography.

overnight fasted rats, and to a greater extent in diaphragms from diabetic rats. 2) Flux through the transaminase may be a major determinant of accelerated branched-chain amino acid oxidation by diaphragms in fasting and diabetes. 3) In diaphragms of fasted and diabetic rats, flux through the branched-chain α-ketoacid dehydrogenase complex is resistant to inhibition by pyruvate, which is normally observed in controls.

Introduction

Unlike most essential amino acids, the branched-chain amino acids, leucine, valine, and isoleucine, are catabolized extensively by skeletal muscle (126,78). Oxidation of the branched-chain amino acids in skeletal muscle is subject to metabolite (28,139) and hormonal control (24,93). In fasting or uncompensated diabetes mellitus, the intracellular concentrations of branched-chain amino acids in skeletal muscle are increased (91,112), despite apparent acceleration of their oxidation under the same conditions (24,27,70,139).

The initial steps in the catabolism of the branched-chain amino acids are a reversible transamination to the corresponding branched-chain α-ketoacid (95) followed by irreversible decarboxylation of the α-ketoacid catalyzed by branched-chain α-ketoacid dehydrogenase complex (131). Skeletal muscle has high levels of branched-chain amino acid transaminase activity but relatively low levels of branched-chain α-ketoacid dehydrogenase activity (131,170). It has therefore been suggested that the activity of the branched-chain α-ketoacid dehydrogenase complex is rate-limiting for the catabolism of the branched-chain amino acids by skeletal muscle (28,90,131,170). The activity of this

enzyme complex can be regulated by the availability of co-factors (NAD, CoA), feed-back inhibition by the products (136,146) and by an ATP-dependent phosphorylation-dephosphorylation cycle (57,143). Recent evidence suggests that the complex is in a more activated state in mitochondria prepared from muscles of fasted or diabetic rats than from fed rats (140). In muscles of diabetic or fasted rats, the glutamate/α-ketoglutarate ratio is increased (30,159). This may limit the rate of transamination of the branched-chain amino acids since α-ketoglutarate is the most effective amino group acceptor for transamination (131). Flux through the branched-chain α-ketoacid dehydrogenase complex may also be limited by substrate availability since the intracellular concentrations of branched-chain α-keto acids are less than saturating for the activity of the complex (131,136,146).

In the experiments described here we investigated the role of the availability of amino group acceptors in regulating the flux of the branched-chain amino acid carbon skeleton through the branched chain α-ketoacid dehydrogenase complex. Pyruvate was added to stimulate the formation of α-ketoglutarate through the coupled reaction of alanine aminotransferase (84). Furthermore, we investigated whether in intact diaphragms the diabetes-induced stimulation of branched-chain amino acid oxidation is primarily a function of accelerated transamination or stimulated decarboxylation of the branched-chain α-ketoacids.

Materials and Methods

Animal care. Male Wistar WI(BR) rats (Charles River Laboratories, Wilmington, MA) weighing 80-100 g were used in these studies. Fasted rats were without food overnight (18 hrs). All other animals were

allowed access to standard laboratory chow (Wayne Rodent Blox), and water ad lib. Diabetes was induced by intraperitoneal injection of streptozotocin (85 mg/kg) after an overnight fast. Rats were considered diabetic if their plasma glucose was greater than 300 mg/dl and they gained less than 1 g per day in weight. Diabetic animals were sacrificed 5 to 7 days after induction of diabetes.

Experimental. Rats were sacrificed by decapitation and their diaphragms dissected, weighed and incubated as described previously (26). Quarter diaphragms (30-50 mg wet weight), were incubated in the balanced salt solution of Gey and Gey (68) equilibrated with O_2/CO_2 (95:5,v/v) pH 7.4 at 37°C, containing a complete amino acid mixture at concentrations found in plasma of normal, ad lib fed rats (114); thus the branched-chain amino acids were always present at physiological concentrations, 0.17, 0.2 and 0.1 mM for leucine, valine and isoleucine respectively. Quarter diaphragms were preincubated 30 min in 2 ml of the appropriate medium, then gently blotted and transferred to a vessel containing 2 ml of fresh medium in which branched-chain amino or α-ketoacid catabolism was assessed over the following 60 min. In experiments where α-ketoisocaproate (KIC; 4-methyl-2-oxopentanoate; the ketoanalogue of leucine) served as the oxidative substrate, the complete amino acid mixture was not included during incubation or preincubation except where indicated in results.

Oxidation and transamination of valine and leucine were determined by including [1-^{14}C]L-valine or [1-^{14}C]L-leucine (0.02 mCi/l) in the incubation medium. Following incubation 0.3 ml Hyamine hydrochloride was injected into small cups suspended over the medium to trap $^{14}CO_2$,

and 0.3 ml 20% perchloric acid was injected into the medium to release $^{14}CO_2$ from bicarbonate, to quantitate oxidation (decarboxylation) of the amino acid. Free branched-chain α-keto[1-^{14}C]acid release into the medium was estimated by suspending a fresh Hyamine hydroxide containing cup over the medium and injecting 0.3 ml 30% H_2O_2 into the medium, the latter results in oxidative decarboxylation of α-ketoacids but not 2-amino acids (131). After each addition of Hyamine hydroxide incubation was continued for 1 hr at 37°C allow complete trapping of $^{14}CO_2$. $^{14}CO_2$ trapped in Hyamine hydroxide was measured in a Beckman LS 7000 scintillation spectrometer and corrected to 75% counting efficiency by automatic external standardization. Samples were counted for 10 min or until sufficient counts were accumulated to reduce counting error to less than 2%. In typical one hour incubations in glucose containing medium, quarter diaphragms released about 1000 cpm from [1-^{14}C]leucine and about 900 cpm were released after H_2O_2 treatment. Background radioactivity from incubations in the absence of tissue were typically less than 40 cpm and less than 120 cpm, respectively. Oxidation and transamination rates were calculated based on the initial specific radioactivity of the substrate in the medium and related to the wet weight of the diaphragm determined before incubation. Total transamination was calculated as the sum of $^{14}CO_2$ released after acid and after H_2O_2 treatment. These calculations underestimate the absolute rates of oxidation, transamination and KIC release, since they do not take into account tracer dilution by intracellular substrate. The isotopic measurements are presented in this manner because the specific radioactivities of the precursor pools were not determined in all experi-

ments. However, studies where the specific radioactivity of KIC released by the diaphragm and that of intracellular leucine were measured, we found no evidence for differences in the specific radioactivity of the precursor pools among treatment groups (see Results and Discussion).

Initial experiments established that oxidation and transamination of [1-^{14}C]leucine by diaphragms from normal or diabetic rats were essentially linear when incubated up to 120 min. Additionally, after 30 min of preincubation, oxidation and transamination rates were constant during each of three successive 30 min incubation periods suggesting that isotopic steady-state was achieved and maintained over this time period. Oxidation and transamination of leucine were also found to be independent of glucose concentration when 5.5 or 16.5 mM glucose was included in the incubation medium.

In experiments to determine the specific radioactivities of leucine and KIC, hemidiaphragms were incubated in 2.5 ml medium as described above, supplemented with 0.1 mM [^{3}H]mannitol (0.5 mCi/l). The specific radioactivity of leucine in the medium was increased to 0.1 mCi/0.17 mmol/l. Diaphragms were incubated 30 or 60 min, the tissues removed, frozen between two blocks of dry ice and 0.5 ml of 25% (w/v) trichloroacetic acid was added to the media. The acidified medium samples were frozen at -20°C until analysis. Leucine was extracted by homogenizing the frozen tissue in 3 ml/g ice-cold 5% trichloroacetic acid, the sample was centrifuged at 12000xg for 15 min and the supernatant frozen at -20°C until analysis. Specific radioactivities of leucine and α-ketoisocaproate were determined using high performance liquid

chromatography (HPLC) to separate and quantitate the compounds; the corresponding eluate fractions were collected and radioactivity determined by scintillation counting. The equipment used consisted of a Perkin-Elmer Series 10 liquid chromatograph, LC10 fluorescence detector (370nm excitation, >418nm emission filter), LCI-100 integrator, and "3x3"C18 reversed phase column preceded by a 2cmx2.1mm i.d. precolumn containing C18 reversed phase packing. The derivatization and separation methods for leucine and α-ketoisocaproate were adapted from Wassner and Li (190) and Kieber and Mopper (103), respectively. Briefly, for leucine the o-phthalaldehyde derivative was prepared and immediately chromatographed using a solvent program of 3 min isocratic with 76% 0.05 M Na-citrate, pH 5.6/12% acetonitrile/12% 2-propanol, a linear gradient to 50% Na-citrate/25% acetonitrile/25% 2-propanol over 1 min, 1.5 min isocratic at this composition followed by a linear gradient to the initial solvent composition over 2 min. Leucine eluted at 4.5 min and was quantitated by comparison to an external standard. KIC was determined as the quinoxalinol derivative prepared using o-phenylenediamine. The solvent program consisted of 3 min isocratic with 75% Na-citrate, pH 5.6/15% acetonitrile/10% 2-propanol followed by a linear gradient to 10% Na-citrate/ 80% acetonitrile/10% 2-propanol over 3 min; this condition was maintained isocratic for 0.5 min then a linear gradient (2 min) to the starting conditions was run. KIC eluted at 3.7 min and was quantitated using an external standard. Preliminary experiments established that recovery of radiolabelled leucine or KIC was quantitative using these methods. Incubation with muscle did not significantly alter the concentration or specific radioactivity of the

medium leucine; KIC concentrations in the medium ranged from 0.002 to 0.012 mM with the limit of detectability being 0.0005 mM. In calculations of intracellular leucine specific radioactivity, it was assumed that the medium was at equilibrium with the extracellular space in which mannitol and extracellular leucine were distributed identically.

Oxidation of KIC (0.01-0.50 mM) was determined using α-keto[1-^{14}C] isocaproate (0.02 mCi/l) prepared from [1-^{14}C]leucine as described previously (120). Oxidation of glucose was determined using [U-^{14}C]-glucose. $^{14}CO_2$ was trapped as described above and calculations were based on the initial specific radioactivity in the medium.

Sources of Materials. Radioactive amino acids, glucose and mannitol were from New England Nuclear (Boston, MA) or ICN Chemical and Radioisotope Division (Irvine, CA). Sodium α-ketoisocaproate was from Aldrich Chemical Company, Milwaukee, WI. Sodium pyruvate was from Boehringer Mannheim, Indianapolis, IN. L-amino acids,aminooxyacetic acid, and C18 reversed-phase column packing were from Sigma Chemical Company, St. Louis, MO; Hyamine hydroxide from Research Products International, Mount Prospect, IL. Streptozotocin was kindly supplied by Dr. William Dulin of Upjohn Research Laboratories, Kalamazoo, MI. HPLC grade solvents, and other chemicals were from Fisher Scientific, Norcross, GA.

Statistics. Tabulated values are means ± S.E.M. Differences between mean values were determined by Student's t test (two-way). Differences between ratios were determined by a Mann-Whitney rank order test. Multiple comparison analyses were performed using the Peritz F test (80). Significance was assigned at $p < 0.05$.

Results

Catabolism of leucine and valine by diaphragms of normal and diabetic rats.

Diaphragms from diabetic rats, incubated in glucose containing medium, produced 25-50% more $^{14}CO_2$ from 0.17mM [1-^{14}C]leucine than did control diaphragms (Table 1). Total transamination by diabetic diaphragms incubated in glucose was 33% greater than transamination by normal diaphragms (Table 1). The ratio of leucine oxidation to total transamination, in glucose containing medium, was the same (about 65%) for diaphragms from normal or diabetic rats (Table 1). Using 0.20 mM [1-^{14}C]valine, $^{14}CO_2$ production by control or diabetic diaphragms was similar while transamination by diabetic diaphragms was 30% greater than by control diaphragms when both were incubated in glucose containing medium (Table 1).

Effects of pyruvate on the catabolism of leucine and valine.

If the rate of leucine transamination is limited by the glutamate /α-ketoglutarate ratio then addition of pyruvate would be expected to stimulate leucine transamination by its effect on glutamate-pyruvate aminotransferase. In pyruvate containing medium (Table 1) transamination of leucine was increased 60% in normal diaphragms and 110% in diabetic diaphragms when compared to glucose containing medium. $^{14}CO_2$ production from leucine by normal diaphragms was inhibited about 40% in pyruvate containing medium while in diabetic diaphragms it was stimulated by about 40%. The ratio of oxidation/transamination decreased in the presence of pyruvate in the control and the diabetic group, but less in the latter. Thus, when incubated with pyruvate,

diaphragms from diabetic rats transaminated 80% more leucine and oxidized 200% more leucine than the controls (Table 1). The effects of pyruvate on oxidation and transamination of valine were similar to those observed with leucine but the magnitude of the effects was smaller (Table 1).

An 18-hour fast affected leucine metabolism similarly to diabetes. When diaphragms were incubated in glucose containing medium, fasting increased leucine transamination more than $^{14}CO_2$ production from leucine (Table 1). The effect of pyruvate on leucine catabolism by diaphragms from fasted rats was qualitatively similar to that observed in diaphragms from diabetic rats (Table 1). Incubation with pyruvate stimulated transamination of leucine by fasted diaphragms 60% compared to glucose containing medium; oxidation of leucine was stimulated slightly and the amount of α-ketoacid released was increased more than 2-fold. Diaphragms from fasted rats therefore oxidized only 30% more leucine than fed controls during incubation with glucose, while they oxidized 100% more than the controls when incubated in pyruvate.

To assess possible effects of fasting, diabetes and pyruvate on the specific radioactivity of the intracellular leucine pool available for transamination and subsequent oxidation, the ratio of the specific radioactivities of extracellular KIC and extracellular leucine was determined. The specific radioactivity of extracellular KIC in perfused rat hindlimbs (93) and of plasma KIC in rats infused with leucine (109) was previously found to closely approximate the intracellular specific radioactivity of leucine in muscles. As shown in Table 2, although the specific radioactivity of extracellular KIC was less

than the specific radioactivity of the extracellular leucine pool (range 0.49 to 0.64 of leucine specific radioactivity) there was no significant difference after 30 or 60 min incubation between any of the groups.

KIC release by incubated diaphragms was calculated from direct measurements (by HPLC) of KIC concentrations in the medium (Table 2). The values for KIC release over 60 min are about 2-fold higher than those estimated radioisotopically based on the specific radioactivity of leucine in the medium (Table 1). This is consistent with the value of 0.57 ± 0.02 (overall mean for 65 observations) obtained for the ratio of extracellular KIC to extracellular leucine specific radioactivities.

In experiments of identical design to those in Table 2, the specific radioactivity of intracellular leucine was measured; multiple comparison analysis of the ratios of the specific radioactivities of intracellular and extracellular leucine found no significant difference between the values obtained from diaphragms of fed, fasted or diabetic rats incubated with glucose or glucose + pyruvate, nor were there differences between measurements obtained after 30 or 60 min incubation. The overall mean of the ratios of the specific radioactivities of intracellular and extracellular leucine was 0.59 ± 0.04 (n = 73) which is not different from the ratio of the specific radioactivities of extracellular KIC and extracellular leucine (0.57 ± 0.02).

Oxidation and transamination of valine at normal physiological concentration by diaphragms of normal fed rats, when incubated in lactate or a mixture of pyruvate and lactate (1.1 mM and 4.4 mM,

respectively), were similar to the values obtained with diaphragms incubated in equimolar glucose (Table 3). Compared to glucose containing medium, the ratio of oxidation to transamination was decreased in the presence of lactate (0.64) and was lowest in the pyruvate-lactate mixture (0.56). These values, however, are still much greater than the ratio of oxidation to transamination (0.30) observed with medium containing 5.5 mM pyruvate (Table 1).

Effect of pyruvate on oxidation of α-keto-[1-^{14}C]isocaproate.

Since the data (Table 1) suggested that pyruvate may have a differential effect on the oxidation of branched chain α-ketoacids by diaphragms of normal fed and fasted or diabetic rats, the effect of pyruvate on $^{14}CO_2$ production from [1-^{14}C]KIC was studied (Table 4). Values for KIC oxidation using 0.01 or 0.05 mM KIC (near physiological concentrations; 91) should estimate flux through the branched-chain α-ketoacid dehydrogenase complex while oxidation using 0.5mM KIC (close to the saturating concentration for oxidation of KIC by rat diaphragm; 186) may serve as an indicator of enzyme activity, if it can be assumed that enzyme activity rather than transport limits oxidation. In glucose containing medium, $14CO_2$ production by diaphragms of fasted or diabetic rats tended to be lower than that by muscles of fed controls, regardless of the concentration of KIC, although there was no significant difference between groups in some experiments (Tables 4-6). Diaphragms from fed controls incubated in pyruvate or glucose + pyruvate oxidized 42-67% less KIC than those incubated with glucose, at all KIC concentrations tested (0.01-0.5 mM). In diaphragms from diabetic and fasted rats, pyruvate inhibited the oxidation of KIC

significantly less than in controls. At the highest KIC concentration tested (0.5 mM), $^{14}CO_2$ production by diabetic diaphragms was identical in glucose or in pyruvate while at lower concentrations of KIC, pyruvate caused slight inhibition of $^{14}CO_2$ production by diaphragms of diabetic or fasted rats in some experiments. During incubation with pyruvate, diaphragms from diabetic and fasted rats oxidized 40-80% more KIC than controls when this substrate was at low, physiological concentrations (0.01-0.05 mM), but oxidation rates were identical in the two groups at 0.5 mM concentrations of the branched-chain α-ketoacid. Inclusion of normal physiological concentrations of all the amino acids did not affect KIC oxidation significantly under any condition tested.

Since the estimates of KIC oxidation could be biased by dilution of the ^{14}C-labelled substrate with unlabelled KIC produced by transamination of endogenous leucine, $^{14}CO_2$ production from 0.05 mM [1-^{14}C]KIC was measured in the presence of aminooxyacetate (Table 5), an inhibitor of aminotransferases (88). These experiments were conducted using 0.05 mM KIC in order to assess oxidation at near physiologic substrate concentration and to avoid activation of the branched-chain α-ketoacid dehydrogenase which has been observed with higher concentrations (0.5 mM) of KIC (33,193). In glucose containing medium, $^{14}CO_2$ production by diaphragms of normal or diabetic rats was similar and was stimulated by about 80% in the presence of aminooxyacetate. Pyruvate caused about 65% inhibition of $^{14}CO_2$ production in control diaphragms and significantly less (about 35%) in diabetic diaphragms. $^{14}CO_2$ production in pyruvate containing medium was

slightly stimulated (about 30%) in the presence of aminooxyacetate but this effect was not significant in diaphragms from either control or diabetic rats.

The activity of branched-chain α-ketoacid dehydrogenase complex in several tissues (e.g. heart, muscle, liver, kidney, and adipose tissue) is regulated by an ATP-dependent phosphorylation-dephosphorylation cycle (for review see 150), therefore the effect of the uncoupler 2,4-dinitrophenol on $^{14}CO_2$ production from 0.05 mM KIC was tested (Table 6). Addition of dinitrophenol stimulated $^{14}CO_2$ production from KIC by 65% in diaphragms of fed rats regardless of the presence of pyruvate and in diaphragms of fasted rats when incubated in glucose alone. In the presence of pyruvate, addition of dinitrophenol stimulated $^{14}CO_2$ production in fasted diaphragms by 37%. The relative inhibition of $^{14}CO_2$ production by pyruvate in diaphragms of fed rats (about 60%) was constant in the presence or absence of dinitrophenol. The inhibitory effect of pyruvate in diaphragms of fasted rats was 30% in the absence of dinitrophenol and 40% in its presence. In parallel experiments this concentration of dinitrophenol was found to stimulate oxidation of [U-^{14}C]glucose ten-fold in fasted and control diaphragms (data not shown), indicating that 0.1 mM dinitrophenol uncoupled respiration in the diaphragms.

Discussion

These data are in agreement with previous studies showing that apparent oxidation of leucine by skeletal muscle is increased in diabetes and fasting (27,70,139). Transamination of leucine was increased in diaphragms from diabetic and fasted rats despite the

increased glutamate/α-ketoglutarate ratio (30,159). Since the proportion of available transamination product of leucine which was oxidized was unchanged in diabetes and fasting, the amount of free α-keto acid available for export was elevated in fasting and diabetes. The studies of Hutson et al. (93) in perfused hindlimbs of rats, found increased branched chain α-keto acid export in fasting.

The data presented here and studies of cultured myocytes (167) indicate that the intracellular specific radioactivity of leucine is less than the specific radioactivity in the medium. We found no evidence to suggest differences in the dilution of labelled substrates by intracellular pools among diaphragms from fed, fasted or diabetic rats or that pyruvate affected leucine distribution. In diaphragms incubated without insulin, protein synthesis is depressed and net protein degradation prevails. In two studies from our laboratory, net protein degradation by diaphragms from diabetic rats incubated without insulin was found to be the same as that by diaphragms from normal rats when determined as tyrosine release (30) or slightly increased when determined as leucine release (27). The intracellular concentration of leucine in diaphragms from normal and diabetic rats was the same after preincubation as was the specific radioactivity of intracellular leucine in diaphragms incubated with [1-^{14}C]leucine, in agreement with the present study. Based on values for leucine release by incubated diaphragms previously obtained in our laboratory (27), the relative dilution of the medium leucine by leucine released from quarter diaphragms would be negligible and possible differences in the release of leucine/mg tissue would be offset by the diminished weight (about

20% less) of the diaphragms obtained from diabetic rats. Thus we would not expect the results to be biased by dilution of the medium branched-chain amino acid pool with endogenous branched-chain amino acids supplied by protein degradation. Since the intracellular specific radioactivity of leucine was lower than that of the medium, the values shown in the Tables, based on the specific radioactivity of leucine in the medium, underestimate the actual rates of leucine transamination and oxidation. This is further supported by the finding that KIC release by diaphragms was greater in the experiments where it was measured directly by HPLC (Table 2), than in experiments where it was estimated radioisotopically (Table 1). Qualitatively the effects of pyruvate, fasting and diabetes on KIC release were identical, using either method of analysis. Since neither fasting nor diabetes, nor the presence of pyruvate or glucose affected the specific radioactivity of released KIC or of intracellular leucine, the relative changes observed in transamination and oxidation in response to the experimental manipulations seem valid. Since substrate specific radioactivities were not measured in all experiments, for consistency, the values for oxidation and transamination were not corrected but were based on the medium specific radioactivity of the added substrate.

The marked stimulation of leucine and valine transamination when diaphragms were incubated with 5.5 mM pyruvate suggests that transamination of the branched-chain amino acids is restrained by the availability of endogenous amino group acceptors. In the presence of glucose or when excess amino group acceptors were provided, intact diaphragms from diabetic and from fasted rats transaminated significantly more

leucine than those of fed controls, suggesting that the amount or activity of the transaminase is increased under these conditions; the effect of diabetes was greater than that of the short fast. Adibi et al. (3) reported that leucine aminotransferase activity in muscle homogenates increased with the duration of fasting. Our data suggest that increased flux through the transaminase resulting from increased transaminase activity and/or elevated branched-chain amino acid concentrations may be a major determinant of accelerated branched-chain amino acid oxidation in fasting or diabetes, due to increased substrate supply to the branched-chain α-ketoacid dehydrogenase complex.

The measurements of KIC oxidation may be complicated by differences between the medium and intracellular specific radioactivities. It seems likely that the relative values of KIC oxidation are valid since the presence of physiological concentrations of exogenous leucine, which readily enters the intracellular KIC pool, did not significantly affect the apparent KIC oxidation (Table 4). As for leucine, at low KIC concentrations, the actual oxidation rates are probably greater than those estimated from the extracellular specific radioactivity. At high concentrations of KIC in the medium, the contribution of intracellular leucine to the KIC pool was presumably negligible. The increased $^{14}CO_2$ production from [1-^{14}C]KIC in the presence of aminooxyacetate also suggests that intracellular KIC specific radioactivity was lower than that of KIC in the medium (at low KIC concentration), presumably due to dilution by KIC arising from endogenous leucine. Alternative mechanisms which may contribute to the observed stimulation of $^{14}CO_2$ production include decreased flux of intracellular KIC (both ^{12}C and

^{14}C) into the intracellular leucine pool (by inhibiting reverse transamination) as well as metabolic effects other than inhibition of branched-chain amino acid transaminase; e.g. blockade of the malate-aspartate shuttle altering the intramitochondrial redox potential (164).

Wagenmakers and Veerkamp (187) have noted a differential effect of aminooxyacetate on oxidation of KIC by diaphragms of normal rats and rats fasted for 3 days. They suggest that estimates of KIC oxidation in diaphragms from fasted rats are greater than the true rate of KIC oxidation due to intracellular compartmentation of the precursor pool. As regards the present study, Wagenmakers and Veerkamp did not demonstrate a differential effect of aminooxyacetate on KIC oxidation by diaphragms from rats fasted for one day and the magnitude of increase in oxidation and transamination of leucine in the diaphragms from one-day fasted rats were similar to our observations in overnight fasted rats (Table 1). Furthermore, the apparent stimulation of KIC oxidation by aminooxyacetate in diaphragms from normal or diabetic rats was similar in the present studies. Thus we have no evidence to suggest that our estimates of KIC oxidation are biased by intracellular compartmentation.

In our studies, during incubation with glucose, oxidation of physiological concentrations of KIC by diaphragms from fasted or diabetic rats was not increased and at the highest KIC concentration tested (0.5 mM, which may activate the branched-chain α-ketoacid dehydrogenase complex (193) but minimizes pool dilution), $^{14}CO_2$ production by diabetic diaphragms was significantly less than that by

normals. Thus in intact diaphragms, incubated with glucose, we were unable to obtain evidence for increased flux through the branched-chain α-ketoacid dehydrogenase complex associated with diabetes, at any KIC concentration tested. Alterations of KIC transport across the cell membrane or into the mitochondria or changes in the level of cytoplasmic inhibitor(s) of branched-chain α-ketoacid dehydrogenase complex (131,141) may have prevented a reflection of altered enzyme activation state in flux measurements.

It has recently been reported that the activation state of the branched-chain α-ketoacid dehydrogenase in diaphragms is increased during incubation (183) with a concomitant decrease in the total amount of complex present in the muscle. The present studies have not determined the activation state of the branched-chain α-ketoacid dehydrogenase complex or total enzyme activity; if there were any changes during incubation, they were not reflected in flux measurements, which were linear over the time period investigated.

The inhibitory effect of pyruvate on leucine oxidation in muscle and its resistance to inhibition by pyruvate in diaphragms of diabetic rats has previously been observed in our laboratory (27,28). Pyruvate induced inhibition is not mediated by increased α-ketoglutarate since previous studies found that leucine oxidation was stimulated when diaphragms were incubated in vitro with 1-5 mM α-ketoglutarate (28). The present data suggest that in diabetes or fasting the branched-chain α-ketoacid dehydrogenase complex is resistant to pyruvate induced inhibition. Studies of perfused hearts (193) and isolated heart mitochondria (22) indicate that addition of pyruvate results in a

modification of branched-chain α-ketoacid dehydrogenase which persists when the complex is solubilized and reduces its activity. The inability of dinitrophenol to prevent inhibition of KIC oxidation by pyruvate suggests that pyruvate induced inhibition may not be mediated solely by ATP-dependent phosphorylation of branched-chain α-ketoacid dehydrogenase (140). The apparent resistance of branched-chain α-ketoacid dehydrogenase to inhibition by pyruvate in muscles of diabetic and fasted rats may be a reflection of increased activation state of the enzyme (140), although we have no direct evidence for such activation. The mechanism causing pyruvate resistance is not clear. The role of pyruvate in regulating branched-chain amino acid catabolism under physiological and pathological conditions, i.e. exercise and diabetes, needs further evaluation, since the concentration of pyruvate in muscle during exercise can attain the concentrations used in these studies (50).

Our data emphasize the complexity of regulation of branched-chain amino acid catabolism. Earlier studies implicated the branched-chain α-keto acid dehydrogenase complex as rate-limiting for branched-chain amino acid degradation in muscle (131,170). The present results suggest that under certain conditions flux through either the branched-chain amino acid aminotransferase or through branched-chain α-ketoacid dehydrogenase complex can limit their rate of degradation. Furthermore, it is remarkable that despite the increased NADH/NAD and acyl CoA/CoASH ratios in diabetes and fasting (71,75) the apparent flux of leucine carbons through the branched-chain α-ketoacid dehydrogenase complex is not inhibited but accelerated. The enzyme complex is

largely in the inactive (phosphorylated) state in skeletal muscle from normal or fasted rats (137,184), but direct measurements of the phosphorylation state of the complex in muscles from diabetic animals remains to be accomplished (137,150). At present it is unclear whether the effects of diabetes and fasting on branched-chain amino acid catabolism result from insulinopenia and/or alterations in the metabolic milieu; addition of insulin to diaphragms of diabetic rats incubated in vitro does not reduce leucine oxidation (27). In this context it is noteworthy that a factor which activates pyruvate dehydrogenase kinase is present in heart mitochondria prepared from diabetic or fasted rats thus rendering the pyruvate dehydrogenase complex refractory to activation by pyruvate (89,101). Further studies are needed to elucidate how diabetes and fasting prevent pyruvate induced inhibition of branched-chain α-keto acid oxidation, e.g. is mitochondrial transport of pyruvate inhibited (104) or is the branched-chain α-ketoacid dehydrogenase complex or one of its modifiers affected.

Table 1 Legend.

Quarter diaphragms were incubated in medium containing 5.5mM glucose or 5.5mM pyruvate; oxidation and transamination of either 0.17mM [1-^{14}C]leucine or 0.20mM [1-^{14}C]valine were determined by $^{14}CO_2$ release as described in the Experimental section. Comparisons for diaphragms of diabetic and fasted rats were made to fed control values obtained with concurrently incubated diaphragms rather than the pooled means which are shown.

* All values were calculated based on the initial specific radioactivity of the amino acid substrate in the medium and are underestimates, since the intracellular specific radioactivity was uniformly lower than in the medium (see Table 2 and Results). [a] $p < 0.01$ vs glucose medium; [b] $p < 0.05$ vs. control rats; [c] $p < 0.001$ vs. control rats; [d] $p < 0.05$ vs. glucose.

Table 1. Catabolism of leucine and valine by diaphragms from fed or fasted normal rats and from fed diabetic rats.

Group	Medium	pmol* $^{14}CO_2$ / hr per mg wet weight Acid Release "Oxidation"	H_2O_2 Release "Free α-ketoacid"	Total "Transamination"	Oxidation / Transamination	n
			Leucine			
Fed	Glucose	160 ± 8	87 ± 6	247 ± 10	0.65 ± 0.02	21
	Pyruvate	92 ± 6[a]	304 ± 20[a]	396 ± 20[a]	0.24 ± 0.03	13
Diabetic	Glucose	202 ± 11[b]	126 ± 11[c]	328 ± 16[c]	0.62 ± 0.03	15
	Pyruvate	208 ± 19[d,c]	408 ± 24[a,b]	688 ± 29[a,c]	0.41 ± 0.02	9
Fasted	Glucose	176 ± 19[b]	127 ± 15[b]	304 ± 27[b]	0.58 ± 0.04	5
	Pyruvate	193 ± 13[c]	285 ± 34[a]	477 ± 31[a,b]	0.41 ± 0.04	5
			Valine			
Fed	Glucose	135 ± 11	82 ± 8	216 ± 16	0.62 ± 0.03	10
	Pyruvate	93 ± 7[d]	222 ± 16[a]	314 ± 20[a]	0.30 ± 0.02	10
Diabetic	Glucose	153 ± 11	126 ± 16[a]	279 ± 22[b]	0.56 ± 0.04	6
	Pyruvate	185 ± 23[c]	339 ± 37[a,b]	524 ± 35[a,c]	0.36 ± 0.04	6

Table 2. Specific radioactivity of extracellular α-ketoisocaproate released by diaphragms incubated with leucine.

Group	Medium	KIC Specific Radioactivity / Leu Specific Radioactivity 30 min	60 min	KIC Release pmol/60 min per mg wet weight
Fed	Glucose	0.49 ± 0.04 (7)	0.61 ± 0.03 (8)	187 ± 13 (8)
	Pyruvate	0.62 ± 0.08 (8)	0.62 ± 0.06 (7)	509 ± 45[a] (7)
Diabetic	Glucose	0.56 ± 0.10 (5)	0.52 ± 0.09 (4)	254 ± 17[b] (4)
	Pyruvate	0.59 ± 0.03 (5)	0.55 ± 0.03 (3)	800 ± 40[a,b] (4)
Fasted	Glucose	0.56 ± 0.11 (5)	0.55 ± 0.06 (4)	255 ± 11[b] (4)
	Pyruvate	0.48 ± 0.04 (5)	0.67 ± 0.06 (4)	507 ± 60[a] (4)

Hemidiaphragms were incubated in medium containing 5.5 mM glucose or 5.5 mM pyruvate with 0.17 mM [1-^{14}C]leucine. After 30 min or 60 min incubation diaphragms were removed from the medium and quickly frozen. The concentrations of leucine and KIC in the medium (extracellular) were determined by HPLC and the specific radioactivities calculated as described in the Experimental section. [a] $p < 0.01$ vs glucose; [b] $p < 0.05$ vs control.

Table 3. Effect of lactate and a mixture of lactate and pyruvate on the catabolism of valine by diaphragms from fed rats.

Medium	pmol* $^{14}CO_2$/hr per mg wet weight		Oxidation / Transamination
	Oxidation	Transamination	
Glucose	151 ± 7	218 ± 12	0.69 ± 0.01
Lactate	165 ± 17	257 ± 22	0.64 ± 0.02[a]
Pyruvate + Lactate	135 ± 14	241 ± 24	0.56 ± 0.02[b,c]

Quarter diaphragms were incubated in medium with 5.5mM glucose or 5.5mM lactate or 1.1mM pyruvate and 4.4mM lactate. Oxidation and transamination of 0.20mM [1-^{14}C]valine was determined by $^{14}CO_2$ release as described in the Experimental section. Values shown are the means of five observations.

* Values were calculated as in Table 1 based on the initial specific radioactivity of valine in the medium.

[a] $p < 0.05$ versus glucose medium

[b] $p < 0.01$ versus glucose medium

[c] $p < 0.025$ versus lactate medium

Table 4 Legend.

$^{14}CO_2$ production from α-keto[1-^{14}C]isocaproate by quarter diaphragms incubated in 5.5mM glucose was compared to $^{14}CO_2$ production during incubation with 5 mM pyruvate (in experiments with diabetic rats) or 5.5 mM glucose + 5 mM pyruvate in experiments with fasted rats. Fed controls were studied in both series; since the effects of pyruvate in the presence or absence of glucose were identical, the data in fed controls were pooled. Incubations included normal physiological concentrations of all the serum amino acids (86) where noted. * Values were calculated as in Table 1 based on the specific radioactivity of KIC in the medium.

[a] $p < 0.005$ versus glucose; [b] $p < 0.05$ versus glucose; [c] $p < 0.05$ versus fed rats.

Table 4. Oxidation of α-ketoisocaproate by diaphragms from fed, fasted and diabetic rats.

Group	[1-^{14}C]α-KIC mM	Amino Acids	pmol* $^{14}CO_2$ / hr per mg wet weight Glucose medium	Pyruvate medium	%Change	
Fed	0.01	-	39 ± 4	12 ± 1[a]	-67±2	(18)
	0.05	-	337 ± 46	106 ± 12[a]	-67±3	(6)
	0.05	+	331 ± 51	108 ± 11[a]	-62±6	(11)
	0.50	-	4152 ± 508	2324 ± 301[a]	-42±8	(6)
	0.50	+	3440 ± 359	1899 ± 253[a]	-46±5	(6)
Diabetic	0.01	-	24 ± 4[c]	20 ± 4[c]	-8±18	(6)
	0.05	-	264 ± 36	193 ± 34[c]	-26±7	(8)
	0.05	+	228 ± 24[c]	154 ± 20[b,c]	-33±6	(8)
	0.50	-	2468 ± 425	2468 ± 306	+18±20	(7)
	0.50	+	1988 ± 226[c]	1825 ± 190	-13±6	(8)
Fasted	0.01	-	31 ± 4	20 ± 2[b,c]	-28±6	(9)
	0.05	-	230 ± 29[c]	173 ± 21[c]	-22±6	(11)

Table 5. Effect of aminooxyacetate on the oxidation of α-ketoisocaproate by diaphragms from normal or diabetic rats.

Pyruvate Added	Aminooxy-acetate	pmol* $^{14}CO_2$/hr per mg wet weight	
		Normal Rats	Diabetic Rats
-	-	320 ± 39	353 ± 10
-	+	582 ± 100[a]	627 ± 45[a]
+	-	110 ± 20[b]	229 ± 45[b,c]
+	+	146 ± 17[b]	295 ± 42[b,c]

Quarter diaphragms from fed normal or diabetic rats were incubated in medium containing 5mM glucose or 5mM glucose + 5mM pyruvate, in the presence or absence of 1mM aminooxyacetate. Oxidation of 0.05mM α-keto[1-^{14}C]isocaproate was determined by $^{14}CO_2$ release as described in the Experimental section. Values are the means for 4 or 5 observations. * Values were calculated as in Table 1 based on the specific radioactivity of KIC in the medium. [a] $p < 0.001$ versus no aminooxyacetate; [b] $p < 0.05$ versus glucose; [c] $p < 0.05$ versus control rats.

Table 6. Effect of 2,4-dinitrophenol on the oxidation of α-ketoisocaproate by fed or fasted rats.

Group	Pyruvate Added	Dinitrophenol	pmol* $^{14}CO_2$/hr per mg wet weight
Fed Rats	-	-	206 ± 34
	-	+	347 ± 35[a]
	+	-	79 ± 6[b]
	+	+	132 ± 13[b,c]
Fasted Rats	-	-	163 ± 25
	-	+	269 ± 43[a,d]
	+	-	116 ± 9[d]
	+	+	160 ± 11[a,b,d]

Quarter diaphragms were incubated in medium containing 5.5mM glucose or 5.5mM glucose + 5mM pyruvate as described in the Experimental section. Oxidation of 0.05mM α-keto[1-^{14}C]isocaproate was determined as $^{14}CO_2$ release in the presence or absence of 0.1mM 2,4-dinitrophenol. Values are the means of six observations. * Values were calculated as in Table 1 based on the specific radioactivity of KIC in the medium. [a] $p < 0.05$ versus no dinitrophenol; [b] $p < 0.02$ versus glucose; [c] $p < 0.005$ versus no dinitrophenol; [d] $p < 0.02$ versus fed rats.

Chapter III

Leucine and Isoleucine Activate Skeletal Muscle Branched-Chain α-Keto Acid Dehydrogenase In Vivo

The contents of this chapter have been previously published in similar form in American Journal of Physiology 250 (Endocrinol. Metabol. 13): E599-E604, 1986.

Summary

The response of rat skeletal muscle branched-chain α-ketoacid dehydrogenase to administration of branched-chain amino acids in vivo was determined using a soluble preparation of the enzyme. After detergent extraction of the complex in the presence of kinase and phosphatase inhibitors, initial in vivo activity was typically 1 nmol/min/g muscle, with 0.1 mM α-keto[1-^{14}C]isocaproate as substrate. Total activity of the dephosphorylated complex, measured after preincubation with 15 mM Mg^{2+}, typically reached a maximum of 29 nmol/min/g. Thus, in overnight fasted rats the complex was 2-3% active. Initial activity increased 3-5 fold after leucine or isoleucine (at higher concentrations) but not valine administration in vivo. After intravenous leucine injection (0.25 mmol/kg) initial muscle enzyme activity increased rapidly and subsequently declined, paralleling plasma leucine concentrations, while plasma valine and isoleucine declined. Conclusions: Muscle branched-chain α-keto acid dehydrogenase complex is rapidly activated when circulating leucine is increased to concentrations which may occur after meals. During hyperleucinemia accelerated valine and isoleucine degradation by muscle may account for the observed "antagonism" among the branched-chain amino acids.

Introduction

Skeletal muscle has been considered an important site for the disposal of the branched-chain amino acids (BCAA)* since the early studies of Miller (136) in eviscerated rats. Studies of the oxidation of BCAA by skeletal muscle have found high activities of the BCAA transaminase but quite low activities of branched-chain α-keto acid dehydrogenase complex (BCDH) EC 1.2.4.4 (77,131,150). BCDH catalyzes the first committed step in BCAA degradation, and is regulated by a phosphorylation-dephosphorylation cycle analogous to that of pyruvate dehydrogenase (reviewed in 150). In homogenates of skeletal muscle from fed or fasted rats only a small fraction of the BCDH complex was in the active state and was essentially unresponsive to physiological manipulations (184,185). However, Kasperek et al. (100), using detergent-treated homogenates, recently reported marked activation of skeletal muscle BCDH by exercise. Studies of muscle homogenates (184, 185) and isolated intact mitochondria (140) cannot distinguish between changes in mitochondrial transport and BCDH activation. Attempts to measure the activity state of BCDH solubilized from rat skeletal muscle mitochondria have been unsuccessful due in part to the low initial activity of the complex, but it was estimated that less than 20% of the complex was active (77,137,150). Since several groups failed to demonstrate significant activation of BCDH in muscle in vivo

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* The abbreviations used in this chapter are: BCAA, branched-chain amino acids; BCDH, branched-chain α-keto acid dehydrogenase; PEG, polyethylene glycol.

the relative contribution of skeletal muscle to whole body BCAA catabolism has been questioned (77,184,200).

Administration of leucine to rats and man results in decreased circulating valine and isoleucine, as well as increased valine oxidation by the intact organism (reviewed in 77). Oxidation of valine by perfused rat hindlimbs (200) and by isolated epitrochlearis muscles (124) was markedly increased by the addition of increasing concentrations of leucine. These results suggest that muscle BCDH may be activated in the presence of leucine, as reported in adipose tissue (64). Due to its mass, skeletal muscle contains a large pool of inactive BCDH which could be recruited to oxidize the BCAA in the presence of increased circulating BCAA, such as occurs after a protein meal (52,145). In this communication we describe a method to determine the activity state of BCDH in a solubilized preparation from skeletal muscle. Using this method we show for the first time rapid, in vivo activation of the muscle BCDH complex after administration of leucine and to a lesser extent isoleucine.

Materials and Methods

Male Wistar (WI)BR rats (Charles River Laboratories, Wilmington, MA) were allowed access to standard rodent chow (Wayne Rodent Blox, Continental Grain Co., Chicago, IL) and water ad libitum, food was removed 14-18 hours prior to sacrifice, between 9 and 11 AM. Rats receiving i.v. treatments had chronic intravenous catheters inserted into a jugular vein the afternoon prior to infusion, using the procedure of Harms and Ojeda (76). Intravenous infusions were performed

with the animals under light anesthesia induced with methoxyflurane vapor.

Rats were anesthetized with methoxyflurane and the hindlimb skinned to expose the muscles. The Achilles tendon was secured with a hemostat and severed, the gastrocnemius and soleus were dissected free of underlying tissue, frozen with metal tongs cooled in liquid nitrogen, cut free of their origins and immersed in liquid nitrogen until further processed, usually within 4 hr. The frozen muscles were powdered with a porcelain mortar and pestle precooled with liquid nitrogen. A sample of the frozen powdered muscle was weighed into a precooled glass homogenizer tube, 4 ml of ice-cold extraction buffer per g of tissue added and the slurry homogenized with 10 strokes of a motor-driven Teflon pestle. Extraction buffer consisted of: 100 mM KH_2PO_4, 5% W1, 2 mM Na_2EDTA, 5 mM dithiotreitol, 0.5 mM thiamine pyrophosphate, 1 mM TLCK, 20 μg/ml leupeptin, 50 mM KF, (phosphatase inhibitor), 5 mM dichloroacetic acid (kinase inhibitor; 143), adjusted to pH 7.4 at 4°C with KOH. (In preliminary studies, dichloroacetic and α-chloroisocaproic acids were both effective in inhibiting the kinase.) Insoluble material was removed by centrifugation (5 min at 27000xg), and 0.5 volume of 27% PEG-6000 was added to the supernatant, resulting in a final PEG concentration of 9%. After standing 20 min on ice the precipitate was collected by centrifugation (10 min at 12000xg), and resuspended in 1 ml assay buffer per g tissue homogenized. Assay buffer consisted of: 25 mM Hepes, 0.2 mM Na_2EDTA, 0.4 mM thiamine pyrophosphate, 1 mM dithiothreitol, 20 μg/ml leupeptin, 0.1% Brij 58, adjusted to pH 7.4 with KOH at 37°C.

BCDH activity in muscle preparations was measured as $^{14}CO_2$ released from 0.1 mM α-keto[1-^{14}C]isocaproate (1.5 Ci/mol). Assays were conducted in triplicate, at 37°C, in 0.35 ml total volume of the assay buffer described above, supplemented with 0.5 mM CoASH, 0.5 mM NAD, and 1.2 mM $MgSO_4$. Assays were quenched with 0.2 ml 25% trichloroacetic acid, $^{14}CO_2$ collected in 0.2 ml Hyamine hydroxide, and radioactivity determined by liquid scintillation spectrometry as described previously (6,120). Initial BCDH activity (an estimate of in vivo activity) was determined using the enzyme preparation from muscle directly in the assay described above; for measurements of total enzyme activity (an estimate of enzyme amount) the preparation was diluted with assay buffer supplemented with $MgSO_4$ (final concentration 15 mM) and pre-incubated 60 min at 37°C to activate the complex before assay (see Results). The activation state of BCDH was determined as the ratio of initial activity to total activity.

Blood was removed by heart puncture immediately after removal of muscle samples and added to heparinized tubes. After centrifugation plasma samples were deproteinized by addition of 0.1 volume of 50% sulfosalicylic acid and stored frozen at -20°C until analysis. BCAA were measured by reversed-phase high-performance liquid chromatography separation and fluorescent detection of their o-phthalaldehyde derivatives as described previously (6).

Materials were obtained as described previously (7). W1, a linear polyoxyethylene ether detergent, and hog kidney D-amino acid oxidase were obtained from Sigma Chemical Co. (St. Louis, MO). Methoxyflurane was obtained from Pitman-Moore, Inc. (Washington Crossing, N.J.).

α-Keto-[1-^{14}C]isocaproate was prepared from DL-[1-^{14}C]leucine (ICN Radiochemicals, Irvine, CA), as described previously (120) but with the addition of D-amino acid oxidase. Dr. Ronald Simpson of Sandoz Pharmaceuticals (E. Hanover, NJ) kindly donated α-chloroisocaproate.

Results and Discussion

Published methods for determining the activity state of muscle BCDH either did not have adequate sensitivity to determine initial activities of the complex or were complicated by membrane barriers between the substrate and the enzyme complex. We therefore developed a method to circumvent these difficulties, guided by several earlier studies (69,131,144). Attempts to prepare detergent extracts directly from skeletal muscle in the presence of detergent and inhibitors of kinase (dichloroacetate) and phosphatase (F^-) failed to detect BCDH activity. Only after precipitation of the complex with PEG could we measure initial BCDH activity. Measurable initial activity was obtained by precipitation with greater than 6% PEG while the maximal rate of activation required about 9% PEG and diminished as the PEG concentration was increased above 12%. The recovery of measurable BCDH activity after PEG precipitation, as opposed to the lack of activity in detergent-treated homogenates, may result from concentration of the complex by precipitation (about 5-fold) or perhaps removal of an inhibitory factor from the homogenates as suggested by Odessey and Goldberg (131), and Paul and Adibi (141). The initial activity of the preparation was stable for at least 90 min when stored on ice and for at least 75 min when incubated at 37°C (data not shown), showing no evidence of activation.

Preincubation of the enzyme preparation with 15 mM Mg^{2+} at 37°C resulted in a time-dependent increase in the activity of the complex, from an initial activity of 0.7 nmol/min/g muscle to a maximum of 29 nmol/min/g muscle after 40-60 min of incubation; maximal activity remained stable until at least 80 min of incubation (Figure 1A). The increase in activity upon incubation with Mg^{2+} presumably reflected conversion of the complex from the phosphorylated (inactive) to the dephosphorylated (active) form (77,137). This suggests that in skeletal muscle of rats fasted overnight about 2% of the BCDH was in the active state. Activation of the complex was unaffected by 10 µg/ml protamine sulfate, 1 µM insulin or 2 mM $MnCl_2$ (data not shown). The relatively high PEG concentration (9%) necessary to maximize activation of the complex may be due to coprecipitation of a specific BCDH phosphatase, although the rate of activation in our preparation was unaffected by protamine sulfate which activated the specific phosphatase purified from bovine kidney (33).

The assay of BCDH activity was linear up to 7.5 min for initial BCDH activity and up to 3 min for assay of total activity after activation of the complex with Mg^{2+} (Figure 1B); measurements were independent of the volume of enzyme preparation assayed in the range of 0.06 to 0.18 ml of initial enzyme preparation and 0.008 to 0.02 ml of enzyme preparation after activation with Mg^{2+} (data not shown). In later experiments initial activity was routinely assayed over 5 min using 0.15 ml of enzyme and total activity was assayed over 1 min after 60 min preincubation with 15 mM Mg^{2+}, using 0.025 ml of diluted enzyme preparation (equal parts of initial preparation and assay buffer

supplemented with 30 mM $MgSO_4$). BCDH activity assayed either before or after incubation with Mg^{2+} was unchanged by the addition of lipoamide dehydrogenase (data not shown). Kinetic parameters of the BCDH complex after activation with Mg^{2+} were determined from reaction velocities with eight substrate concentrations between 0.01 and 0.6 mM α-ketoisocaproate. The data were analyzed using non-linear least squares fit to the Michaelis-Menten equation (150) and the apparent parameters found to be: Km 0.09 ± 0.01 mM, Vmax 79 ± 2 nmol/min/g muscle (mean ± SE from 5 preparations). Due to the low activity of the enzyme preparation in its initial state it was not possible to determine the kinetics of the complex prior to activation.

Our method of preparation of branched-chain α-keto acid dehydrogenase may result in some loss of the complex during the precipitation step (133,144), thus the total amount of enzyme complex may be greater than our measurements indicate; losses of complex would not be expected to bias measurements of the activity state of the complex since samples for initial and total activities would be subject to the same losses. Despite this possibility, our measurements of the initial and total BCDH activity and activation state are similar to the values obtained by Wagenmakers et al. in muscle homogenates (184,185) and the total BCDH activity obtained by Patston et al. in detergent solubilized muscle mitochondria (137). The BCDH activities obtained by Kasperek et al. (100) are also similar to ours and other investigators (137,184, 185) although their assay used 1 mM α-ketoisocaproate (a saturating concentration) while the other assays use 0.1 mM substrate (subsaturating). In light of the low values we have obtained for the initial

activity of BCDH, which precluded generation of kinetic data, we cannot distinguish between the possibilities that the initial activities measured represent either 1) the activity solely of a small fraction of active (i.e. dephosphorylated) complex or 2) that initial activity is comprised principally of residual activity of the phosphorylated (i.e. inactive complex) which could exhibit different kinetic properties.

We first attempted to modulate the activity of BCDH in vivo by injecting rats i.p. with leucine or valine (2 mmol/kg) and taking muscle samples for the assay of BCDH complex one hour later. As shown in Figure 2A treatment with leucine resulted in a nearly four-fold increase in the activity state of BCDH (from 2.1% to 7.8%) while after valine treatment the activity state was the same as that in saline treated controls. Since the lack of effect of valine on BCDH activity could have been due to differences in the rate of absorption and clearance, we investigated the effect of i.v. administration of the BCAA on the activity of the muscle complex. Figure 2B shows that valine administration had no effect on the activity state of BCDH even at the highest dose tested (0.5 mmol/kg), which resulted in a 2.2 fold increase in the whole blood valine concentration (data not shown). Within 15 min of administering a large dose (0.5 mmol/kg) of isoleucine or leucine the complex was activated from 3% in controls to 12% and 22% active, respectively; whole blood isoleucine and leucine concentrations increased 3.5- and 1.9-fold, respectively, with these treatments (data not shown). With smaller doses (0.25 mmol/kg) only leucine activated BCDH, to about 10% active.

Branched-chain α-keto acids, the products of the transamination of BCAA, are known inhibitors of BCDH kinase, and their levels in plasma and muscle generally parallel those of circulating BCAA (52). Our results are consistent with the potency of the α-keto analogues of the branched-chain amino acids as inhibitors of BCDH kinase: α-ketoisocaproate > α-keto-β-methylvalerate >> α-ketoisovalerate (107,142). Rapid activation of BCDH after leucine administration in our studies may explain the accelerated valine oxidation observed in muscles exposed to high leucine concentrations (124,200).

The time course of activation of muscle BCDH by leucine is shown in Figure 3A. The initial activity of the complex increased rapidly (within 10 min) after i.v. administration of leucine, from 0.7 to 2.1 nmol/min/g muscle (1.8% to 6.6% active complex). BCDH activity declined within 20 min after leucine treatment, decreasing to about 1 nmol/min/g (about 3.3% active) at 40 min and remained unchanged at 60 min. The changes in muscle BCDH activity with time after administration of leucine closely parallel the changes in the plasma leucine concentration shown in Figure 3B; plasma leucine increased from 0.165 mM in saline controls to 0.329 mM by 10 min after leucine injection and then declined to the initial value at 40 min. The plasma concentrations of valine and isoleucine declined steadily after leucine administration reaching a nadir at 40 min with valine concentrations depressed by 25% and isoleucine concentrations depressed by 30%; no further change occurred at 60 min.

The circulating concentrations of leucine which resulted in activation of muscle BCDH were not very great and should be easily

attained post-prandially after protein ingestion (52,145). Indeed, the activity of muscle BCDH responds to consumption of a meal and to the protein content of the diet (unpublished observations). Thus, the contribution of skeletal muscle to BCAA catabolism may be markedly increased post-prandially.

Feeding animals diets supplemented with excessive amounts of leucine results in growth impairment, increased concentrations of circulating leucine and depressions in valine and isoleucine; feeding excesses of isoleucine or valine does not result in depression of plasma leucine or growth impairment. This apparent antagonism among the BCAA has been extensively studied (for review see 77). In our experiments an i.v. bolus of leucine resulted in decreased circulating valine and isoleucine and concomitant activation of muscle BCDH; the activation state of BCDH appeared to parallel changes in circulating leucine. Thus, during hyperleucinemia, accelerated oxidation of valine and isoleucine by muscle may result in the observed depressions of these amino acids in plasma. We cannot exclude however possible changes in protein turnover contributing to the altered amino acid profile observed here after leucine injection (reviewed in 77).

The precise role of the various tissues in branched-chain amino acid catabolism is not well understood. The non-uniform distribution of transaminase and BCDH in different tissues suggests the existence of interorgan cooperativity in their catabolism (reviewed in 77,150). In contrast to muscle, BCAA transaminase is low in liver, but BCDH is abundant. Hepatic BCDH in rats fed an adequate protein diet is nearly fully active and is not further activated by increasing the protein

content of the diet (69) or administering BCAA (16). We confirmed the latter in the present study. In the experiments shown in Fig. 2 the activation state of hepatic BCDH was measured as described in (4); it was found to be 80-83% active and unchanged after the administration of leucine, isoleucine, or valine. However a role for the liver in the disposal of excess BCAA cannot be discounted since increased branched-chain α-keto acid export from peripheral tissues rich in BCAA transaminase may increase available substrate for liver BCDH (112).

Recent studies suggest that extrahepatic tissues (e.g. muscle) must contribute to the leucine induced stimulation of BCAA oxidation in vivo (16,77); the present observations, showing activation of the rate limiting enzyme in muscle after leucine administration, support this hypothesis. Our method for measuring the activity state of BCDH in muscle should prove a valuable adjunct to studies of BCAA degradation and its regulation by dietary and hormonal treatments.

Figure 1. Activation of muscle branched-chain α-keto acid dehydrogenase by Mg^{2+} and assay linearity.

Branched-chain α-keto acid dehydrogenase was prepared as described under "Materials and Methods". In panel A 0.15 ml of enzyme preparation was assayed for 5 min as described under "Materials and Methods" to obtain the zero time point. A sample of enzyme preparation was mixed with an equal volume of assay buffer supplemented with 30 mM $MgSO_4$ and incubated at 37°C; at the indicated times 0.025 ml samples were taken and assayed for 1 min. Values shown are the means ± SEM from 6 enzyme preparations. In panel B initial (unactivated) BCDH activity (■) was determined using 0.15 ml of enzyme preparation and assayed for the times indicated. Total BCDH activity (●) was measured using 0.025 ml of diluted enzyme after incubation for 60 min with Mg^{2+} as described above, and assays terminated at the times indicated. Results shown are the means ± SEM from 4 enzyme preparations.

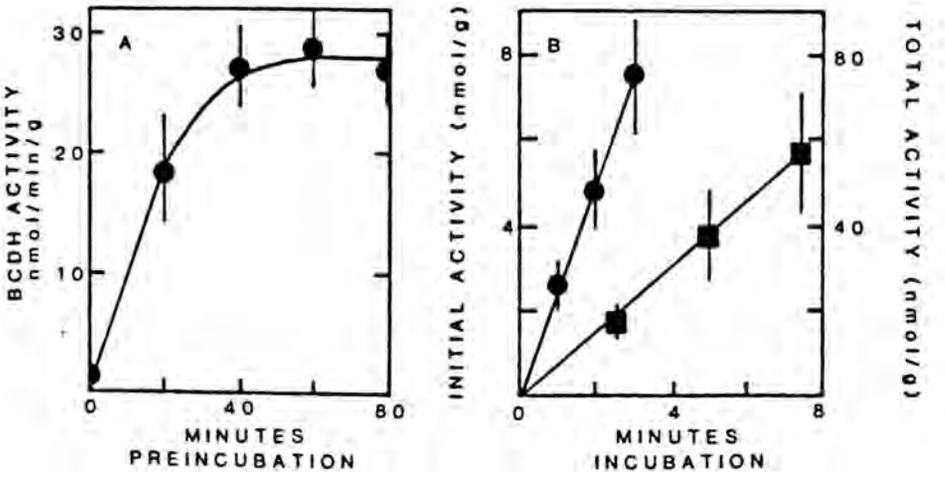


Figure 2. Activity state of muscle branched-chain α-keto acid dehydrogenase after treatment with branched-chain amino acids in vivo.

In panel A rats were injected i.p. with leucine or valine (2 mmol/kg) or an equal volume of saline. One hour after injection muscle samples were taken and initial and total BCDH activity assayed as described under "Materials and Methods" and Fig 1. Percent activity was determined as the ratio of initial activity to total activity, results shown are means ± SEM; 6 animals were treated with valine or saline and 11 with leucine. Total activities were the same for all groups, 18 ± 2 nmol/min/g muscle (n=23). In panel B rats were injected i.v. through a jugular venous catheter as described under "Materials and Methods" with amino acids at the dose shown or an equal volume of saline. Muscle samples were taken 15 min after treatment for the determination of BCDH activity as described above. Total activities were the same for all groups, 28 ± 1 nmol/min/g muscle (n=39), with 4 to 9 animals in each group. * $p < 0.05$ compared to saline control; ** $p < 0.001$ compared to saline control, $p < 0.05$ compared to 500 μmol/kg isoleucine, $p < 0.05$ compared to 250 μmol/kg leucine.

Figure 2.

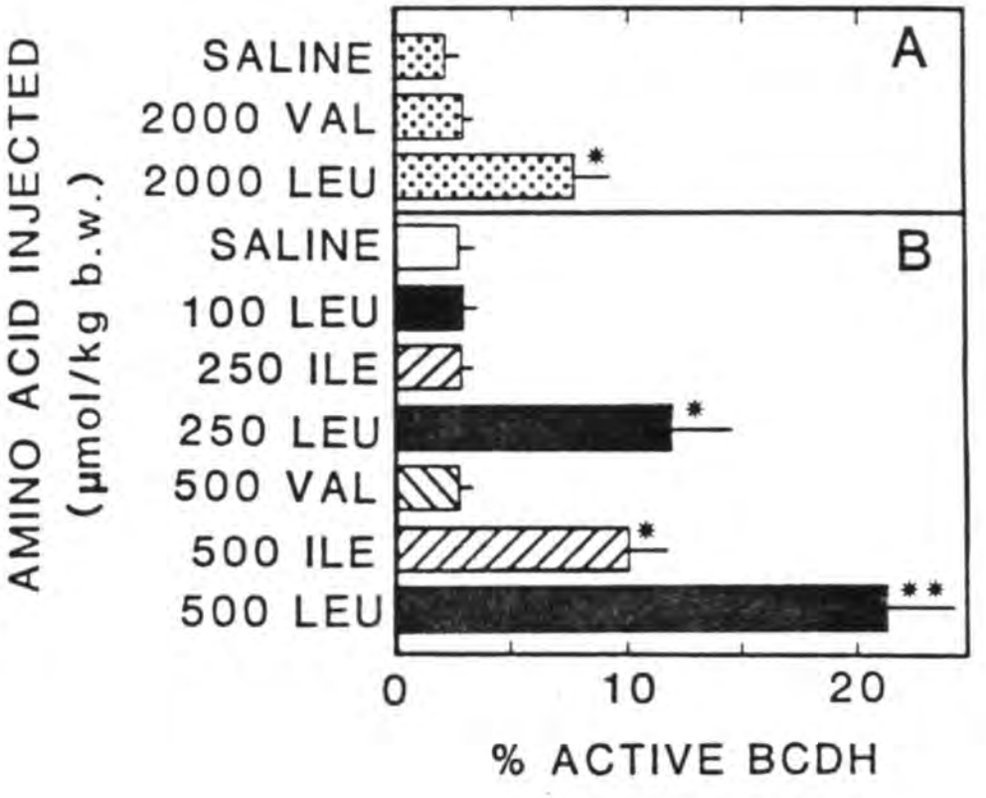
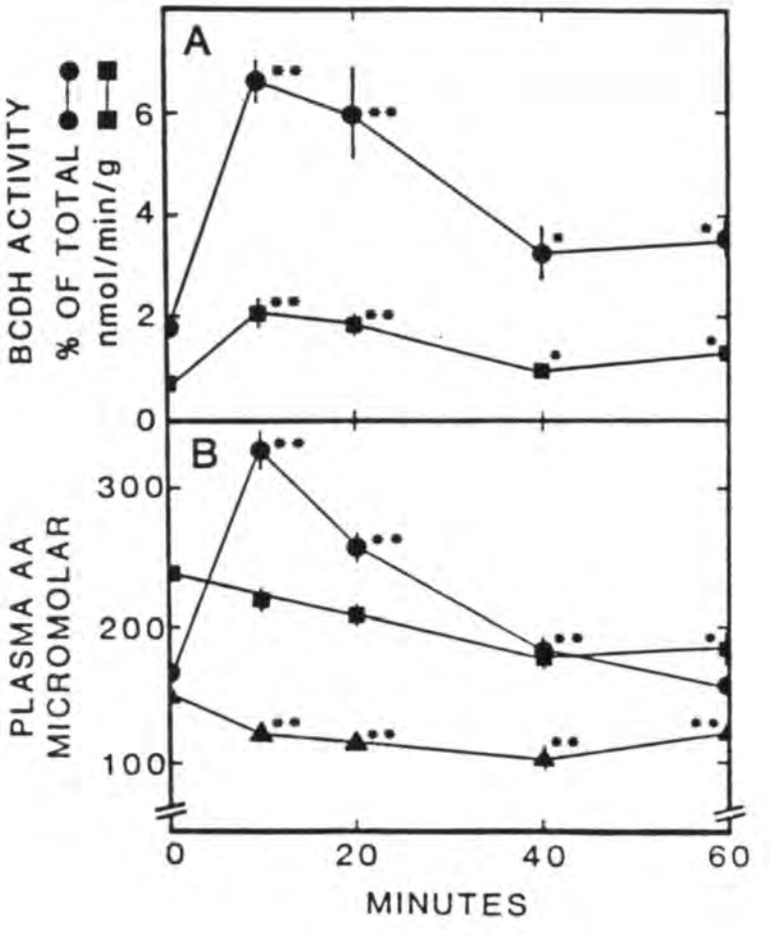

AMINO ACID INJECTED
(μmol/kg b.w.)
SALINE
2000 VAL
2000 LEU
A
SALINE
100 LEU
250 ILE
250 LEU
500 VAL
500 ILE
500 LEU
B
*
*
*
**
0
10
20
% ACTIVE BCDH

Figure 3. Time course of muscle branched-chain α-keto acid dehydrogenase activation and plasma branched-chain amino acid concentrations after leucine infusion in vivo.

Rats were injected i.v. with 0.25 mmol/kg leucine or an equal volume of saline (time 0 points), muscle and blood samples were taken at various times after injection as indicated. A. Initial activity (■), total activity, and % activity (●) of branched-chain α-keto acid dehydrogenase were determined as described in Fig. 2B. Total activities were the same for all groups, 33.9 ± 1.3 nmol/min/g tissue (n = 28). B. Leucine (●), valine (■) and isoleucine (▲) in plasma were determined as described under "Materials and Methods". In both panels values shown are the means ± SEM from 4-8 animals. * $p < 0.05$ compared to time 0; ** $p < 0.01$ compared to time 0.

Figure 3.


A
B
BCDH ACTIVITY
% OF TOTAL ●—●
nmol/min/g ■—■
PLASMA AA
MICROMOLAR
0
2
4
6
100
200
300
0
20
40
60
MINUTES

Chapter IV

Modulation of Rat Skeletal Muscle Branched-Chain α-Keto Acid Dehydrogenase In Vivo: Effects of Dietary Protein and Meal Consumption

The contents of this chapter have been published in similar form in Journal of Clinical Investigation 79: 1349-1358, 1987.

Summary

The effects of dietary protein on the activity of skeletal muscle branched-chain α-keto acid dehydrogenase (BCDH)* were investigated. BCDH is rate-limiting for branched-chain amino acid (BCAA) catabolism by muscle; its activity is modulated by phosphorylation-dephosphorylation. In rats fed an adequate protein (25% casein) diet, BCDH was ~2% active postabsorptively and increased to 10% or 16% active after a 25% or 50% protein meal, respectively. Feeding a 50% protein diet (10 days) increased postabsorptive BCDH activity to 7% with further increases to 40% active postprandially. On a low protein (9% casein) diet (10 days) BCDH remained ~2% active regardless of meal feeding. Dose-dependent activation of BCDH by intravenous leucine in postabsorptive rats was blunted by a low protein diet. Conclusions: Excesses of dietary protein enhance the capacity of skeletal muscle to oxidize BCAA; when protein intake is inadequate, muscle conserves BCAA; skeletal muscle may play an important role in whole body BCAA homeostasis.

Introduction

Chronic consumption of high protein diets results in induction of rate-limiting degrading enzymes for most essential amino acids (79, 106). Hence, the concentrations of most amino acids do not increase markedly in body fluids after long term high protein feeding and may actually decrease as more protein is consumed (145). When dietary

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* Abbreviations used in this chapter are: BCDH, branched-chain α-keto acid dehydrogenase; BCAA, branched-chain amino acids; BCKA, branched-chain α-keto acids.

protein intake is restricted to less than the requirement for growth and maintenance, these enzymes decrease (79,106), resulting in conservation of essential amino acids. The catabolic system for the branched-chain amino acids (BCAA) - leucine, isoleucine and valine - appears to be an exception to this homeostatic scheme for essential amino acids in that a) their concentrations in blood and tissues rise in direct proportion to the protein content of the diet (109,118,145) and b) BCAA aminotransferase, the initial enzyme in BCAA catabolism, is relatively refractory to changes in dietary protein (96). This apparent lack of adaptation has prompted some investigators to conclude that the body is unable to maintain the free body pools of BCAA by increasing oxidation during times of surfeit or by decreasing oxidation during times of deficiency. However, studies in man and rat have shown that BCAA oxidation in vivo is increased markedly after high protein feeding (109,129,182), and is severely depressed after consumption of low protein diets (109,129). The mechanisms responsible for these alterations in BCAA oxidation are incompletely understood. Recent studies suggest that fluctuations in BCAA oxidation with dietary manipulations cannot be explained based solely on the mass action principle (129a). Control of BCAA metabolism by dietary protein is of particular interest as branched-chain amino and keto acids are being used in the treatment of hepatic encephalopathy and renal insufficiency, conditions in which low protein diets are usually indicated (19,189).

The first committed reaction in the metabolism of BCAA is catalyzed by branched-chain α-keto acid dehydrogenase (BCDH), a multienzyme

complex located on the inner surface of the inner mitochondrial membrane of most cells (77,150). BCDH catalyzes the irreversible oxidative decarboxylation of branched-chain α-keto acids (BCKA) - α-ketoisocaproate (ketoleucine), α-keto-β-methylvalerate (ketoisoleucine), and α-ketoisovalerate (ketovaline) - which are formed from their respective BCAA through the action of BCAA aminotransferase. Both BCDH and BCAA aminotransferase are distributed ubiquitously but non-uniformly throughout the body. BCDH activity is highest in liver, kidney, and heart, but very low in skeletal muscle (77,150). In contrast, BCAA aminotransferase activity is highest in skeletal muscle but very low in liver (77,150). BCDH is believed to limit the rate of BCAA catabolism in vivo (117).

BCDH is unusual among amino acid-degrading enzymes in that it is regulated by a phosphorylation (inactivation)-dephosphorylation (activation) mechanism similar to that which exists for the pyruvate dehydrogenase complex (195). As with pyruvate dehydrogenase, a protein phosphatase and kinase are believed to be associated with BCDH in vivo. Alteration of the phosphorylation state of BCDH permits rapid changes in enzyme activity which are not possible via an induction-repression mechanism (77). The physiological significance of BCDH modulation by phosphorylation is currently an active area of study (199).

In normally fed rats, BCDH from liver is essentially fully active (i.e. dephosphorylated) (69,77,81,185). In contrast, less than 20% of skeletal muscle BCDH is in the active form (5,77,137,150,185). As skeletal muscle comprises approximately 40% of the rat's body weight (73), changes in the phosphorylation state of muscle BCDH could have

profound effects on BCAA metabolism in vivo. However, efforts to detect changes in the activity state of muscle BCDH in response to physiological manipulations have been largely unsuccessful (77,137, 184). In view of the high BCAA aminotransferase and low BCDH activity of muscles, it was proposed that muscle may primarily deaminate BCAA and release BCKA into the circulation for eventual oxidation by liver where BCDH is found in abundance (77,112,150). Uncertainty about the role of muscle in BCAA metabolism has stemmed in part from the difficulty in measuring activities of BCDH from solubilized muscle preparations (131,137,140,181). Therefore, intact muscle mitochondria were used in most studies to assay BCDH activity (77,140,150,184). Problems associated with substrate transport, quantitation of precursor specific radioactivity, and possible activation of the enzyme during mitochondrial isolation or incubation complicate interpretation of such studies. Recently, we described an assay for measuring BCDH in solubilized rat muscle tissue and showed that the enzyme was activated in vivo after intravenous leucine administration (5). We have used this assay in the present study and found that muscle BCDH is activated rapidly in vivo after consumption of meals containing adequate to high levels of protein, and that the activation state of the enzyme is responsive both acutely and chronically to the protein content of the diet.

Materials and Methods

Animals. Male Wistar (WI) BR rats (Charles River Laboratories, Wilmington, MA), initially weighing 140-160 g, were used in all studies. Rats were kept in a room maintained at 24°C with a reverse 12

hour light - 12 hour dark cycle. The dark period was from 3:00 AM to 3:00 PM. Rats were offered their daily allotment of food within the last 6 hours of the dark cycle (9:00 AM - 3:00 PM). This meal-feeding regimen minimized variations in enzyme and metabolite measurements due to fluctuations in food intake (145). Water was supplied ad libitum.

Diets and Treatments. All diets were specially prepared in pellet form by United States Biochemical Corp. (Cleveland, OH) and contained in percentage by weight: Rogers and Harper's mineral mix, 5 (155); AIN 76 vitamin mix, 1; corn oil, 5; choline chloride, 0.2; vitamin-free casein, 0, 9, 25 or 50; and equal amounts of glucose monohydrate (A.E. Staley Manufacturing Co., Oak Brook, IL) and cornstarch (American Maize, Hammond, IN) to make up 100%. The 9% casein diet was supplemented with methionine, 0.3% (w/w) (48).

Rats were first acclimated to the 25% casein diet ad libitum for 2 days; then rats were trained to consume the 25% casein diet during the last 6 hours of the dark cycle for 2 weeks (meal-feeding). After this training period, both chronic and acute effects of dietary protein on BCAA metabolism were studied. In the chronic study, rats were meal-fed the 0, 9, 25 or 50% casein diets for 10 days. Body weights were measured each day. On the tenth day, rats were killed before or 3 hours after presentation of the meal. Previous studies have shown that circulating levels of BCAA are greatest 3 hours postprandially in meal-fed rats (48). In the acute studies, rats previously fed the 25% casein diet for 2 weeks were offered a single meal containing 9, 25 or 50% casein. Rats failed to consume a single meal of the 0% casein

diet, hence this group was omitted. Rats were killed before and 3, 6 and 12 hours after presentation of the meal.

Previous studies (17,48,145) have shown that during the first few days of offering an adequate protein diet for 6-8 hrs a day (meal-feeding), food consumption is less than that of ad libitum-fed rats. However, food intake increases gradually and after one week, meal-fed and ad libitum-fed rats consume equal amounts of food. Rats maintained chronically on casein diets similar to those used here consume equal amounts of food except for those given a protein-free diet, which consume 43% less (17). In the present study, food intake was measured in the acute experiments and was not significantly different between rats fed 9%, 25% or 50% casein diets (14 ± 1 g/meal).

In a separate experiment, the effects of leucine infusions on muscle BCDH activity were investigated in rats fed 25% or 9% casein diets ad libitum for 2 weeks. Rats were anesthetized with methoxy-flurane (Metofane) after an overnight fast (5) and L-leucine was infused via the tail vein at doses of 0, 10, 25, 50, and 100 μmol/100 g body weight. Muscles were excised 15 minutes after injections.

BCDH Assay. Our method for determining BCDH activity in muscle has been described in detail previously (5,15). Briefly, the method involved freeze-clamping whole muscle in situ. BCDH was extracted from powdered frozen muscle in the presence of protease, phosphatase, and kinase inhibitors and then precipitated with polyethylene glycol. Basal (in vivo) BCDH activities were determined immediately after redissolving the precipitated proteins and total (fully-active) enzyme activities were measured after preincubation of the extract at 37°C

with 15 mM Mg^{2+} prior to assay. In these studies BCDH activity was determined by $^{14}CO_2$ release from 0.1 mM α-keto[1-^{14}C]isovalerate at 37°C. Percentage of BCDH in the active form was calculated as the ratio of basal to total BCDH activities.

Amino Acids, Protein and DNA. Heparinized plasma was deproteinized by adding ice cold sulfosalicylic acid (0.1 volumes of a 50% w/v solution). After standing 20 minutes on ice, samples were centrifuged at 30,000 X g for 10 minutes. Muscle samples were deproteinized by adding 4 volumes of ice cold 5.9% w/v sulfosalicylic acid to powdered muscle samples. Muscles were then homogenized with a Polytron tissue homogenizer and left on ice for 20 minutes. The mixture was then centrifuged at 30,000 X g at 4°C for 10 minutes. Plasma and muscle BCAA concentrations in deproteinized samples were measured using reversed-phase high performance liquid chromatography separation of the o-phthalaldehyde derivatives and fluorescent detection (6). BCAA concentrations (μmol/g) were expressed per milliliter of intracellular water assuming that muscle contains 80% water: 60% intracellular and 20% extracellular, the latter at equilibrium with plasma (30).

Muscle protein concentrations were determined according to the method of Lowry (113) with bovine serum albumin as the standard. Muscle DNA concentrations were measured using a modification of the Burton assay (191) with herring sperm DNA as the standard.

Statistical analyses of differences between groups were performed by Student's t test or by the Peritz multiple range F test (80) and were considered significant if $p < 0.05$. Values for % active complex were subjected to the inverse sine transformation prior to statistical

analysis as percentages are distributed binomially rather than normally (173). This transformation is especially recommended for values in the range of most of the present observations (0-20%). Regression analysis was performed by the linear least squares method and slopes compared by Student's t test (173). All values shown are means ± SEM.

Results

BCDH Assay. As others have shown diet-mediated changes in BCDH activities from liver, kidney and heart (69,77,81,137,150), we first sought to optimize the assay for BCDH isolated from muscles of rats fed differing levels of protein. Rats were meal-fed 0%, 25% (normal) or 50% (high) casein diets for approximately 2 weeks. Muscles were excised 3 hours after presentation of the meal. As shown in Figure 1, basal (in vivo) BCDH activity increased as the protein content of the diet increased. The assay for basal BCDH activity was linear up to 5 minutes in all diet groups whether 100 or 150 µl of muscle extract was used. To ensure that no more than 10% of α-ketoisovalerate was utilized during the reaction (168), basal activities from 0% and 9% casein groups were routinely assayed for 5 minutes using 150 µl of extract. Basal BCDH activities from 25% and 50% casein groups were assayed for 2-5 minutes, utilizing 100 µl of extract. Total BCDH activities were linear up to 3 minutes in all diet groups; therefore, total activities were routinely assayed over 1 minute (5,15).

Recently, Randle and associates have shown that BCDH from liver of rats fed low protein diets is refractory to dephosphorylation (138). To investigate whether dietary protein might affect the rate of muscle

BCDH activation, total BCDH activities were measured 20, 40, 60 and 80 minutes after preincubation at 37°C in a buffer containing 15 mM Mg^{2+}. The time required to attain maximal BCDH activities in muscle extracts from rats fed diets containing 0%, 25% or 50% casein are shown in Figure 2. Maximal activities for rats fed the 25% or 50% casein diets were obtained after 40 minutes of preincubation. In contrast, maximal BCDH activities in muscles of rats fed the 0% casein diet were not obtained until 60 minutes of preincubation. This lag phase of muscle BCDH activation was also observed in rats fed the 9% casein diet (data not shown). Fully activated (total) enzyme activities (nmol/min/g tissue) were not significantly different among the groups.

Chronic Effects of Dietary Protein. Rats fed the protein-free diet failed to thrive and lost approximately 40 grams of weight during the 10 day study (Table 1). Addition of 9%, 25% or 50% casein to the diet resulted in growth rates of 4-6 grams/day. Rats fed the 9% casein diet attained 60% of the weight gain observed in rats fed the 25% casein diet. Continued growth on the 9% casein diet was most probably due to its supplementation with methionine, the first limiting amino acid in casein; hence this diet constituted a protein restricted rather than an essential amino acid deficient diet. The changes in growth due to dietary protein were accompanied by alterations in the protein and DNA concentration in skeletal muscle (Table 1). The protein/ DNA ratio, an estimate of cell size, was significantly depressed when protein was omitted from the diet (g protein/g DNA = 336 ± 16; 418 ± 16; 458 ± 18; and 464 ± 17 for rats fed 0%, 9%, 25% or 50% casein respectively).

The effect of consuming 0%, 9%, 25% or 50% casein diets for 10 days on the percentage of active BCDH in muscle are shown in Figure 3A. Measurements were made before (postabsorptive) or 3 hours after (postprandial) presentation of the meal. All postprandial rats had food in their stomachs at sacrifice. Compared to postabsorptive values, consumption of the 0% or 9% casein diets had no significant effect on the activity state of the complex. In contrast, the activity state of BCDH increased markedly (~7-fold) after consumption of the 25% or 50% casein diets. The postabsorptive activity of BCDH was identical in rats fed 9% or 25% casein; however the postprandial activity was 80% lower in the former group. Consumption of the diet containing 50% casein resulted in greater than 3-fold increases in both postabsorptive and postprandial BCDH activities when compared to the group fed the normal protein diet (25% casein). The postabsorptive BCDH activity in rats fed 50% casein was significantly higher than the activity in the 9% or 25% casein-fed rats. Interestingly, consumption of a protein--free diet resulted in significantly higher postabsorptive activities than observed in the 9% and 25% casein groups.

The effects of dietary protein on total BCDH activity (an estimate of enzyme content) are shown in Table 1. Total BCDH activity was not significantly different in rats fed the 25%, 9% or 0% casein diets. In contrast, consumption of high levels of protein resulted in increases in total BCDH activity when expressed per mg DNA or per g protein (Table 1). Hence, the apparent amount of BCDH complex per muscle cell increased ~35% after feeding a high protein diet for 2 weeks.

We have recently demonstrated that infusions of leucine result in

activation of rat muscle BCDH (5). Therefore, plasma BCAA concentrations were measured in the present study. As shown in Figure 3B, the concentration of plasma leucine increased in direct proportion to the protein content of the diet. In general, the increases in plasma leucine paralleled the changes in the activation state of the complex ($r = 0.781$; $n = 62$; $p < 0.001$). Similar relationships were observed between the activity state of BCDH and the plasma concentrations of isoleucine or valine ($r = 0.783$ for both; $n = 62$; $p < 0.001$). However, a causal relationship between these amino acids and the activity of the complex is unlikely as we have shown previously that infusions of equimolar amounts of valine or isoleucine fail to activate BCDH (5). The intracellular concentrations of leucine were measured in muscles of some rats before and after consuming the four diets (Table 2). Intracellular leucine concentrations were significantly correlated ($r = 0.498$; $n = 40$; $p < 0.01$) with the activity of muscle BCDH. The intracellular concentrations of leucine increased postprandially in rats fed 9%, 25% or 50% casein, but not in rats fed the protein free diet. The postabsorptive intracellular leucine concentrations were similar among the groups fed 0%, 9% or 25% casein diets, but were increased in the group fed the 50% casein diet. The postprandial intracellular leucine concentrations were significantly higher in the groups fed 25% and 50% casein, than in those fed 0% casein. Meal consumption had no significant effect on the ratio of leucine concentrations between muscle and plasma in any of the diet groups. Interestingly, the highest muscle/plasma ratios were observed in the group fed the protein-free diet. Postprandially this ratio was significantly

higher in the 0% casein group than in any of the groups fed protein containing meals.

Acute Effects of Dietary Protein. To examine the effects of acute protein consumption on muscle BCDH, rats previously fed a 25% casein diet for 2 weeks were offered a single meal of 9%, 25%, or 50% casein. No differences in food intake were observed between the three groups. As shown in Figure 4A, consumption of the 9% casein meal had no effect on muscle BCDH activity 3, 6, or 12 hours after feeding. In contrast, consumption of the 25% and 50% casein meals resulted in 6 and 11-fold increases in the percentage of active complex 3 hours after presentation of the meal (postabsorptive 1.6 ± 0.1% active; postprandial 9.4 ± 2.0 and 17.2 ± 3.6% active respectively). By 6 hours, the proportion of BCDH in the active form in rats offered the 25% casein meal returned towards baseline and changed little after 12 hours. The percentage of active complex in rats offered the 50% casein meal decreased in a linear fashion between 3 and 12 hours after presentation of the meal. By 12 hours, BCDH activity in the high protein group was still 50% greater than baseline.

The effect of consuming single meals of varying protein content on the plasma concentrations of leucine is illustrated in Figure 4B. A strong correlation ($r = 0.852$; $n = 45$; $p < 0.001$) was observed between the percentage of active BCDH and the concentration of leucine in plasma. Significant correlations were also observed between the activity of BCDH and the plasma concentrations of isoleucine ($r = 0.854$; $n = 45$; $p < 0.001$) and valine ($r = 0.906$; $n = 45$; $p < 0.001$).

The relationship between the percentage of active BCDH and circulating leucine in both the chronic and acute studies is shown in Figure 5. Linear regression analysis between % active complex and plasma leucine concentration demonstrated a significant direct correlation between these values ($r = 0.788$, $n = 107$, $p < 0.001$). A better linear least squares fit was obtained by semilog transformation of the data (i.e. log % active complex vs. plasma leucine concentration) which gave a correlation coefficient of 0.848 ($p < 0.001$). The solid line in Figure 5 represents the best fit line for the semilog transformation.

Leucine Infusion Studies. In rats fed a 9% casein diet for 10 days, consumption of the meal resulted in an approximate doubling in plasma and intracellular leucine without a change in the activity of the complex (compare Figures 3A, 3B and Table 2). Also, the enzyme from rats fed a protein-free diet required a longer period of incubation to attain maximal activity (Figure 2). To study the apparent refractoriness of the enzyme from low protein-fed rats to activation, rats previously fed adequate (25% casein) or low (9% casein) protein diets for approximately 2 weeks were infused with varying doses of leucine (Figure 6). BCDH activity was closely correlated with the dose of leucine infused ($r = 0.92$ for each diet group, $p < 0.001$) and leucine infusions at doses of 25 μmol/100g body weight or greater resulted in activation of muscle BCDH in both diet groups (Figure 6A). However, the leucine-mediated activation of muscle BCDH from low protein-fed rats was less than that observed in rats fed adequate amounts of protein ($p < 0.001$), by comparison of the slopes of the regression lines for leucine dose vs. BCDH activity). Plasma BCAA

concentrations were essentially identical in the two groups (Figure 6B and C).

Discussion

The body pools of most amino acids are enlarged in animals immediately after they have consumed a high protein diet (106,145). Continued consumption of amino acids in quantities greatly in excess of requirements would result in death if no homeostatic controls were present. The relatively high K_m's of amino acid-degrading enzymes enable the rate of oxidation of surplus amino acids to increase initially as the result of increased substrate concentrations (106). However, if a high influx of amino acids continues, the organism synthesizes more of many of the rate-limiting amino acid-degrading enzymes found in liver. Prominent among these inductions are: serine-threonine dehydratase; histidase; tryptophan pyrrolase; tyrosine-α-ketoglutarate aminotransferase; glutamate-oxaloacetate and glutamate--pyruvate aminotransferases; and all of the urea cycle enzymes-(reviewed in 63,66,79,106). Hence, excesses of amino acids are catabolized and in the process, essential precursors for gluconeogenesis (i.e. pyruvate, tricarboxylic acid cycle intermediates) are formed. A similar control mechanism for BCAA is believed to be absent (106). Reports of BCAA aminotransferase induction by diet are inconsistent and the observed effects are relatively small compared to other amino acid-degrading enzymes (77,106). Yet, BCAA oxidation in vivo is increased markedly after high protein feeding (109,129a,182a). In rats adapted to a high protein diet, the protein content of the body does not increase, and BCAA excretion in urine and feces is negligible (79).

Based on these considerations, Harper et al. (17,77) calculated that when rats are switched from a 20% to 50% protein diet, 12,000 μmol BCAA are available for degradation, while the increase of BCAA in bodily fluids is only ~100 μmol. Hence, extensive oxidation of BCAA must be occurring. In the present study, a small increase in total BCDH activity/mg DNA was observed in rats after feeding a high protein diet for two weeks. This may represent stimulation of enzyme synthesis and/or decreased degradation of the complex. However, the major effect of protein intake was exerted at the level of enzyme activation.

BCDH activity is thought to be the major regulator of BCAA oxidation in vivo (117). As liver BCDH is fully active in normally-fed rats (69,77,81,185), activation of extrahepatic BCDH would be expected to result in enhanced oxidation of BCAA in vivo. In the present study, skeletal muscle BCDH was only 1-2% active during the postabsorptive period in rats fed 25% casein (Figures 3A and 4A). Increasing the casein content of the diet from 25% to 50% for 10 days resulted in a greater than 3-fold increase in the percentage of active muscle BCDH measured either before or 3 hours after feeding (Figure 3A). Furthermore, compared to postabsorptive values, consumption of the 25% or 50% casein diet resulted in a greater than 7-fold activation of muscle BCDH. The proportion of BCDH in the active form rose from 2% in postabsorptive rats fed adequate amounts of protein to greater than 40% in rats 3 hours after consuming a high protein diet. That a single normal or high protein meal could also activate muscle BCDH is shown in Figure 4A; the duration and magnitude of activation was proportional to the protein content of the diet.

Considering all available data, we observed a strong correlation between circulating leucine and the activity of muscle BCDH. Inspection of the points in Figure 5 suggests that a threshold phenomenon may obtain at leucine concentrations less than 0.2 mM with a fairly steep slope for activation of muscle BCDH at leucine concentrations greater than 0.2 mM. At low circulating leucine concentrations, liver would, therefore, assume greater importance in BCAA homeostasis but as circulating BCAA and muscle BCDH activity increase the latter may become the predominant tissue in BCAA metabolism. The correlation between plasma leucine and muscle BCDH activity (5) is most simply explained by inhibition of BCDH kinase by α-ketoisocaproate (150), the transamination product of leucine. In studies of rats it was found that the concentrations of BCKA in muscle paralleled those of the BCAA in rats fed low, normal or high protein diets (91) and plasma α-KIC was found to increase in man after a protein meal (165). It is clear however that this simple explanation for the relationship between muscle BCDH and plasma leucine is inadequate. Despite similar circulating leucine concentrations, muscle BCDH in rats fed a 9% protein diet activated less than BCDH from rats fed a 25% protein diet (Figure 6). Furthermore, meal-induced activation of BCDH was greater than that observed after leucine infusions achieving similar plasma leucine concentrations (compare Fig. 4 and 6). The two studies are not directly comparable however because the duration of hyperleucinemia before sacrifice was much longer in the former experiments. It is also noteworthy that the postprandial activation of BCDH by a high protein meal (40% active) was ~2.5X greater in rats maintained on a high

protein diet than in those maintained on a normal diet although plasma leucine was only 50% higher in the former group after consumption of the protein meal (compare Fig. 3 and 4). It seems unlikely that meal-induced insulin release played a role in the postprandial activation of BCDH, as treating postabsorptive rats with large doses of insulin and glucose did not activate the complex in muscle (15). Furthermore, in perfused hindlimbs of postabsorptive rats, insulin added to the perfusate decreased flux through BCDH (93).

Despite a marked activation of muscle BCDH in rats fed a high protein diet, plasma and tissue concentrations of BCAA remained elevated (Figures 3,4; Table 2). The BCAA aminotransferase reaction is readily reversible and indeed 80-90% of leucine transaminated to α-ketoisocaproate is reaminated to leucine in humans in vivo (117). High levels of tissue glutamate in animals fed high protein diets would favor maintenance of elevated BCAA by restraining their transamination and favoring reamination of BCKA (77,93). The physiological advantage of maintaining enlarged BCAA pools during high protein feeding is unknown. Elevated BCAA concentrations in blood could act as a protective buffer, competing with other large neutral amino acids (e.g. tyrosine, tryptophan and phenylalanine) for transport across the blood-brain barrier (135,145), thus preventing increased synthesis of certain neurotransmitters (61,197). The toxic effects of elevated brain aromatic amino acids are well documented (11). BCAA have been used clinically to reduce the levels of brain aromatic amino acids in hepatic encephalopathy (189) and phenylketonuria (12).

Consumption of protein deficient diets results in inactivation of

liver BCDH (69,81,137,138). Recently, increased BCDH kinase activity was reported in livers and hearts of rats fed a protein-free diet (55). The decrease in the percentage of active complex in livers of rats fed low protein diets presumably represents a mechanism by which essential BCAA are conserved during periods of protein depletion. Indeed, BCAA oxidation is severely depressed in man and rat after low protein feeding (109,121,129,153,171). In the present study, switching from an adequate to a low protein diet for 10 days had no significant effect on muscle BCDH activity in the postabsorptive state (Figure 3A). This is not surprising as most of the complex was already in an inactive form in postabsorptive rats fed the adequate protein diet (i.e. 2% active). However, the following observations were of interest: 1) muscle BCDH from rats fed protein-free or low protein diets required a longer period of preincubation with Mg^{2+} to attain maximal activity than BCDH from rats fed adequate protein diets (Figure 2) and 2) plasma leucine concentrations increased approximately 2-fold after consumption of the low protein diet, but muscle BCDH activity remained unchanged (Figure 3). To further investigate these differences, varying amounts of leucine were infused into rats fed adequate or low protein diets. Despite similar circulating leucine concentrations, the complex from rats fed the low protein diet was less responsive to leucine activation than the enzyme isolated from rats given adequate amounts of protein (Figure 6). The refractoriness of muscle BCDH to activation in rats fed low protein diets may be of physiological significance. An activation-resistant complex in muscle from low protein-fed rats, along with an inactivated complex in liver, would ensure efficient conserva-

tion of BCAA during periods of protein depletion. The mechanism of this resistance is not clear; in view of the findings of Espinal et al. (55) increased skeletal muscle BCDH kinase activity in response to protein depletion is an attractive hypothesis.

We have no explanation for the increased basal BCDH activity observed in postabsorptive rats maintained on the protein-free diet (Fig. 3). These animals were in a protein-catabolic state but intracellular leucine concentrations were not significantly different from the values in the 9% and 25% casein groups (Table 2). The apparent increase in the muscle/plasma leucine ratio in this group (Table 2) may be a reflection of accelerated net degradation of muscle protein. α-Ketoisocaproate is compartmentalized in muscle cells (94); whether intramitochondrial α-ketoisocaproate is increased in muscles of protein-starved rats or if other mechanisms lead to the relatively modest activation of the complex in the postabsorptive state is not known.

Our studies were carried out in rats trained to consume their daily caloric requirements during a relatively short period of time. Since rats are predominantly nocturnal "nibblers" rather than "meal-eaters", the postprandial activation of BCDH may be less marked when normal feeding patterns are maintained. In rats fed a normal diet ad libitum the plasma leucine concentration cycles between a nadir of 170 μM at 6 PM and a peak of 275 μM at 6 AM (60). Man however is a meal eater; following a protein meal most of the absorbed amino acids (except for the BCAA) are taken up by the liver, while a large fraction of BCAA is taken up by muscle (188). Since BCAA concentrations in muscle generally parallel plasma concentrations (reviewed in 77) and

the latter increase 2-3 fold after a protein meal in man (129,165,188), muscle BCDH may be activated and likely contributes to the postprandial increase in BCAA oxidation (129). Our studies in rats may have practical implications for nutritional therapy in some patients. Distribution or presentation of dietary protein in a manner to avoid large increases in circulating BCAA, or slowing the rate and extending the duration of amino acid infusions, may prevent or decrease activation of muscle BCDH and spare BCAA for protein synthesis.

Our data support an active role for skeletal muscle in the disposal and conservation of BCAA. Muscle releases BCKA to the blood stream for eventual uptake and oxidation by the liver (77,184). In addition, during periods of protein surfeit, the capacity to oxidize BCAA carbon increases markedly in skeletal muscle, while during periods of protein inadequacy, skeletal muscle, along with liver, adapts to conserve essential BCAA.

Table 1. Effects of Dietary Protein on Total Muscle BCDH Activities Expressed on a Wet Weight, Protein, or DNA Basis.

Casein (%)	Body Wt Chg (g/10 days)	DNA (μg/g)	Protein (mg/g)	Total BCDH Activity in (nmol/min) x (g tissue)$^{-1}$	(mg DNA)$^{-1}$	(g protein)$^{-1}$
0	-41 ± 2^{a}	537 ± 16^{a}	174 ± 7^{a}	64 ± 5^{a}	120 ± 10^{a}	368 ± 23^{a,b}
9	+40 ± 4^{b}	448 ± 15^{b}	188 ± 12^{a,b}	63 ± 6^{a}	140 ± 9^{a}	332 ± 14^{a,b}
25	+64 ± 2^{c}	466 ± 13^{b}	222 ± 11^{b}	63 ± 5^{a}	135 ± 14^{a}	304 ± 35^{a}
50	+59 ± 2^{c}	432 ± 16^{b}	200 ± 10^{a,b}	78 ± 4^{a}	185 ± 15^{b}	406 ± 35^{b}

Rats were trained to meal-feeding on a 25% casein diet as described in Materials and Methods, then changed to diets with varying casein content as shown for a period of 10 days. Starting weights were not different between rats fed the four diets (205 ± 1 g, n = 64). DNA, protein, and total BCDH activities expressed per gram of tissue were unaltered 3 hours after feeding. Therefore, postabsorptive and postprandial values for each diet group were pooled. Numbers represent mean ± SEM for n = 13 (0% casein), 15 (9% and 50% casein) or 21 (25% casein) rats/group. DNA, protein and total BCDH activities were measured as described in Materials and Methods. Values in vertical columns with different superscript letters are significantly different ($p < 0.05$).

Table 2. Effects of Dietary Protein on Muscle Intracellular Leucine Concentrations and Intracellular Muscle/Plasma Leucine Ratios Before or After the Meal.

	Intracellular Leucine Concentration* (nmol/ml)		Muscle/Plasma Leucine	
Casein (%)	B	A	B	A
0	134 ± 24[a]	106 ± 16[a*]	1.61 ± 0.26[a]	1.64 ± 0.35[a]
9	94 ± 10[a]	162 ± 8[a*]	1.25 ± 0.31[a,b]	1.02 ± 0.13[b]
25	128 ± 24[a]	300 ± 17[b*]	1.00 ± 0.12[b]	0.95 ± 0.03[b]
50	244 ± 53[b]	371 ± 52[b*]	1.02 ± 0.14[b]	0.70 ± 0.07[b]

Casein diets were fed for 10 days and values listed are from before (B) or three hours after (A) presentation of the meal. Plasma and muscle leucine concentrations were determined as described in Materials and Methods. Values are means ± SEM for 5 rats/group. Values in vertical columns with different superscript letters are significantly different ($p < 0.05$).

* Intracellular concentrations of leucine were calculated according to the following formula:

$$\text{intracellular [leu]} = \frac{(\text{nmol leu/g muscle}) - (0.2)(\text{nmol leu/ml plasma})}{0.6}$$

* $p < 0.05$ compared to before meal values.

Figure 1. Linearity of Basal Muscle BCDH From Rats Fed Differing Levels of Protein.

Rats were meal-fed diets containing 0, 25 or 50% casein for approximately 2 weeks. Muscles were frozen in situ after 3 hours of feeding. Basal BCDH activity was measured for 1, 2 or 5 min using 150 µl of enzyme extract, as described in Materials and Methods. Values represent mean ± SEM for enzyme activities from 4 rats per diet group. Identical values were obtained when 100 µl of enzyme extract was used (data not shown).

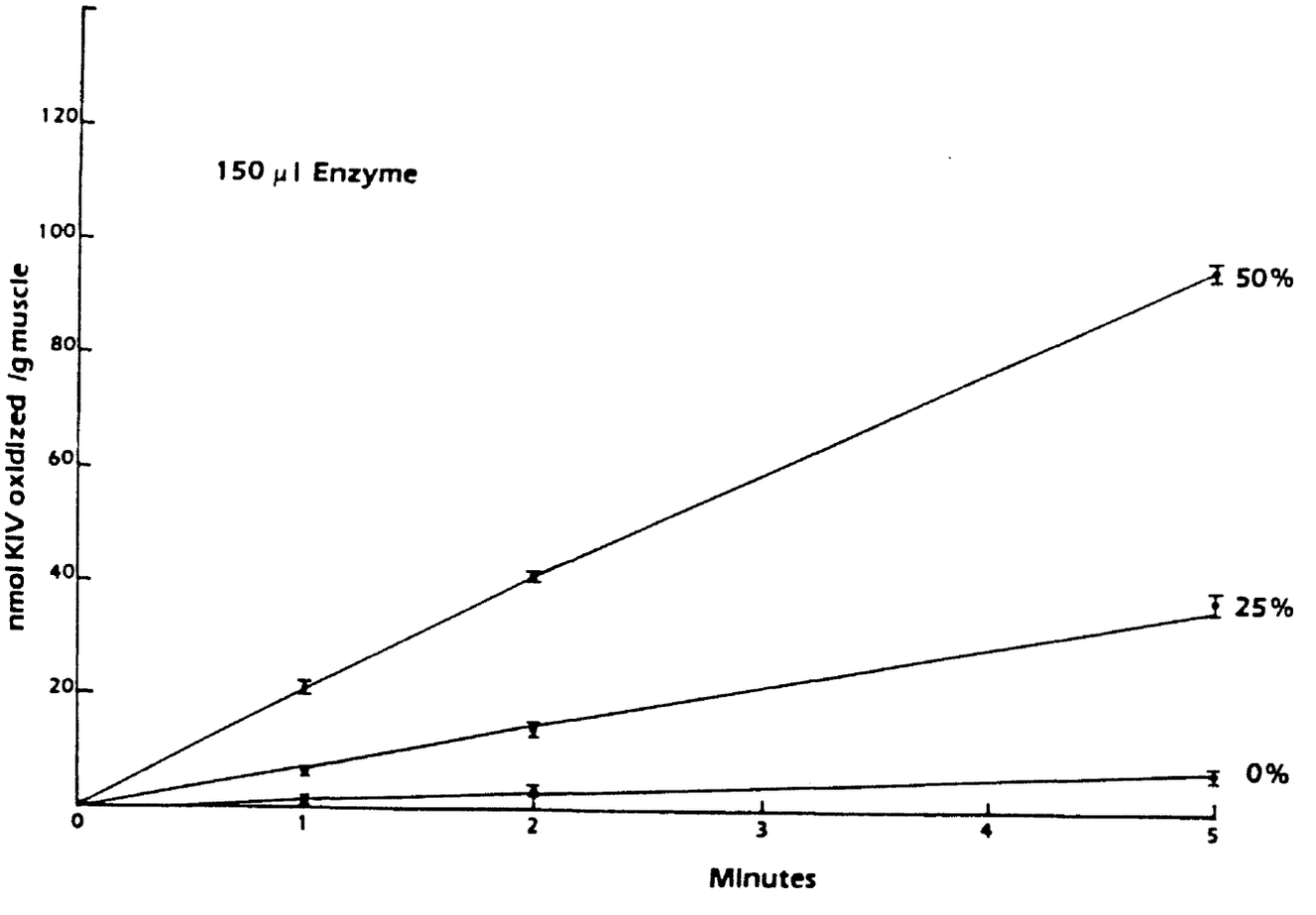


Figure 2. Effect of Preincubation Time on The Activation of Muscle BCDH Isolated From Rats Fed Differing Levels of Protein.

Rats were treated as described in Figure 1. Total BCDH activity was measured as described in Materials and Methods. Enzyme activities were measured after 20, 40, 60 or 80 minutes preincubation with 15 mM Mg+. Values represent mean ± SEM for enzyme activities from 3-4 rats per diet group.

Figure 2.

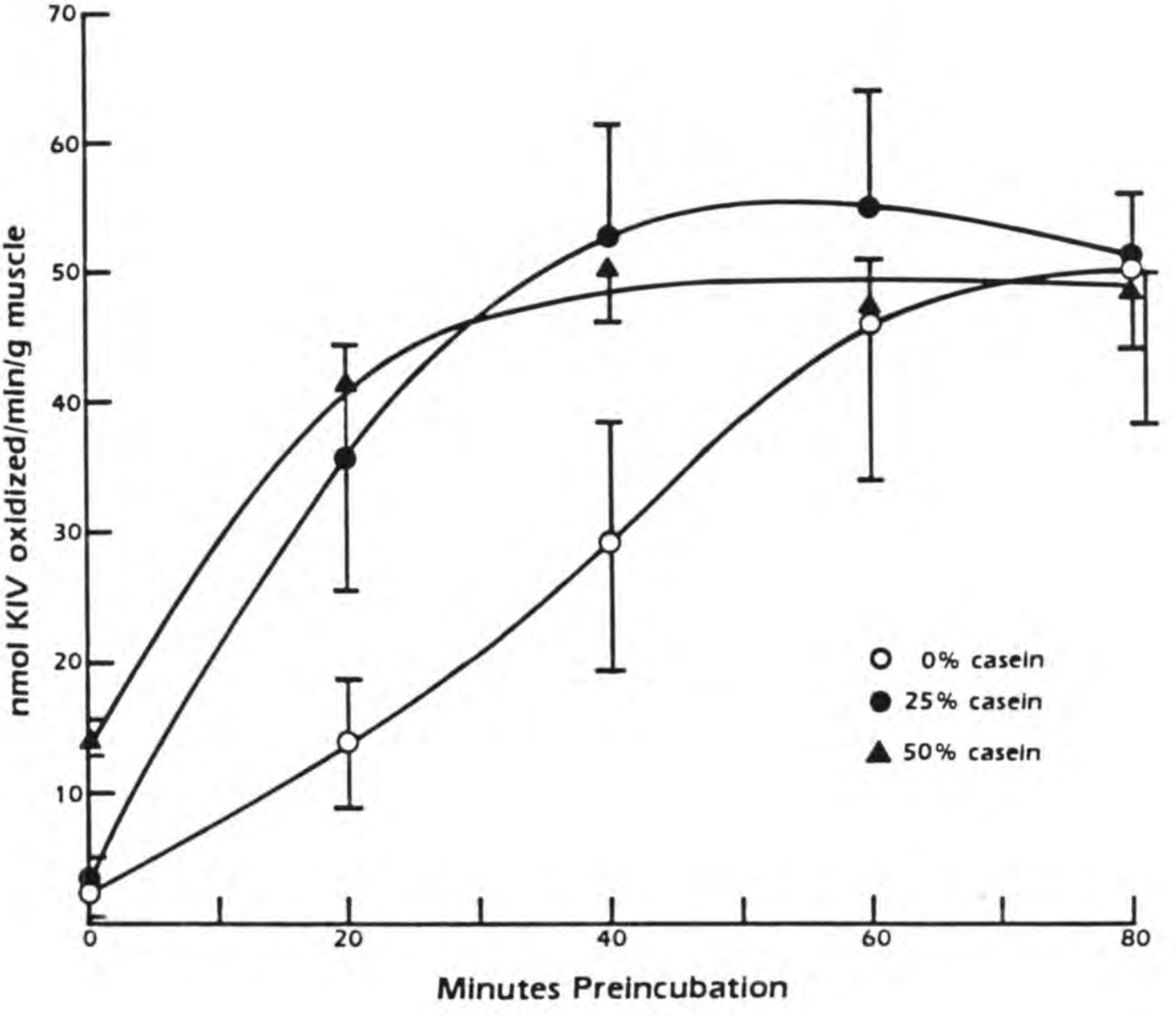

70
60
50
40
30
20
10
0
20
40
60
80
nmol KIV oxidized/min/g muscle
Minutes Preincubation
0% casein
25% casein
50% casein

Figure 3. Chronic Effects of Dietary Protein on the Percentage of Active Muscle BCDH and the Plasma Concentration of Leucine.

Rats were meal-fed diets containing 0, 9, 25 or 50% casein for 10 days. On the tenth day, rats were killed before (open bars) or 3 hours after (hatched bars) presentation of the meal. All postprandial rats had food in their stomachs at the time of sacrifice. Muscle BCDH activity was measured as described in Materials and Methods and the percent active complex (Panel A) computed as basal (in vivo) activity divided by total (fully-active) activity x 100. Total BCDH activities for each diet group are given in Table 1. Plasma leucine concentrations (Panel B) were measured as described in Materials and Methods. Before meal values represent means ± SEM for n = 5 (0%, 9%, 50% casein) or 11 (25% casein) rats per group. After meal values represent means ± SEM for 8-10 rats per group. * $p < 0.01$ compared to before meal values.

Figure 3.

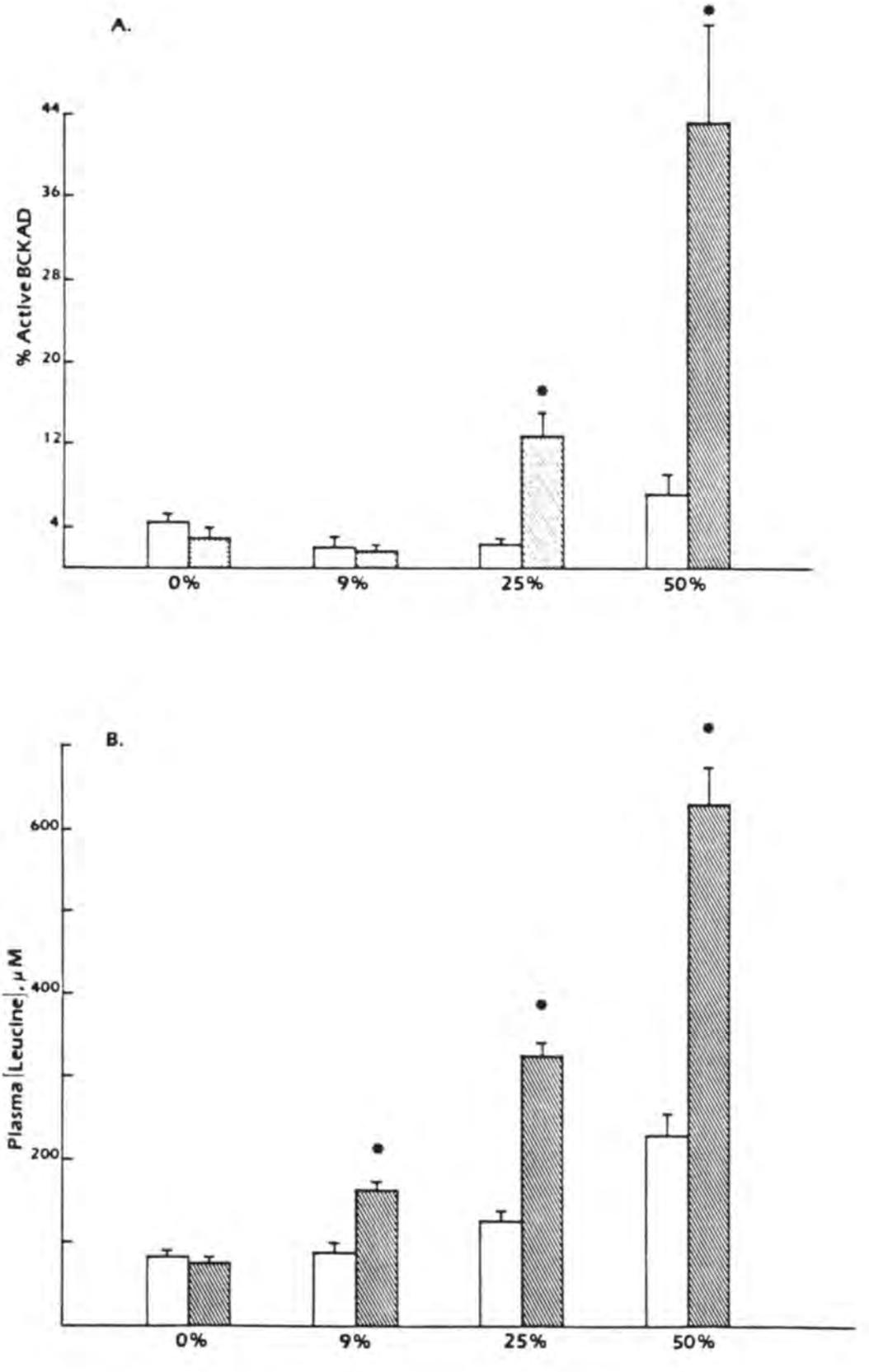

A.
% Active BCKAD
44
36
28
20
12
4
0%
9%
25%
50%
B.
Plasma [Leucine], μM
600
400
200
0%
9%
25%
50%

Figure 4. Effects of Single Meals of Varying Protein Content on Muscle BCDH and the Plasma Concentration of Leucine.

Rats were meal-fed a diet containing adequate amounts of protein (25% casein) for approximately 2 weeks, then killed before or 3, 6 or 12 hours after presentation of meals containing 9%, 25% or 50% casein. Food was withdrawn 3 hours after presentation. All rats had food in their stomachs after 3 hours and no difference in food intake was observed (14 ± 1 grams consumed, n = 15 rats). Muscle BCDH activity (Panel A) and plasma leucine concentrations (Panel B) were measured as described in Materials and Methods. Values represent mean ± SEM for 4-5 rats per group.

Figure 4.

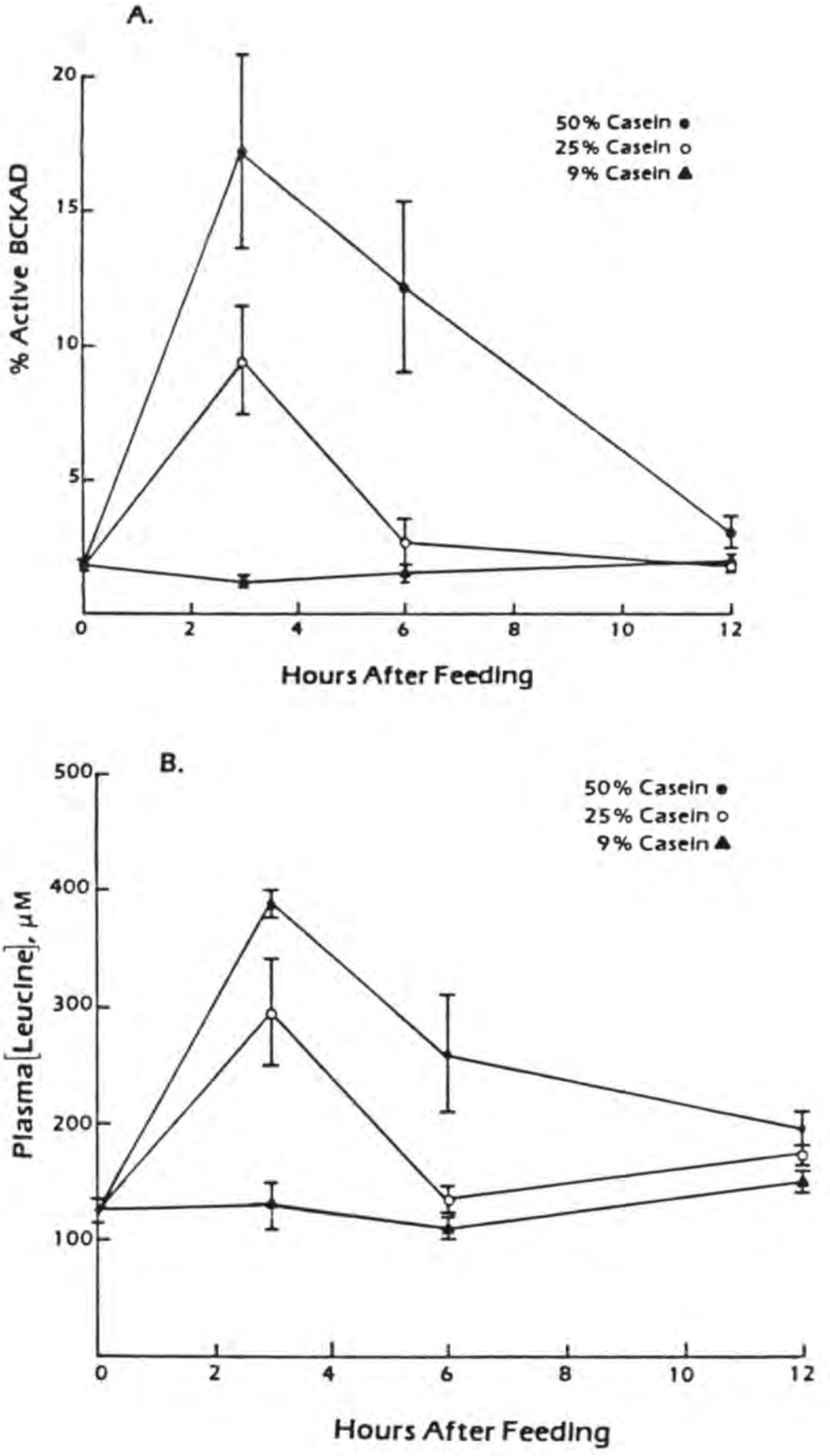

A.
% Active BCKAD
20
15
10
5
0
2
4
6
8
10
12
Hours After Feeding
50% Casein •
25% Casein o
9% Casein ▲
B.
Plasma[Leucine], μM
500
400
300
200
100
0
2
4
6
8
10
12
Hours After Feeding
50% Casein •
25% Casein o
9% Casein ▲

Figure 5. Relationship of Skeletal Muscle BCDH Activity to the Concentration of Circulating Leucine.

Values from the chronic (closed circles) and acute (open circles) protein studies were taken from Figures 3 and 4 respectively. The curve shown was calculated from the best linear least squares fit which was obtained after semilog transformation of the data (i.e. log % active complex vs plasma leucine) and yielded a linear correlation coefficient of 0.848 ($p < 0.001$; $n = 107$). Analysis of the non-transformed data by linear regression yielded a correlation coefficient of 0.788 ($p < 0.001$, $n = 107$).

Figure 5.

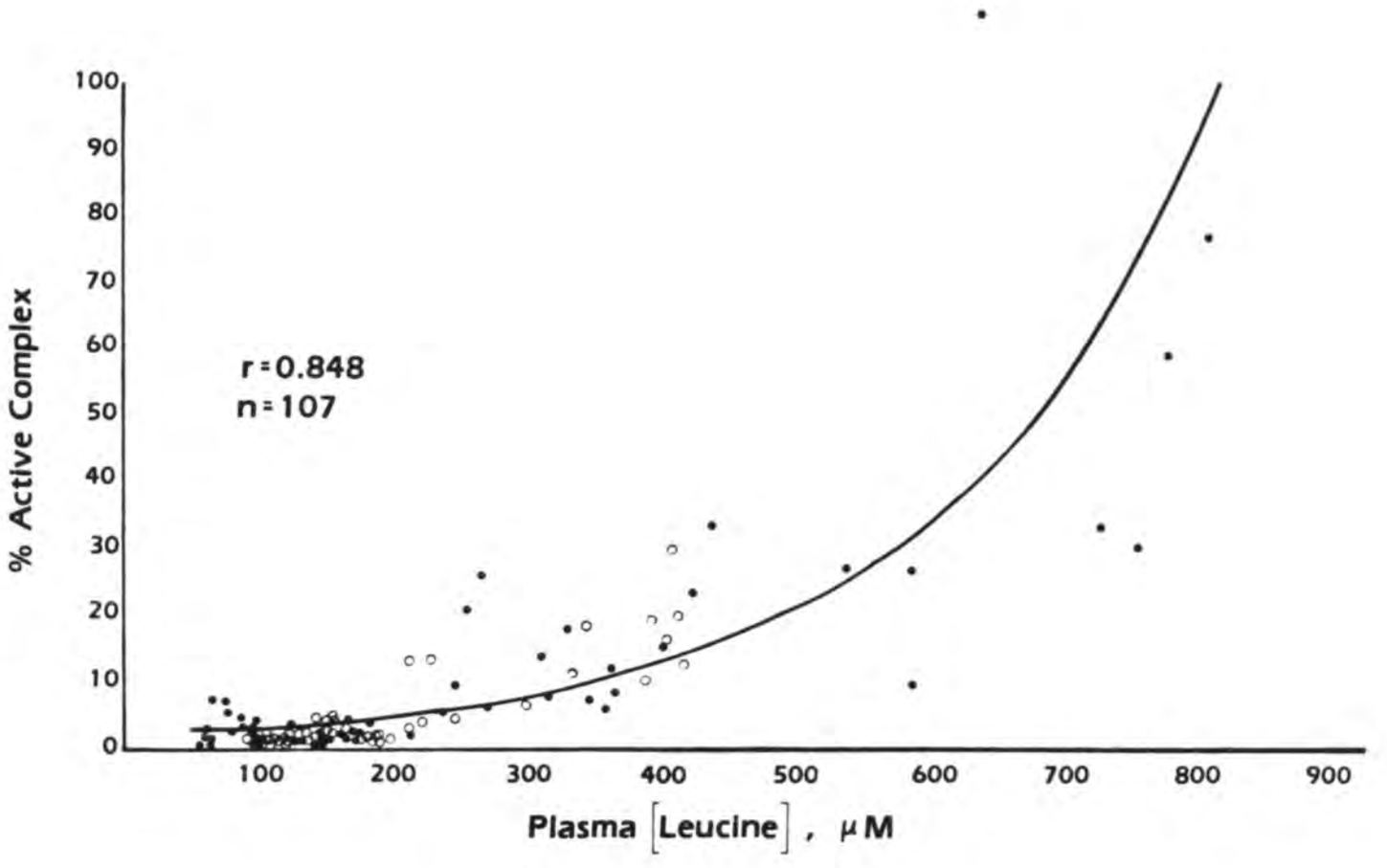
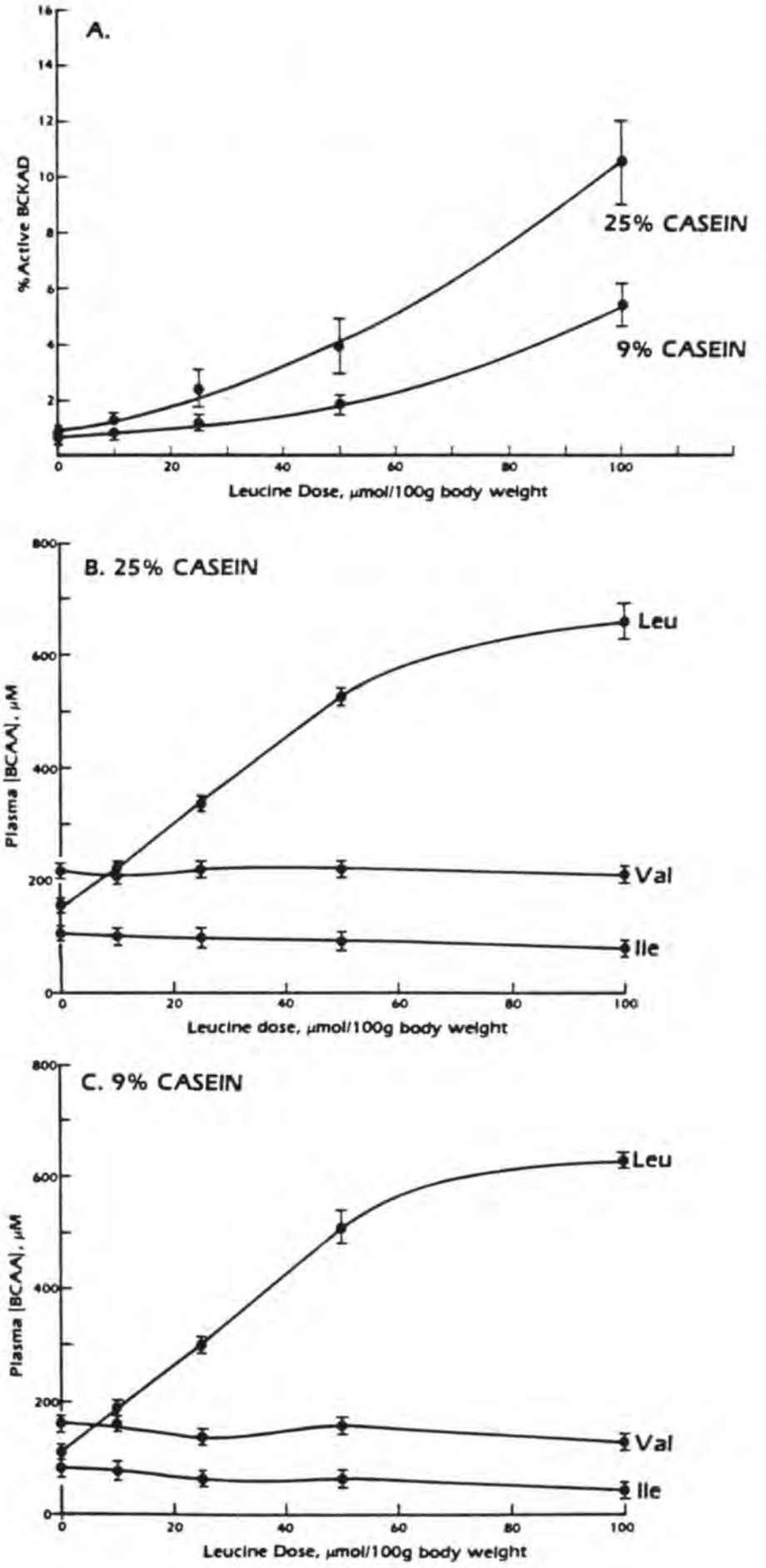

% Active Complex
100
90
80
70
60
50
40
30
20
10
0
r = 0.848
n = 107
100
200
300
400
500
600
700
800
900
Plasma [Leucine], μM

Figure 6. Leucine-Mediated Activation of Muscle BCDH Isolated From Rats Fed Adequate or Low Protein Diets.

Rats were fed a 9 or 25% casein diet ad libitum for approximately 2 weeks. After an overnight fast, rats were infused with saline or 10, 25, 50 or 100 μmol L-leucine per 100 g body weight via the tail vein. Rats were killed 15 minutes after infusion and the percentage of BCDH in the active state (Panel A) and plasma BCAA concentrations (Panels B and C) were measured as described in Materials and Methods. Values represent means ± SEM for 4 rats per group. BCDH activation in response to leucine was significantly depressed in rats fed the low protein diet ($p < 0.01$ after injection of 50 or 100 μmol leucine/100 g; $p < 0.001$ by comparison of the slopes of the regression lines for leucine dose vs. BCDH activity).

Figure 6.


A.
%Active BCKAD
25% CASEIN
9% CASEIN
Leucine Dose, μmol/100g body weight
B. 25% CASEIN
Plasma [BCAA], μM
Leu
Val
Ile
Leucine dose, μmol/100g body weight
C. 9% CASEIN
Plasma [BCAA], μM
Leu
Val
Ile
Leucine Dose, μmol/100g body weight

Chapter V

Effects of Diabetes and Starvation on Skeletal Muscle Branched-Chain α-Keto Acid Dehydrogenase Activity

Summary

Branched-chain α-keto acid dehydrogenase (BCDH)* is rate limiting for branched-chain amino acid (BCAA) catabolism in muscle and is regulated by phosphorylation-dephosphorylation. Circulating BCAA increase with insulinopenic diabetes and starvation despite accelerated oxidation. The activation state of BCDH was studied in rat hindlimb muscles. Diabetes (streptozotocin-induced and spontaneous BB/W) increased circulating BCAA 4-5 -fold and BCDH activity ~3-fold. Insulin treatment caused near normalization of circulating BCAA without correcting BCDH activity. Adrenalectomy of diabetics decreased (without normalizing) circulating BCAA and BCDH activation. Starvation caused mild, progressive increases in circulating BCAA and significant activation of BCDH only after 4 days. Leucine infusion activated BCDH in control, fed rats; the response was blunted by diabetes and to a lesser degree by starvation. In isolated perfused hindlimbs (control and diabetic) insulin did not affect BCDH significantly; perfusion with leucine activated BCDH, this response was blunted in diabetics. Activation of muscle BCDH may contribute to increased BCAA catabolism in diabetes; blunted activation response to hyperleucinemia may spare BCAA and contribute to their persistent elevation in plasma.

Introduction

Starvation and diabetes mellitus are conditions associated with elevated circulating concentrations and increased metabolism of the essential branched-chain amino acids (BCAA), leucine, valine and

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* Abbreviation used in this chapter are: BCAA, branched-chain amino acids; BCDH, branched-chain α-keto acid dehydrogenase.

isoleucine, in humans (154,178,179) and animal models (6,139,182). BCAA are unique among the essential amino acids as they are catabolized extensively by extrahepatic tissues (77); skeletal muscle is thought to play a major role in their disposal (5,67,77). Skeletal muscles from diabetic or starved rats incubated in vitro (6,27,139) and perfused hindlimbs from starved rats exhibit accelerated oxidation of BCAA (93). Insulin addition had no effect on the oxidation of medium leucine by diaphragms from diabetic (27) or normal (115) rats incubated in vitro but in perfused hindlimbs insulin caused an immediate increase in leucine oxidation in preparations from starved rats but not from fed rats (93).

In humans with type I diabetes mellitus the total body oxidation of BCAA is markedly increased after insulin withdrawal (154,179). Yet, BCAA uptake by tissues of the leg (predominantly muscle) after a protein meal was greatly diminished in insulinopenic patients (188). In normal humans BCAA uptake by the leg was found to be independent of the insulin concentration (53); but in experiments using tracer techniques insulin decreased leucine oxidation and turnover in a dose-dependent manner in normal humans (175). Similarly, insulin decreased leucine release by liver and muscle in normal dogs (1).

The activity of branched-chain α-keto acid dehydrogenase complex (BCDH), which catalyzes the irreversible oxidative decarboxylation of the three branched-chain α-keto acids produced by reversible transamination of the BCAA, is considered rate-limiting for BCAA catabolism by skeletal muscle (77,131). BCDH, is an intramitochondrial multienzyme complex analogous to other α-keto acid dehydrogenase complexes

(e.g. pyruvate). The activity of BCDH is regulated by a reversible phosphorylation (inactive)-dephosphorylation (active) cycle, catalyzed by specific BCDH kinase (77,142,150) and BCDH phosphatase (152) enzymes. In the postabsorptive state, BCDH is nearly totally inactive in skeletal muscle of fed rats (Chapter 4,5,15,77,150,184), but muscle BCDH activity increases rapidly following a protein meal (Chapter 4) or i.v. infusion of small doses of leucine (5). The activity of muscle BCDH also increases after administration of large doses of isoleucine but valine administration had no effect; this presumably reflects the relative efficacy of the ketoanalogues of the BCAA at inhibiting BCDH kinase (ketoleucine > ketoisoleucine >> ketovaline; refs. 142,150).

The activity of BCDH in rat kidney, and liver decreased while the heart activity was unaffected by alloxan diabetes (151), but BCDH activity decreased in each of these tissues after 2 days of starvation (151). BCDH activity in rat muscle homogenates was unaffected by 3 days of starvation (184). Considering that under physiological conditions muscle BCDH is activated in response to increases in circulating BCAA (Chapter 4,5), increased muscle BCDH activity would be expected in diabetes or starvation. Using diaphragm muscles incubated in vitro no evidence for this could be obtained (6). As mentioned above, the role of insulin in regulating BCAA oxidation in muscle is not clear (1,27, 93,151); there is considerable evidence however that insulin retards whole body BCAA catabolism (1,65). Whether this is due solely to the protein anabolic effects of insulin (i.e. stimulation of protein synthesis and inhibition of protein degradation) decreasing BCAA flux through BCDH, or BCDH activity is also affected remains unresolved. In

the present study a recently developed method for determining the activity state of skeletal muscle BCDH in vivo was used to assess changes in the activity of this enzyme complex during insulinopenic diabetes and starvation, and to determine whether the activity of BCDH is subject to acute regulation by insulin.

Materials and Methods.

Animals and Animal Treatments. Male Wistar rats, strain WI(BR), were obtained from Charles River Laboratories, Wilmington, MA. In some studies a spontaneously diabetic strain of Wistar rats (BB/W) was used (116); these were kindly supplied by Dr. Arthur Like of the University of Massachusetts, Worcester, MA. Rats were housed in an animal facility maintained at 25°C with a 12 hour light-12 hour dark cycle (light from 0600 - 1800). Animals were maintained on Wayne Rodent Blox (Continental Grain, Chicago, IL) chow (24% protein) and water, both supplied ad libitum.

Diabetes mellitus was induced by a single intraperitoneal injection of streptozotocin (120 mg/kg body weight in 0.05 M sodium citrate, pH 4.5). Rats were tested for glycosuria with Tes-Tape (Eli Lilly & Co., Indianapolis, IN) the day following streptozotocin injection. All rats having greater than 2+ glycosuria were started on a regimen of 2 units Humulin R insulin + 2 units Humulin N insulin (Lilly) injected subcutaneously each morning and evening. Insulin therapy was maintained for at least 60 hours then was discontinued 72 hours prior to sacrifice when untreated diabetic animals were studied; these animals were presumed to have been insulinopenic for about 60 hours. Insulin-treated animals were continued on the above insulin therapy but the

morning of the experiment they received only 2 units Humulin R insulin 2-3 hours prior to sacrifice.

BB/W rats were checked daily for glycosuria; after glycosuria developed plasma glucose was determined on blood obtained from the tail veins. Diabetic BB/W rats were treated with single daily injections of 1.2-2 units of protamine zinc insulin (Lilly), with the dose being adjusted to maintain glycosuria less than 3+ by Tes-tape and no ketonuria as determined with Ketostix (Ames Division, Miles Laboratories, Elkhart, IN). When untreated BB/W diabetic rats were studied insulin therapy was discontinued 72 hours prior to sacrifice.

Adrenalectomy of diabetic rats was performed on the day of their last insulin injection under ether anesthesia either by bilateral subcostal approaches or a dorsal approach. Adrenalectomized animals were supplied 0.9% (w/v) NaCl to drink ad libitum, and killed on the third day after operation. Rats weighed between 150 and 220 g at the time of sacrifice, except for BB/W rats and the rats used for hindlimb perfusions which typically weighed 280 to 350 g.

In some experiments central venous catheters were placed, under light ether anesthesia, in an internal jugular vein of rats as previously described (6,76). Catheters were placed ~20 hrs prior to leucine infusions. Leucine (0.1 M in 0.9% NaCl, 250 μmol/kg b.w.) or an equal volume of saline was infused by jugular catheter or by tail vein injection while rats were under light methoxyflurane (Pitman-Moore, Washington Crossing, NJ) anesthesia. Muscle and blood samples were obtained as described below 15 min after leucine or saline infusion.

BCDH Assay. The method to assess BCDH activity in vivo was described in detail previously (5,15). Briefly, rats were anesthetized with methoxyflurane and muscle samples frozen in situ at liquid nitrogen temperature. BCDH complex was extracted from powdered frozen muscle in a detergent solution containing protease, phosphatase and kinase inhibitors. In vivo BCDH activity was estimated using the freshly prepared complex and total (dephosphorylated) activity estimated after incubation at 37°C with 15 mM Mg^{2+}; % active complex was calculated from the ratio of in vivo to total BCDH activities in the same muscle sample. Enzyme activity was estimated as $^{14}CO_2$ release from 0.1 mM α-keto[1-^{14}C]isocaproate, as described previously (5,15). Preliminary experiment established that total BCDH activity in muscle extracts prepared from diabetic rats reached a maximum after 60 min incubation with Mg^{2+}, as previously found in normal rats (5).

Hindlimb Perfusions. Isolated rat hindlimb preparations were prepared under pentobarbital anaesthesia and perfused essentially as described by Jefferson (98). The perfusion medium consisted of Krebs-Henseleit buffer containing aged, rejuvenated human erythrocytes (12 g hemoglobin per 100 ml), 4% (w/v) bovine serum albumin, and 5.5 mM D-glucose; insulin when present was added at a concentration of 10 mU / ml. During preparation an additional tie was loosely placed around the internal iliac vessels on one side. After 15 min of perfusion one limb was skinned, the gastrocnemius freeze-clamped in situ and removed, then the flow rate was reduced, the internal iliac tie knotted and the limb isolated by an umbilical tape tie at the level of the hip. Leucine infusion was started via a second pump connected piggy-back to the main

perfusion line set to produce a final concentration of 1 mM in the perfusate. After 15 min additional perfusion the remaining gastrocnemius was frozen in situ and removed.

Amino acid and glucose determinations. Blood samples were collected by cardiac puncture using a heparin-treated syringe and plasma deproteinized by addition of 0.1 volume of 50% (w/v) sulfosalicylic acid. Amino acids were extracted from frozen powdered muscle samples by addition of ~4 ml/g ice-cold 6% sulfosalicylic acid. Particulate material in all preparations was removed by centrifugation and the samples stored at -20°C or below until amino acid concentrations were determined by reversed-phase high performance liquid chromatography of the o-phthalaldehyde derivatives with fluorescent detection as described previously (6). Plasma glucose concentrations were determined by a glucose oxidase method using a Beckman Glucose Analyzer 2 (Beckman Instruments Inc., Fullerton, CA). Reagents were obtained from sources described previously (5,6,15) and were reagent grade or higher quality.

Statistics. All values are presented as mean ± SEM. Comparisons were conducted using either Student's t test or analysis of variance followed by a multiple comparison test (Peritz F test; 80), where appropriate. Analysis of values expressed in per cent was conducted after angular transformation, to assure normal distribution (173).

Results

Muscle BCDH activity in stretozotocin-diabetic rats. The in vivo activity of muscle BCDH in rats rendered diabetic with streptozotocin was about 2.5-fold greater than in normal, fed rats whether expressed as actual activity or % active complex (Table 1, without leucine

infusion). Plasma leucine was increased more than 5 fold by diabetes, similar increases were observed in plasma valine and isoleucine (data not shown). Insulin treatment of diabetic rats markedly decreased plasma glucose concentrations (from 512 ± 37 mg/dl in diabetic rats to 70 ± 13 mg/dl in insulin treated diabetic rats compared with 171 ± 8 in normal rats) and decreased plasma leucine concentrations to normal values (Table 1). BCDH activity remained about 2-fold greater in insulin-treated diabetic rats compared to normal rats; BCDH activity in insulin-treated diabetic rats was greater than in normals, while the % active complex in insulin-treated diabetics was between the values for normals and diabetics and not different from either. Total BCDH activities were the same in control, diabetic and insulin-treated rats.

Muscle BCDH activity in spontaneously diabetic rats. Considering the possibility that streptozotocin could have a direct effect on BCDH or one of its regulatory enzymes, causing activation of BCDH which could not be reversed with insulin therapy, the activity of BCDH was investigated in a spontaneously diabetic strain of rats. In the BB/W diabetic rat BCDH activity was increased more than 3-fold over the activity in non-diabetic litter-mate rats (Table 2). Insulin therapy did not return BCDH activity to the low levels present in non-diabetic BB/W rats; in vivo BCDH activity in insulin-treated diabetics was not different from activities in either diabetic or non-diabetic rats, while the % active complex in insulin-treated diabetics was different from non-diabetic values only. In BB/W diabetic rats total BCDH activity tended to be lower than in non-diabetic and insulin-treated diabetic rats but this effect was not significant (Table 2).

As in the streptozotocin-diabetic rats plasma BCAA were dramatically elevated in the BB/W diabetic rats compared to the non-diabetic controls (Table 3). Insulin therapy restored the plasma concentrations of valine and isoleucine to normal. The plasma leucine concentration in insulin-treated BB/W diabetic rats was much lower than in the diabetic rats but remained about 30% greater than in the non-diabetic rats. The calculated intracellular concentrations of BCAA in muscles of non-diabetic rats were lower than the plasma concentrations, while in the BB/W diabetic rats the calculated intracellular BCAA were not significantly different from the plasma concentrations. Insulin therapy decreased intramuscular BCAA concentrations to values that were not different from the values in the non-diabetic rats.

Muscle BCDH activity in adrenalectomized streptozotocin-diabetic rats. Glucocorticoid administration was reported to activate muscle BCDH (15) suggesting that endogenous corticosterone might contribute to the activation of BCDH in diabetes; this possibility was investigated in adrenalectomized, streptozotocin-diabetic rats (Table 4). Total BCDH activity was unaffected by adrenalectomy. In vivo BCDH activity in diabetic rats was reduced by adrenalectomy but remained greater than in normal, fed rats. Similarly, plasma leucine in diabetic rats was markedly reduced by adrenalectomy but remained greater than in the control rats.

Effect of leucine infusion on muscle BCDH activity in streptozotocin-diabetic rats. In previous studies muscle BCDH was activated by moderate increases in circulating leucine concentration from dietary protein intake (Chapter 4) or intravenous administration (Chapter 4,5).

In view of the marked elevations in circulating and intracellular BCAA in diabetic rats a greater increase in muscle BCDH activity than was observed might be expected. This suggests that diabetes renders muscle BCDH refractory to activation by increased leucine. Therefore, a bolus of leucine (250 μmol/kg) was administered i.v. and BCDH activity determined in muscle samples taken 15 minutes later (Table 1). In normal, fed rats leucine infusion increased BCDH activity 4-fold; plasma leucine increased about 50% from 151 μM to 234 μM. In streptozotocin-diabetic rats leucine infusion increased BCDH activity and plasma leucine by about 50% but neither increase was significant; BCDH activity increased only about 20% when expressed as % active complex. Muscle BCDH activity increased greater than 2.5-fold in insulin-treated streptozotocin-diabetics after leucine infusion and was indistinguishable from the activity observed in normal rats after leucine infusion. Plasma leucine increased from 164 μM to 219 μM in insulin-treated diabetic rats after leucine infusion which was similar to the increase in normal rats.

Muscle BCDH activity in starved rats. BCDH activity increased slightly after one day of starvation, significant only when comparing % active complex. BCDH activity then was constant until the fourth day of starvation when it increased abruptly (Table 5). Plasma leucine increased slowly over the time course of starvation and was significantly different from the value in the fed rats on the fourth day of starvation (Table 5); a significant positive correlation was observed between plasma BCAA and duration of starvation from 0 to 4 days (r =

0.48, 0.56, 0.56 for valine, isoleucine and leucine, respectively; n = 32; $p < 0.01$).

To assure that measurements of total BCDH activity in these studies were not biased by different rates of BCDH activation in vitro as reported for liver BCDH in rats fed a protein-deficient diet (138) and for muscle BCDH in rats fed a low protein diet (Chapter 4), the time course of activation of BCDH in vitro in the presence of Mg^{2+} was determined (Figure 1). No apparent differences in the rate of activation or the total activity attained were observed between fed rats and rats fasted 1 to 4 days; maximum BCDH values were attained at 60 min and all further determinations used 60 min incubations for determination of total BCDH activity. In the small group of animals shown in Figure 1, total BCDH activity per g muscle increased with the duration of starvation; but when larger numbers of animals were analyzed the trend toward increased total BCDH activity per g muscle remained but was not significant (Table 5).

The response of BCDH to i.v. infusion of leucine was determined in fed rats and after 2 or 4 days of starvation. Muscle BCDH activity increased after leucine infusion, regardless of the duration of starvation. The BCDH activity after 4 days of starvation was similar to that in fed rats after leucine infusion and plasma leucine concentrations were similar in these two groups. The BCDH activity attained after leucine infusion was indistinguishable in the fed and starved rats but progressively greater plasma leucine concentrations were found in the rats starved for 2 or 4 days (Table 5).

Effect of insulin and leucine on perfused hindlimbs of normal and streptozotocin-diabetic rats. The acute effects of insulin and leucine on the activity of BCDH in muscle were investigated in isolated, perfused hindlimbs of normal and streptozotocin diabetic rats (Table 6). Addition of insulin to the perfusate resulted in slightly higher BCDH activities both in the absence and presence of leucine in hindlimbs from control and diabetic rats, but the insulin effect was not significant in any of the groups (Table 6). When BCDH activities in all hindlimbs from diabetic rats were compared to all hindlimbs from normal rats, in vivo BCDH activity in diabetic hindlimbs was doubled ($p < 0.01$); the effect of diabetes was not significant when values for % active complex were compared, presumably due to differences in the total BCDH activities. Addition of leucine to the perfusate resulted in activation of BCDH in hindlimbs from both control and diabetic rats; no additional effect of insulin was observed. After leucine infusion, BCDH activity in hindlimbs from diabetic rats was less than in hindlimbs from normal rats; the effect was significant only when values for % active complex for all hindlimbs receiving leucine were compared (Table 6). In this group of experiments total BCDH activity per g muscle was greater in muscles from diabetic rats than in those from normal rats; there was no effect of insulin or leucine on total activity.

Discussion

The present studies establish that muscle BCDH is activated in diabetes as would be anticipated in view of the marked increases in BCAA concentrations in plasma and intracellularly. The degree of BCDH

activation observed in diabetic muscles was less than previously observed in normal rats with similar increases in plasma leucine from dietary protein or leucine infusion. For example, rats maintained on a 50% protein diet had postprandial plasma leucine concentrations greater than 600 μM and muscle BCDH greater than 40% active (Chapter 4) and in fed rats maintained on a 24% protein diet infusion of 250 μmol leucine per kg body weight increased plasma leucine to 200 - 230 μM 15 min post infusion, with BCDH 7-10% active (Tables 1,5; ref. 5). Furthermore, muscle BCDH in diabetic rats did not activate after infusion of a leucine dose which clearly activated BCDH in normal and insulin-treated diabetic rats (Table 1), and BCDH in perfused hindlimbs of diabetic rats activated less than in controls when leucine was added to the perfusate (Table 6). The latter observation suggests that decreased activation of muscle BCDH by leucine in diabetes is not due to alterations in a hormonal response to leucine infusion, and this refractoriness persists for at least 30 min after the muscles are removed from their metabolic milieu. The activation of muscle BCDH in hindlimbs of diabetic rats by perfusion with a leucine concentration which did not activate muscle BCDH in vivo in diabetic rats (compare Tables 1 and 6) suggests however that the metabolic milieu in vivo is at least partly responsible for the blunted response to leucine. Since the isolated hindlimbs were perfused without added amino acids (except for leucine) it is unlikely that the decreased response to leucine in diabetic rats in vivo results solely from competition of leucine with other large neutral amino acids (especially valine and isoleucine) for transport across the plasmalemma. Competition for transport could however

contribute to the blunted response to leucine in vivo. Impaired activation of muscle BCDH by increased BCAA in diabetes may contribute to the decreased clearance of exogenous leucine (51) and the decreased BCAA uptake by legs of diabetic patients following a protein meal (188).

Insulin-therapy of diabetic rats tended to decrease plasma BCAA toward normal values (Tables 1,3,4), but did not restore BCDH activity despite nearly complete normalization of plasma BCAA by insulin in one experiment (Table 1). Persistent activation of muscle BCDH despite insulin therapy was not due to a toxic effect of streptozotocin since similar results were obtained in spontaneously diabetic rats (Table 2). Increased BCDH activity in diabetic rats treated with insulin could be due to elevation of the intracellular leucine concentration as suggested in the studies of BB/W rats (Table 3), coupled with improved response of muscle BCDH to increased leucine (Table 1). Plasma BCAA were considered poor indicators of normalization of amino acid metabolism by skeletal muscle in humans with type I diabetes mellitus since the intramuscular BCAA (and presumably branched-chain α-keto acid) concentrations remained elevated despite their normalization in plasma (18). Furthermore, the intramitochondrial concentration of ketoleucine (the transamination product of leucine, which is the substrate for BCDH and an inhibitor of BCDH kinase) may remain elevated even after intracellular leucine has returned toward normal (92). Our results are consistent with reports in patients with type I diabetes demonstrating that restoration of the metabolic parameters of leucine (catabolism and flux as assessed by tracer techniques in vivo) to normal values was

difficult to achieve with conventional or with insulin pump therapy (154,179). Based on measurements in heart, liver and kidney, Randle estimated that whole body BCDH active complex decreased by 50% in alloxan diabetes (151). The increase in muscle BCDH activity reported here suggests that muscle (and perhaps brain, ref. 21) are the probable sites of increased BCAA oxidation in insulinopenic diabetes.

The near normalization of plasma BCAA in diabetic-adrenalectomized rats highlights the important role of glucocorticoids unopposed by insulin in the dramatic increases in plasma BCAA observed in diabetes. The nature of the glucocorticoid effect on circulating BCAA is not clear. The predominant effect of exogenous corticosterone on skeletal muscle protein turnover in the rat was found to be inhibition of protein synthesis with a much lesser effect on protein degradation (130), although the effect of corticosterone on protein degradation was enhanced in diabetic rats (177). Protein synthesis was depressed by 50% or more in skeletal muscles of diabetic rats after about 72 hours of insulinopenia and protein degradation was increased (134) but the role of endogenous unopposed glucocorticoids in these changes remains undefined. The possibility of differences in food consumption between the diabetic and diabetic-adrenalectomized rats was evaluated in one experiment. During the 24 hour period immediately prior to sacrifice sham-operated diabetic rats consumed significantly more food than the adrenalectomized group; 20.6 ± 1.6 g and 9.1 ± 2.6 g, respectively, with 6 animals per group ($p < 0.01$). During the preceding 24 hour period,(24 - 48 hours after operation) food intakes were similar between the two groups, 8.6 ± 1.9 g for adrenalectomized and 9.3 ± 1.6

g for sham-operated diabetic rats. At present it is not possible to assess the relative importance of these factors (food consumption, protein synthesis and protein degradation) in the contribution of corticosterone to BCAA elevation in the diabetic rat. It appears however that glucocorticoids contribute to diabetes associated hyperphagia.

In starvation, muscle BCDH activity was not increased significantly until the fourth day (Table 5); previous studies also found no change in muscle BCDH activity after 3 days of starvation (184). These results contrast with studies of muscles incubated in vitro and perfused rat hindlimbs where oxidation of leucine was increased earlier in starvation (6,93). Recent studies of leucine metabolism in the rat in vivo have established that whole body oxidation of leucine is indeed accelerated after three days of starvation (182). It seems unlikely that the small increment in muscle BCDH activity observed in rats starved for one to three days accounts for accelerated leucine oxidation early in starvation. Accelerated oxidation of BCAA may be due to increased flux through BCDH resulting from increased substrate supply, since muscle and plasma branched-chain α-keto acids (and BCAA) were increased in rats starved for three days (93,182) and leucine transamination was increased in diaphragm muscles after an overnight fast (6).

The clearance of infused leucine is markedly decreased by three or four days of starvation in man (51). A dose-response relationship between leucine infused and muscle BCDH activity has been reported in rats (Chapter 4;5), and there was a direct correlation between plasma

leucine concentration and BCDH activity (Chapter 4). In the present studies muscle BCDH in starved rats was readily activated by leucine infusion, unlike muscle BCDH in diabetic rats. Comparison of the plasma leucine concentrations in the starved rats after leucine infusion (Table 5) with those in fed rats (Tables 1,5) suggest that in starvation the normal relationship between plasma leucine and muscle BCDH activity may be disturbed (i.e. BCDH in starved rats may be less active after leucine infusion than it would be in fed rats at similar plasma leucine concentrations). Blunted response of muscle BCDH to infused leucine was previously observed in rats fed a protein-restricted diet (Chapter 4). Activation of muscle BCDH may play a greater role in the accelerated oxidation of BCAA in diabetes than in starvation (compare Tables 1 and 2 to Table 5). Although activation of BCDH by increased BCAA (leucine probably being most important) apparently can occur in both diabetes and fasting, the response is less than normal and may contribute to decreased clearance of exogenous leucine in these states.

The tendency toward increased total muscle BCDH activity with duration of starvation (Table 5) is consistent with previous studies which found increased total BCDH activity in the gastrocnemius after 3 days of starvation (184). Since the BCDH values are presented on a tissue weight basis it seems likely that increased total activity represents sparing of BCDH from catabolism relative to other cellular constituents rather than increased synthesis of BCDH during starvation. Increased total BCDH activity per g muscle was also observed in diabetic rats (Table 6) but this was not confirmed in other experiments

(Tables 1,2). There is no apparent explanation for these discrepancies.

We were unable to detect a significant acute effect of insulin on the activity of muscle BCDH or its response to increased circulating leucine in studies of perfused hindlimbs from normal,fed or from diabetic rats (Table 6). Studies of leucine fluxes reported that insulin rapidly stimulated leucine oxidation by perfused hindlimbs of rats starved for three days, but decreased oxidation by hindlimbs of fed rats (93). The latter effect was thought to reflect decreased flux through BCDH secondary to increased leucine utilization for protein synthesis. In a previous study, the BCDH activity of skeletal muscle was unaffected by administering large doses of insulin with glucose to normal, fed rats (15). The present studies suggest that, in fed diabetic or control rats, insulin is not required for activation of muscle BCDH by increased leucine, supporting the concept that leucine catabolism by skeletal muscle is relatively independent of insulin (53). However, as insulin concentrations wane during fasting or after insulin withdrawal in diabetes, and the ensuing net protein catabolism increases BCAA available for oxidation, the normal activation of BCDH by increased BCAA is blunted; this may contribute to the elevation of circulating BCAA observed during starvation and more markedly in insulinopenic diabetes.

The mechanism of diminished muscle BCDH response to leucine in diabetes and starvation is unresolved. The system appears to become refractory to normal regulation, e.g. in incubated diaphragms of fasted and diabetic rats flux through BCDH is resistant to inhibition by

pyruvate, which is normally observed in controls (6). Since activity of BCDH is dependent on the balance of the kinase and phosphatase reactions alterations in the activity of these enzymes may be important. Indeed, increased BCDH kinase activity was demonstrated in heart mitochondria of rats starved for two days (55). BCDH kinase is subject to inhibition by branched-chain α-keto acids (particularly ketoleucine (142), presumably accounting for the direct relationship between BCDH activity and leucine concentration;Chapter 4, ref. 5), hence alterations in mitochondrial branched-chain α-keto acid transport could affect the relationship between leucine and BCDH activity. Recent reports indicate that branched-chain α-keto acid transport across the mitochondrial membrane is a regulated process (92,94) and may be a rate-limiting step for BCAA oxidation (94). The phosphatase reaction appears to be regulated in a complex fashion involving an inhibitory protein (152) but alterations in BCDH phosphatase activity in physiologic or pathologic states have not been reported. Other factors which may be altered in muscle cells by diabetes and fasting and may modulate activation of BCDH directly or indirectly include: redox potential (27), mitochondrial pH (94), acyl-CoAs (142,150,151), and possible protein inhibitors and activators of the complex (131,141,150). Further work is required to elucidate the mechanism(s) of altered muscle BCDH response to leucine in diabetes and fasting.

Legend for Table 1.

Muscle BCDH activity and plasma leucine concentration were determined in normal, streptozotocin-diabetic and insulin-treated diabetic rats as described under Materials and Methods. One group of rats received a leucine infusion (250 μmol/kg) through a jugular venous catheter as described under Materials and Methods; samples were taken 15 min after infusion. As a control for infusion, some rats in each group were infused with an equal volume of saline. The results in saline-infused and non-infused rats were not significantly different therefore values for all rats not receiving leucine were combined. Total BCDH activities were not significantly different between groups or treatments (40 ± 2 nmol / min / g muscle, n = 43). [a] $p < 0.005$ compared to control; [b] $p < 0.025$ compared to control; [c] $p < 0.025$ compared to no leucine infusion; [d] $p < 0.005$ compared to diabetic; [e] $p < 0.05$ compared to no leucine infusion.

Table 1. Effect of streptozocin-diabetes, insulin therapy and leucine infusion on muscle BCDH activity and plasma branched-chain amino acids.

	BCDH activity (nmol/min/g muscle)	% Active complex	Plasma leucine (μM)	n
		Without leucine infusion		
Control	0.9 ± 0.1	2.8 ± 0.3	151 ± 8	7
Diabetic	2.6 ± 0.3[a]	7.1 ± 1.1[b]	819 ± 202[a]	9
Insulin-treated	2.1 ± 0.4[b]	6.0 ± 1.2	164 ± 17[d]	6
		With leucine infusion		
Control	4.1 ± 0.3[c]	11.4 ± 2.7[c]	234 ± 13[c]	6
Diabetic	4.1 ± 0.6	8.6 ± 1.1	1255 ± 251[a]	8
Insulin-treated	5.5 ± 1.3[c]	14.5 ± 2.7[c]	219 ± 13[d,e]	7

Table 2. Muscle BCDH activity in BB/W rats.

Group	BCDH Activity (nmol/min/g muscle) In vivo	Total	% Active complex
Control	1.2 ± 0.2	46 ± 7	2.7 ± 0.4
Diabetic	4.1 ± 0.9[a]	29 ± 5	11.7 ± 3.2[b]
Insulin-treated	2.3 ± 0.6	38 ± 2	6.6 ± 1.6[a]

Muscle BCDH activity was determined in control, diabetic, and insulin-treated diabetic BB/W rats as described under Materials and Methods. Control rats were non-diabetic littermates of the diabetic BB/W rats. Values represent mean ± SEM from 6 animals in each group.

[a] $p < 0.05$ compared to control value; [b] $p < 0.005$ compared to control value.

Table 3. Plasma and muscle branched-chain amino acid concentrations in BB/W rats.

	Amino Acid Concentration (μM)		
	Leucine	Valine	Isoleucine
	Plasma		
Control	156 ± 14	248 ± 22	102 ± 10
Diabetic	648 ± 101[a]	886 ± 141[a]	378 ± 51[a]
Insulin-treated	204 ± 11[b,c]	250 ± 14[c]	120 ± 8[c]
	Muscle		
Control	112 ± 13[d]	158 ± 24[d]	52 ± 9[d]
Diabetic	660 ± 107[a]	837 ± 120[a]	371 ± 48[a]
Insulin-treated	160 ± 23[c]	207 ± 25[c]	81± 13[c,d]

Plasma branched-chain amino acid concentrations were determined as described under Materials and Methods. Muscle intracellular branched-chain amino acids were calculated from amino acid concentrations in muscle extracts according to the formula:

$$[\text{intracellular BCAA}] = \frac{(\text{nmol BCAA/g muscle}) - (0.2)(\text{nmol BCAA/ml plasma})}{0.6}$$

Values represent the mean ± SEM from six animals. [a] $p < 0.001$ compared to control value; [b] $p < 0.05$ compared to control value; [c] $p < 0.005$ compared to diabetic value; [d] $p < 0.05$ compared to plasma value.

Table 4. Effect of streptozotocin-diabetes and adrenalectomy on muscle BCDH activity.

	BCDH activity (nmol/min/g muscle)	% Active complex	Plasma leucine	n
Control	1.0 ± 0.1	2.7 ± 0.2	153 ± 6	12
Diabetic	3.4 ± 0.5[a]	9.3 ± 1.7[a]	719 ± 102[a]	19
Diabetic + Adrenalectomy	1.7 ± 0.2[a,b]	5.2 ± 0.9[a]	226 ± 20[a,b]	12

Muscle BCDH activity and plasma leucine concentration were determined in control, streptozotocin-diabetic, and adrenalectomized streptozotocin-diabetic rats as described under Materials and Methods. Normal and streptozotocin-diabetic rats were subjected to sham operation with no differences in BCDH activity or plasma leucine results compared to unoperated animals, the values shown for control and diabetic animals were pooled from sham-operated and unoperated animals. Total BCDH activities were not different between groups, 38 ± 1 nmol / min / g muscle (n = 43). [a] $p < 0.025$ compared to control; [b] $p < 0.05$ compared to diabetic.

Legend for Table 5.

Muscle BCDH activity and plasma leucine concentration was determined in fed rats and rats starved for 1 to 4 days as described under Materials and Methods. One group of rats received a leucine infusion (250 μmol/kg), samples were taken 15 min after infusion. As a control for infusion, some rats in each group were infused with an equal volume of saline. The results in saline-infused rats were not different from those in rats which received no infusion, therefore values for all rats not receiving leucine were combined. [a] $p < 0.01$ compared to 4 days of starvation; [b] $p < 0.05$ compared to fed rats; [c] $p < 0.05$ compared to no leucine infusion; [d] $p < 0.001$ compared to no leucine infusion; [e] $p < 0.05$ compared to 4 days of starvation; [f] $p < 0.01$ plasma leucine concentration in each group of rats receiving leucine infusion was different from the leucine concentrations in the remaining two groups.

Table 5. Effect of starvation on muscle BCDH and its activation by leucine infusion.

Days of starvation	BCDH activity (nmol/min/g muscle) Initial	BCDH activity (nmol/min/g muscle) Total	% Active complex	Plasma leucine
		Without leucine infusion		
0	0.6 ± 0.1[a]	39 ± 3	1.5 ± 0.2[a] (7)	145 ± 8[e] (7)
1	1.0 ± 0.2	40 ± 2	2.5 ± 0.4[b] (9)	158 ± 10[e] (4)
2	0.9 ± 0.1[a]	40 ± 3	2.2 ± 0.3[a] (14)	160 ± 8 (9)
3	0.9 ± 0.2[a]	43 ± 5	2.0 ± 0.2[a] (7)	178 ± 8 (4)
4	2.2 ± 0.4	48 ± 4	4.6 ± 0.9 (13)	207 ± 20 (8)
		With leucine infusion		
0	2.6 ± 0.4[d]	37 ± 2	7.2 ± 1.0[d] (8)	206 ± 6[d,f] (7)
2	2.2 ± 0.2[d]	40 ± 2	5.4 ± 0.6[d] (8)	266 ± 11[d,f] (8)
4	4.2 ± 0.8[c]	49 ± 2	9.1 ± 2.1 (8)	322 ± 15[d,f] (7)

Legend for Table 6.

BCDH activity was determined in muscle samples from hindlimbs of normal and streptozotocin-diabetic rats perfused as described under Materials and Methods. Hindlimbs were perfused in the presence (+) or absence (-) of 10 mU/ml insulin. A first sample was taken after 15 min of perfusion (before leucine addition) and a second sample was taken 15 min later after exposure to 1 mM leucine. Initial experiments showed no difference in BCDH activity between samples obtained after 15 or 30 min of perfusion. Combined values (+/-) represent the pooled values for normal or diabetic hindlimbs perfused both in the presence and absence of insulin. Total BCDH activity was significantly different ($p < 0.01$) between hindlimbs of normal and diabetic rats, 25.6 ± 1.5 ($n = 22$) vs. 34.9 ± 1.5 nmol/min/g muscle ($n = 20$), respectively; total BCDH activity was unaffected by insulin or leucine in either group. [a] $p < 0.001$ compared to normal rat; [b] $p < 0.001$ compared to before leucine addition; [c] $p < 0.01$ compared to normal rat.

Table 6. Effects of leucine and insulin on muscle BCDH activity in isolated perfused rat hindlimbs.

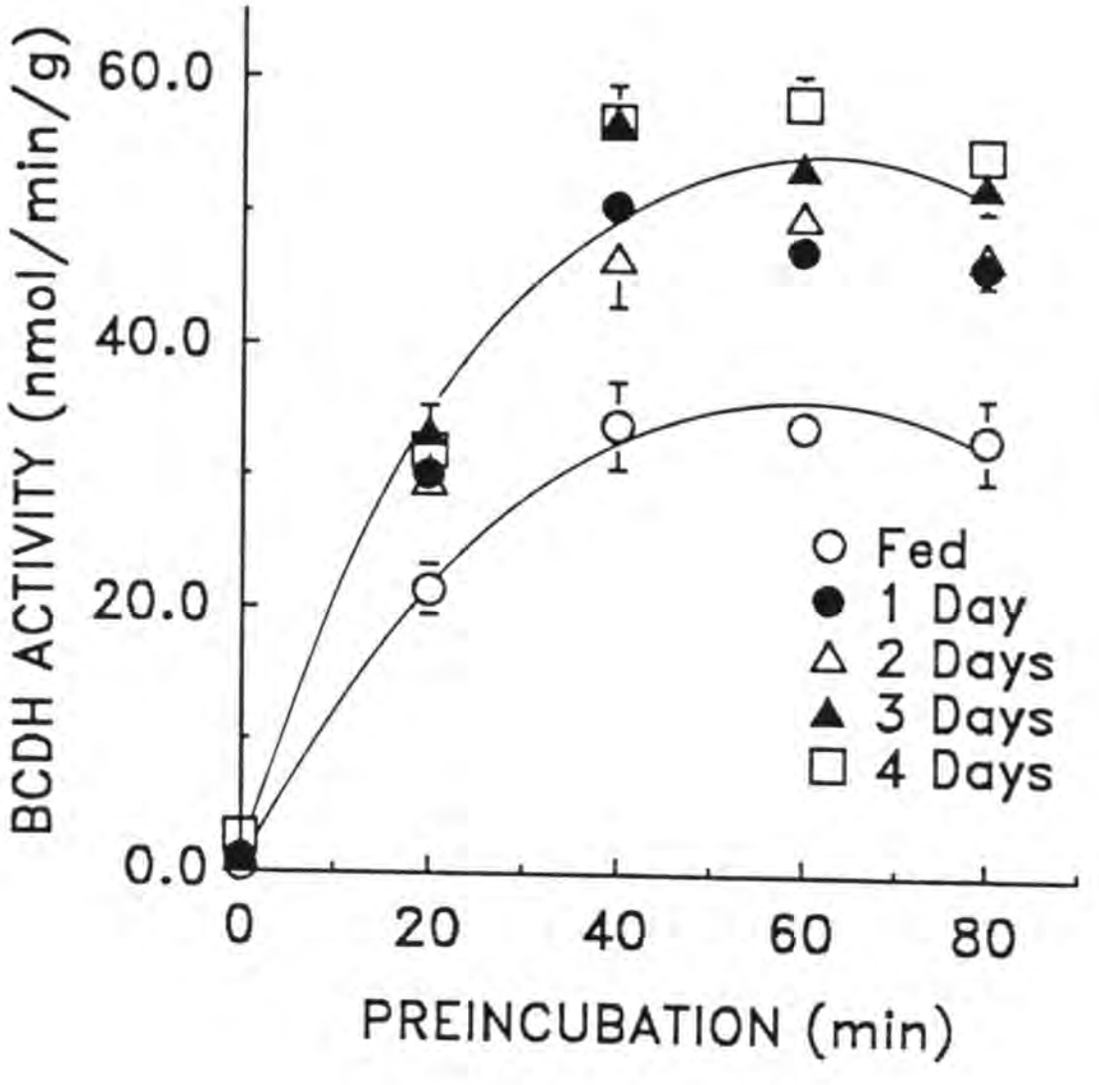
	Insulin	BCDH Activity (nmol/ min /g muscle)	% Active complex	n
		Before leucine addition		
Normal	-	0.9 ± 0.2	3.6 ± 0.7	6
	+	1.3 ± 0.3	4.9 ± 1.0	5
Combined	-/+	1.1 ± 0.2	4.2 ± 0.6	11
Diabetic	-	1.9 ± 0.4	4.9 ± 0.6	5
	+	2.4 ± 0.2	6.5 ± 0.9	5
Combined	-/+	2.2 ± 0.2[a]	5.7 ± 0.6	10
		After leucine addition		
Normal	-	10.8 ± 2.8[b]	42.6 ± 10.4[b]	6
	+	12.4 ± 1.9[b]	52.7 ± 7.0[b]	5
Combined	-/+	11.5 ± 1.7[b]	43.2 ± 3.8[b]	11
Diabetic	-	6.4 ± 0.8[b]	18.6 ± 3.0[b]	5
	+	9.3 ± 2.3[b]	30.6 ± 5.6[b]	5
Combined	-/+	7.7 ± 1.3[b]	24.6 ± 3.6[b,c]	10

Table 6. Effects of leucine and insulin on muscle BCDH activity in isolated perfused rat hindlimbs.

	Insulin	BCDH Activity (nmol/ min /g muscle)	% Active complex	n
		Before leucine addition		
Normal	-	0.9 ± 0.2	3.6 ± 0.7	6
	+	1.3 ± 0.3	4.9 ± 1.0	5
Combined	-/+	1.1 ± 0.2	4.2 ± 0.6	11
Diabetic	-	1.9 ± 0.4	4.9 ± 0.6	5
	+	2.4 ± 0.2	6.5 ± 0.9	5
Combined	-/+	2.2 ± 0.2[a]	5.7 ± 0.6	10
		After leucine addition		
Normal	-	10.8 ± 2.8[b]	42.6 ± 10.4[b]	6
	+	12.4 ± 1.9[b]	52.7 ± 7.0[b]	5
Combined	-/+	11.5 ± 1.7[b]	43.2 ± 3.8[b]	11
Diabetic	-	6.4 ± 0.8[b]	18.6 ± 3.0[b]	5
	+	9.3 ± 2.3[b]	30.6 ± 5.6[b]	5
Combined	-/+	7.7 ± 1.3[b]	24.6 ± 3.6[b,c]	10

Figure 1. Time course of in vitro activation of muscle BCDH from fed and starved rats.

Muscle BCDH was prepared from fed rats and rats starved for 1 to 4 days as described under Materials and Methods. BCDH was incubated at 37°C in the presence of 15 mM Mg^{2+} and samples removed every 20 minutes for determination of BCDH activity as described in Materials and Methods and in references 2 and 4. Values are mean ± SEM for 3-7 rats per group. Curves are shown for fed animals and from the means of all starved animals at each point.



Chapter VI

Concluding Remarks

The previous chapters have presented studies of certain aspects of branched-chain amino acid catabolism by skeletal muscle. At this point some consideration will be given to how these findings relate to the whole body economy of the BCAA. Since synthesis de novo is not an option (BCAA being essential), homeostasis of the BCAA must involve disposal of the amino acids during times of surfeit and conservation during times of deficiency. In this framework it should be clear that regulation of branched-chain α-keto acid dehydrogenase (BCDH) is central to homeostatic control of the BCAA. To better understand the role of skeletal muscle in whole body disposal of BCAA the interactions between muscle and other tissues is also necessary.

In the postabsorptive fed rat, which might be considered a neutral (i.e. no deficiency and no excess) condition with regard to BCAA homeostasis, the activity of BCDH in muscle is very low (Chapters 3-5) while hepatic and renal BCDH is very active (69,137,185). Under postabsorptive conditions it is likely that muscle is a relatively unimportant site for BCAA oxidation and instead is a source of circulating branched-chain α-keto acids which are oxidized by liver, kidney and perhaps other tissues. Once protein is ingested and BCAA enter the systemic circulation BCDH activity in muscle increases rapidly (Chapter 4), but except for adipose tissue (64) there is no evidence indicating that BCDH activity in other tissues increases postprandially. The importance of muscle, relative to other tissues, as a site of BCAA oxidation should therefore increase in the postprandial period. It must be noted however that circulating branched-chain α-keto acids increase in the postprandial period thus indicating that BCAA disposal

by liver may also increase during this period. As circulating BCAA decline during the postprandial period BCDH activity in muscle declines in parallel (Chapter 4) and so should its role in BCAA oxidation.

When rats were fed a high protein diet chronically, muscle BCDH activity, along with circulating BCAA concentrations, increased to a new "steady-state" value during the postabsorptive period (Chapter 4). BCDH activity in liver and kidney however were decreased slightly by a high protein diet (137,151). Muscle therefore may be a central organ in the disposal of excesses of BCAA. The capacity of muscle to dispose of excess BCAA may be counterproductive when leucine alone is in excess. Under these conditions muscle BCDH activity increases (Chapter 2) but since valine and isoleucine are also catabolized by BCDH they may be removed in excess and become limiting. Indeed, this may be the explanation for the observed depression of growth rate in animals fed diets with excesses of leucine alone (77).

In times of diminished supply, such as chronically feeding a low protein diet postabsorptive muscle BCDH activity was the same as in rats fed a normal diet (Chapter 4), it should be recalled though that this activity is very low (1-2% of total). BCDH activity in liver and kidney was markedly decreased by a low-protein diet (137,151). More remarkable though is the observation that the activation response of muscle BCDH to circulating BCAA from meal ingestion or leucine infusion is impaired (Chapter 4). This suggests that muscle adapts to times of diminished BCAA supply in a manner to slow or diminish muscle oxidation of BCAA when they are acquired.

The results of studies of BCAA catabolism in diabetes or during starvation (Chapter 2 and 5) suggest that the response of muscle BCDH to BCAA is blunted as it is in rats fed a low protein diet. Furthermore, BCDH activity in liver and kidney is also decreased in diabetes and during starvation (137,151). Despite the changes in muscle response to BCAA in diabetes and starvation, when BCAA concentrations are markedly elevated, muscle BCDH activity increases. Under these conditions diminished BCDH activity in liver and kidney may be more important to conservation of the BCAA, especially in view of the increased capacity of muscle to transaminate BCAA (Chapter 2). Although whole body BCAA oxidation increases in insulinopenic diabetes (154,179) and starvation (4), plasma BCAA concentrations also increase suggesting that their oxidation must nonetheless be restrained. It is unclear why the balance of oxidation and supply in diabetes and fasting is altered from the steady-state which obtains in normal rats, thus increasing plasma BCAA. It was postulated that increased BCAA may be beneficial in these conditions by maintaining basal insulin secretion which along with direct effects of BCAA could serve to maintain or augment protein synthesis (151). Furthermore, increased catabolism of BCAA in muscle may serve to restrain proteolysis (127) which is increased in these conditions (154,178).

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