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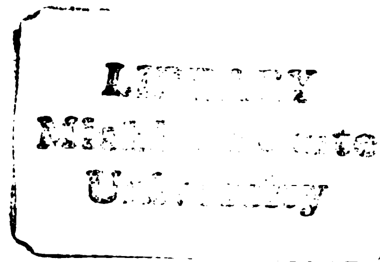

This is to certify that the

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EFFECTS OF AGE AND NUTRITIONAL STATE ON BRANCHED CHAIN AMINO ACID DEGRADATION IN SHEEP

presented by

Jan Roger Busboom

has been accepted towards fulfillment of the requirements for

Ph.D. degree in Animal Science

Robert A. Merkel
Major professor

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EFFECTS OF AGE AND NUTRITIONAL STATE ON BRANCHED CHAIN AMINO ACID DEGRADATION IN SHEEP

By

Jan Roger Busboom

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
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1984

ABSTRACT

EFFECTS OF AGE AND NUTRITIONAL STATE ON BRANCHED CHAIN AMINO ACID DEGRADATION IN SHEEP

By

Jan Roger Busboom

Four experiments were conducted to examine the effects of: 1) age on leucine aminotransferase (LAT) and alpha-ketoisocaproate dehydrogenase (KICDH) activities in rams; 2) dietary crude protein (CP) and fasting on leucine degradation in wether lambs; 3) fasting on the relative rates of leucine and valine degradation by sheep tissues; and 4) several species on the tissue and subcellular distribution of LAT and KICDH activities. In Experiment 1, five lambs were slaughtered at 28 d intervals from 1 to 224 d of age and four at d 365. LAT and KICDH activities were measured in muscle, liver, kidney and adipose tissue. In the three youngest groups, muscle was the predominant site of LAT activity and an important site of KICDH activity. Adipose tissue was the predominant site of LAT activity and an important site of KICDH activity in the older lambs. Liver was an important site of KICDH activity at all ages. In the second experiment, five lambs were assigned to each

of five treatments: unfasted lambs fed diets containing 8, 12 or 18% CP or lambs fed 12% CP were fasted for either 48 or 96 h. Dietary CP did not alter (P>.05) LAT activity in the tissues studied, but activity tended to increase as protein content increased. KICDH activity was increased (P<.05) in muscle and liver, and tended to (nonsignificantly) increase in adipose tissue when CP was increased from 8 to 18%. LAT and KICDH activities were decreased by 96 h of fasting in all tissues. Adipose tissue was the most important tissue for LAT activity for all groups and for KICDH activity for all except the 96 h fasted group. Three lambs were fasted for 12 h and three for 84 h in Experiment 3. The rates of transamination and decarboxylation of L-[1-^{14}C]-leucine and L-[1-^{14}C]-valine were measured. The relative changes in leucine and valine transamination caused by fasting length were similar. Leucine was more rapidly transaminated than valine in all tissues, although the difference was not always significant. In Experiment 4, LAT activity and [1-^{14}C]-alpha-ketoisocaproate decarboxylation were measured in cell-free preparations of tissues from rats, pigs, cattle and sheep. Rats had the highest (P<.05) LAT and KICDH activities in muscle and kidney; adipose tissue activities were similar in all species and pigs had the highest liver LAT activity.

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LITERATURE REVIEW

There has been much recent interest in branched-chain amino acid (BCAA) metabolism because of their purported role in regulating muscle protein turnover (Buse and Reid, 1975; Fulks et al., 1975). However, initial interest in the degradation of leucine, valine and isoleucine was stimulated from the observation that metabolism of these BCAA was impaired by several inborn errors in metabolism, including maple syrup urine disease (Dancis and Levitz, 1972, 1978). In addition, Miller (1962) showed with perfused hepatectomized rats that BCAA are unique among essential amino acids, in that they are primarily metabolized by extrahepatic tissues.

The major pathways in the degradation of BCAA have been identified (Meister, 1965) and reviewed (Dancis and Levitz, 1972). The first step in the degradative metabolism of BCAA is the loss of the amino group primarily by reversible transamination with an alpha-ketoacid amino group acceptor (Mathews et al., 1981, see figure 1a). Following transamination of leucine, valine and isoleucine to form alpha-ketoisocaproate (KIC), alpha-ketoisovalerate (KIV) and alpha-keto-beta-methylvalerate (KMV), respectively, these branched chain alpha-keto acids (BCKA) are irreversibly

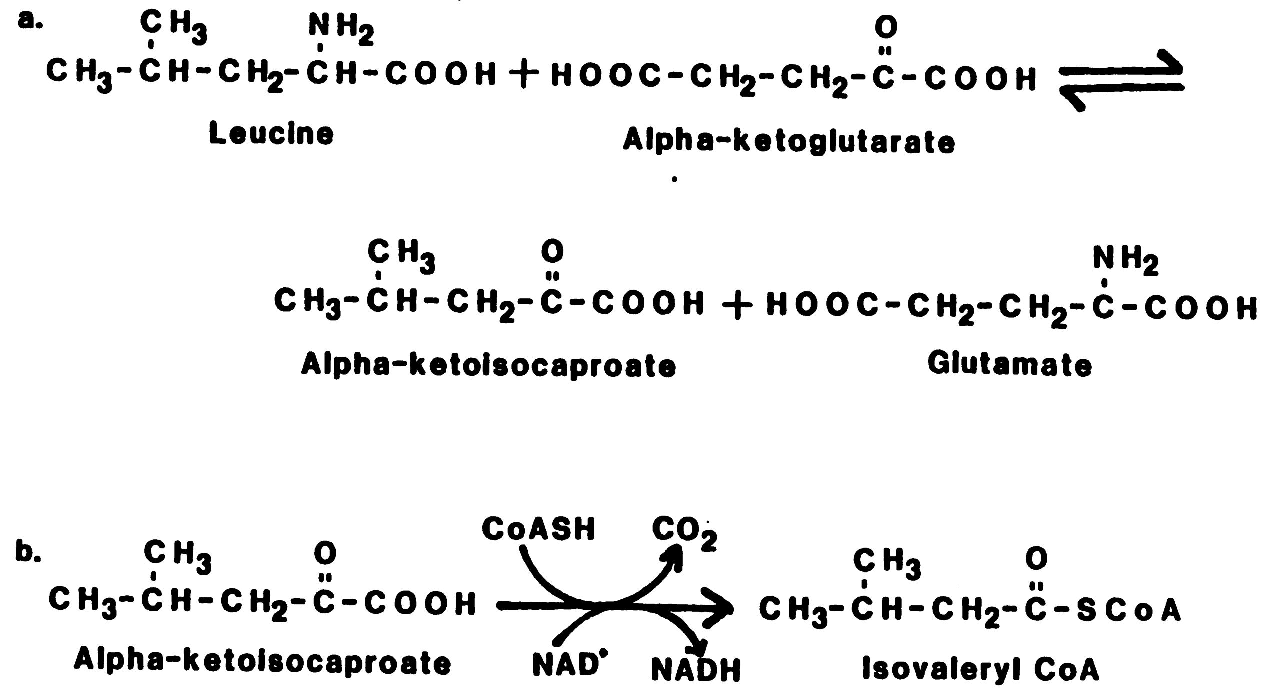


Figure 1. The reversible transamination (a) of the branched chain amino acid, leucine, and the irreversible decarboxylation (b) of the branched chain alpha-ketoacid, alpha-ketoisocaproate.

decarboxylated to their homologous branched chain fatty acyl CoA (figure 1b). Isovaleryl CoA (from leucine), isobutyryl CoA (from valine) and alpha-methylbutyryl CoA (from isoleucine) are subsequently catabolized through a series of steps to acetyl CoA and acetoacetyl CoA, propionyl CoA, and acetyl CoA and propionyl CoA, respectively.

Isoenzymes Involved in the Degradation of BCAA

BCAA Aminotransferase Activity

Ichihara (1975) summarized the findings of several of his studies relating to properties of three isoenzymes of BCAA aminotransferase (Ichihara and Koyama, 1966; Ichihara et al., 1967, 1973; Aki et al., 1968). Isoenzymes I (which is fairly ubiquitous in rat tissues) and III (found in brain, ovary and placental tissue) are similar in substrate specificity and equal in activity for the three BCAA, while isoenzyme II (found only in liver) is specific for leucine. Isoenzymes I, II and III are primarily localized in the cytosolic fraction, however, Ikeda et al.(1976) and Ichihara et al.(1981) described a mitochondrial isoenzyme which like isoenzyme II is specific for leucine and is found primarily in the liver, but can be differentiated from isoenzyme II by

molecular weight, K_m values, electrophoretic mobility, chromatographic behavior and heat stability. In addition, Kadowaki and Knox (1982) isolated a mitochondrial enzyme similar to isoenzyme I that differed in heat stability and activation by mercaptoethanol.

Rowsell (1956) reported that pyruvate would serve as an amino acceptor for leucine, but Krebs (1975) concluded that there is very little direct transfer of amino groups from BCAA to pyruvate. Crabb and Harris (1978) demonstrated that pyruvate stimulated leucine aminotransferase activity and suggested as one possible explanation that pyruvate serves directly as an amino acceptor. However, their second suggestion that pyruvate serves as an amino acceptor for glutamate and thereby increases the concentration of alpha-ketoglutarate is in agreement with most other reports in the literature (figure 2). Ichihara and Koyama (1966), Ichihara (1975), Krebs (1975), Ikeda et al.(1976) and Odessey and Goldberg (1979) agreed that alpha-ketoglutarate and the BCKA are the primary acceptors for BCAA amino groups. Odessey and Goldberg (1979) found that skeletal muscle supernatant BCAA aminotransferase had a K_m of about .1 mM for alpha-ketoglutarate compared to a K_m of 3 mM for pyruvate, and saturating concentrations of pyruvate supported only one-tenth of the maximal rate of leucine transamination with alpha-ketoglutarate present.

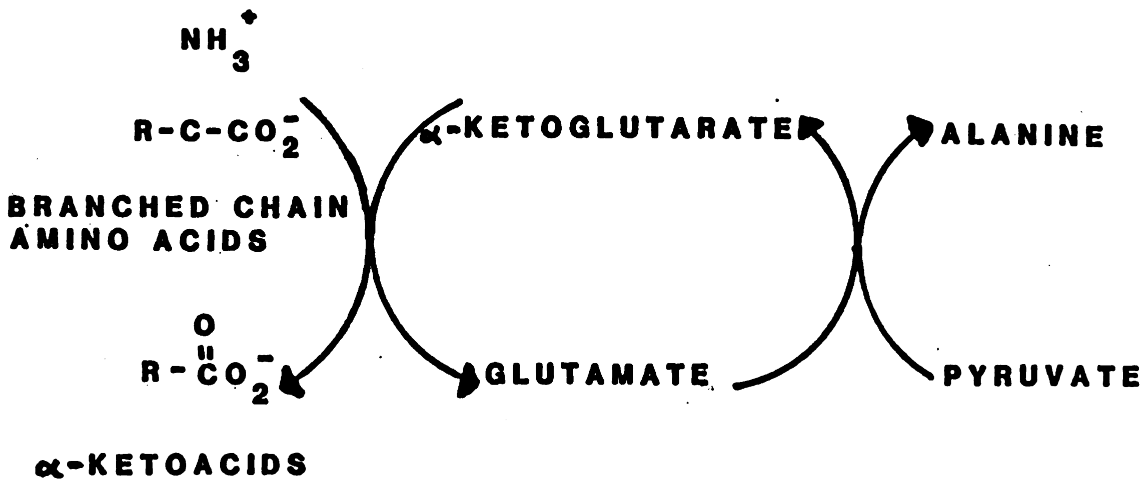


Figure 2. Alpha-ketoglutarate is the primary acceptor for branched chain amino acid amino groups.

Although an asparagine aminotransferase (Cooper, 1977) and glyoxylate aminotransferase (Hsieh and Tolbert, 1976), which have a broad specificity including leucine, have been isolated from rat liver, overall, isoenzymes I, II and III described by Ichihara (1975) appear to be responsible for the vast majority of BCAA aminotransferase activity. Isoenzymes I and III are specific for the three BCAA and isoenzyme II is only reactive with leucine. While pyruvate at very high concentrations may serve directly as amino group acceptor, alpha-ketoglutarate and the BCKA are the physiologically important amino group acceptors for BCAA.

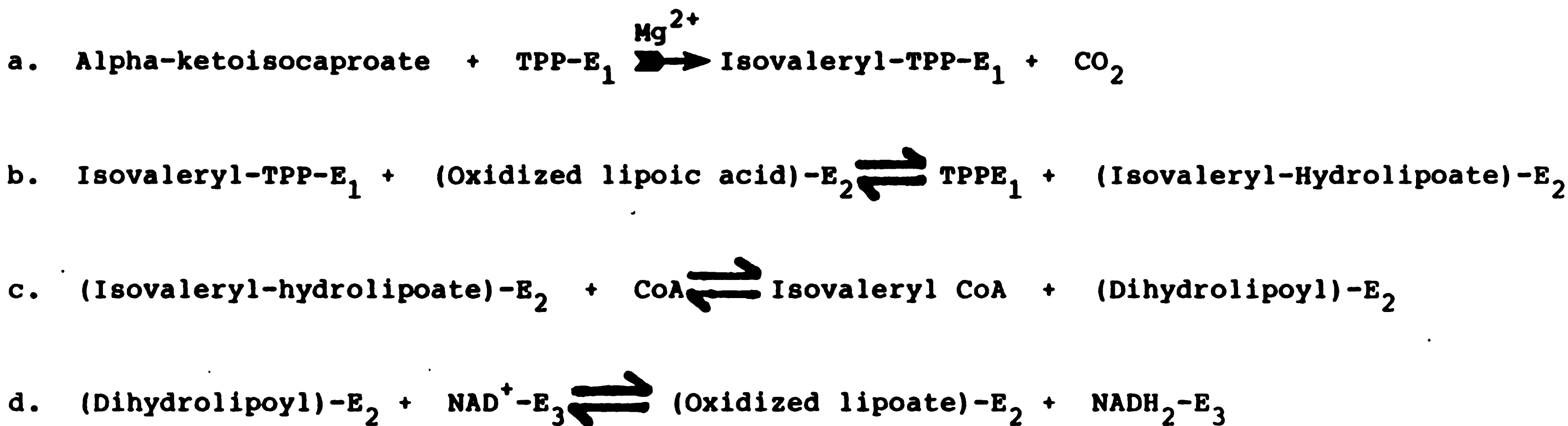
BCKA Dehydrogenase Activity

Currently, the data in the literature are not clear as to whether multiple enzymes are involved in the decarboxylation of BCKA (Danner and Bowden, 1966; Bowden and Connelly, 1968; Sullivan et al., 1976; Kean and Morrison, 1979; Sabourin and Bieber, 1981) or if a single enzyme complex is responsible for the decarboxylation of all three BCKA (Wohlhueter and Harper, 1970; Danner et al., 1975, 1978, 1979, 1981; Khatra et al., 1977a; Parker and Randle, 1978a; Pettit et al., 1978; Odessey and Goldberg, 1979; Frick and Goodman, 1980; Odessey, 1979, 1980; Williamson et

al., 1979; Randle et al., 1981; Morrison and Mullings, 1983). However, the bulk of the evidence suggests that a single enzyme complex degrades the BCKA with K_m values of 20 to 50 .M (Danner et al., 1978; Parker and Randle, 1978a; Pettit et al., 1978; Odessey and Goldberg, 1979). There are at least five lines of evidence supporting this supposition: 1. BCKA acidemia is inherited as a monogenic trait since the degradative pathways for all three BCAA are blocked at the BCKA decarboxylation step (Dancis and Levitz, 1972). 2. Feeding a diet high in just one of the BCAA or high in thiamine caused coordinate induction of activity for all three BCKA (Wohlhueter and Harper, 1970; Danner et al., 1975; Khatra et al., 1977a). 3. Measurements of competitive inhibition suggested that KIC, KIV and KMV were decarboxylated by the same enzyme since K_i values were approximately equal to K_m(Danner et al., 1978; Odessey and Goldberg, 1979). 4. When enzyme preparations were deactivated by heating or treatment with sodium arsenite, decarboxylase activity with each of the BCKA fell off in concert (Wohlhueter and Harper, 1970; Frick and Goodman, 1980). 5. During purification, dehydrogenase activities with KIC, KIV and KMV were co-purified and the relative ratio of activities with the three BCKA remained constant (Danner et al., 1979).

The BCKA dehydrogenase is a high molecular weight multienzyme complex much like pyruvate dehydrogenase, which may be dissociated and reassociated, and each component is responsible for one of three steps in the pathway (Dancis and Levitz, 1972). The first enzyme component of BCKA dehydrogenase is a decarboxylase that uses thiamine pyrophosphate and Mg^{2+} as cofactors (figure 3a). A transacylase (dihydrolipoyl transacylase) with covalently bound lipoic acid, which accepts the acyl moiety from the decarboxylase bound thiamine pyrophosphate and transfers it to CoASH, constitutes the second component (figures 3b and c). A NADH:lipoamide oxidoreductase also referred to as dihydrolipoyl dehydrogenase, which reoxidizes the lipoate of dihydrolipoyl transacylase and transfers the electrons to NAD^{+}, is the third component (Pettit et al., 1978; Danner et al., 1979; Odessey, 1981; Randle et al., 1981; see figure 3d).

The theory for separate substrate specific complexes was based primarily on the report of Connelly et al.(1968). This paper showed that dehydrogenase activity with KIV remained particle bound while activity for KIC and KMV was solubilized during isolation. Sullivan et al.(1976) reported that liver from hypophysectomized rats had three- and fourfold greater KIV dehydrogenase and KMV dehydrogenase activities, respectively, than controls while KIC



a. Alpha-ketoisocaproate + $TPP\text{-}E_1 \xrightarrow{Mg^{2+}}$ Isovaleryl-$TPP\text{-}E_1$ + CO_2

b. Isovaleryl-$TPP\text{-}E_1$ + (Oxidized lipoic acid)-$E_2 \rightleftharpoons TPPE_1$ + (Isovaleryl-Hydrolipoate)-E_2

c. (Isovaleryl-hydrolipoate)-E_2 + CoA $\rightleftharpoons$ Isovaleryl CoA + (Dihydrolipoyl)-E_2

d. (Dihydrolipoyl)-E_2 + $NAD^{+}\text{-}E_3 \rightleftharpoons$ (Oxidized lipoate)-E_2 + $NADH_2\text{-}E_3$

Figure 3. The proposed steps performed by the branched chain alpha-ketoacid dehydrogenase enzyme complex. Thiamin pyrophosphate, decarboxylase, dihydrolipoyl transacylase and NADH:lipoamide oxidoreductase are abbreviated TPP, E_1, E_2 and E_3, respectively.

dehydrogenase activity was not changed by hypophysectomy. These two studies indicate a multiple enzyme situation, but these results could be made compatible with the later work of Danner et al.(1978, 1979, 1981), Odessey (1980) and Randle et al.(1981) if we include in the BCKA dehydrogenase complex three separate decarboxylase components which share the dihydrolipoyl transacylase and dihydrolipoyl dehydrogenase components. While Danner et al.(1979) agreed that the selective solubilization of individual decarboxylases could occur, they stated that thus far, we have found no evidence suggesting that separate decarboxylases exist.

In addition to the BCKA dehydrogenase complex which is primarily found to be bound to the inner surface of the inner mitochondrial membrane (Hinsbergh et al., 1978; Danner et al., 1979; May et al., 1980; Patel et al., 1980), Sabourin and Bieber (1981) isolated a soluble KIC decarboxylase. This soluble enzyme, which the authors referred to as KIC oxidase did not require CoA or NAD^+, was inactive with KIV and KMV, and the major product of the reaction catalyzed by the enzyme was beta-hydroxyisovalerate.

The evidence indicates that a single enzyme complex (BCKA dehydrogenase) is primarily responsible for the degradation of all three BCKA, although the possibility

exists for three separate branched chain decarboxylase subunits associated with that single BCKA dehydrogenase complex (Danner et al., 1979). In addition, there is an oxidase in the soluble fraction of liver that decarboxylates KIC (Johnson and Connelly, 1972; Sabourin and Bieber, 1981).

Regulation of Enzymes Responsible for BCAA Oxidation

BCAA Aminotransferase

In work with purified preparations, BCAA aminotransferase required pyridoxal phosphate and had an optimal pH ranging from 8.3 to 8.5 (Taylor and Jenkins, 1966). In agreement with that observation, Aki et al.(1968) reported that purified isoenzymes I and II had pH optima of 8.2 and 8.6. It is also known that BCAA aminotransferase requires BCAA as amino donors and alpha-ketoglutarate or BCKA as amino group acceptors. Although pyruvate has been shown to increase leucine transamination in liver (Crabb and Harris, 1978) and skeletal muscle (Hedden and Buse, 1981), pyruvate apparently accomplished this increase in rate of transamination by increasing the concentration of alpha-ketoglutarate (Odessey and Goldberg, 1979).

Mitch and Chan (1978) and Mitch (1980) reported that preincubation of kidney or skeletal muscle homogenates with KIC stimulated BCAA aminotransferase activity, but preincubation with KIV, KMV or alpha-ketoglutarate had no effect on BCAA aminotransferase activity. However, Khatra et al.(1977a) found that feeding KIC, KIV or KMV had no effect on liver BCAA aminotransferase activity. While BCKA dehydrogenase is very tightly controlled, BCAA aminotransferase enzymes do not appear to be closely regulated, except by substrate concentration (Odessey and Goldberg, 1979).

BCKA Dehydrogenase

BCKA dehydrogenase requires the presence of CoA, NAD^+ and thiamine pyrophosphate and is stimulated by Ca^{2+}, Mg^{2+} and K^+ in muscle (Odessey and Goldberg, 1979), liver (McFarlane and von Holt, 1969a; Johnson and Connelly, 1972; Danner et al., 1979; May et al., 1980) and adipose tissue (Frick and Goodman, 1980). However, ATP and reaction end products (NADH and branched chain acyl CoA) inhibit its reaction (Johnson and Connelly, 1972; Parker and Randle, 1978b; Odessey and Goldberg, 1979; Odessey, 1980, 1981). Data from several groups of authors have shown the pH optima

range from 6.8 to 7.8 (Wohlhueter and Harper, 1970; Johnson and Connelly, 1972; Khatra et al., 1977b; Odessey and Goldberg, 1979), but most researchers have utilized assay media at pH 7.4 to 7.8 (Odessey and Goldberg, 1972; Buse et al., 1976; Dohm et al., 1976; Paul and Adibi, 1976; Shinnick and Harper, 1976; Odessey and Goldberg, 1979).

Concentrations of BCKA regulate the flux through BCKA dehydrogenase by inducing the enzyme in liver (Wohlhueter and Harper, 1970; Khatra et al., 1977a; Zapalowski et al., 1981), activating or preventing the deactivation of the enzyme complex (Odessey, 1980; Waymack et al., 1980) and by simple substrate availability (Randle et al., 1981). Khatra et al.(1977a) showed that rats that were tube fed BCKA had increased hepatic BCKA dehydrogenase activity two- to sixfold but did not affect BCKA dehydrogenase in skeletal muscle or kidney. The presence of KIC prevented the inhibition of BCKA dehydrogenase caused by ATP (Odessey, 1980) and pyruvate (Waymack et al., 1980). Liver and heart mitochondrial concentration of BCKA (2 to 4μM) is well below the Km (20 to 50μM) for the BCKA complex, indicating their concentration could be an important determinant of the rate of BCKA decarboxylation (Livesey and Lund, 1980; Randle et al., 1981).

The first step (decarboxylation) in the reaction catalyzed by BCKA dehydrogenase is irreversible, but the

remaining two steps involving the formation of branched chain acyl CoA and NADH are reversible. Randle et al.(1981) suggested that end product inhibition results from reversal of the latter two reactions, but NADH may also act by potentiating the inhibitory response of BCKA dehydrogenase to ATP (Odessey, 1980). Buse et al.(1976, 1980) demonstrated that the redox state of the mitochondria may, in and of itself, be an important regulator of BCKA dehydrogenase. Addition of ketone bodies or NADH (both of which increase the state of reduction) decreased BCKA dehydrogenase, but addition of methylene blue (an electron acceptor) or NAD^+ increased leucine oxidation (Buse et al., 1976, 1980).

Many properties of BCKA dehydrogenase indicate that it, (Goodman, 1977; Parker and Randle, 1978b, 1980; Odessey, 1979, 1980, 1981; Odessey and Goldberg, 1979; Hughes and Halestrap, 1980; Sans et al., 1980; Buffington et al., 1981; Paul and Adibi, 1981) like other alpha-keto acid dehydrogenases such as pyruvate dehydrogenase and alpha-ketoglutarate dehydrogenase (Linn et al., 1969; Severson et al., 1974; Roche and Reed, 1974; Cooper et al., 1974; Chiang and Sactor, 1975) may be regulated by a phosphorylation - dephosphorylation cycle. In support of this hypothesis, ATP markedly inhibits BCKA dehydrogenase activity in isolated mitochondrial preparations from muscle (Odessey and Goldberg, 1979), heart (Parker and Randle,

1978b), liver, kidney (Hughes and Halestrap, 1980) and adipose tissue (Goodman, 1977; Frick and Goodman, 1980). The activity remains low after removal of the nucleotide unless reactivated by Mg^{2+} and this reactivation is blocked by NaF (Odessey, 1980). Odessey and Goldberg (1979) demonstrated with skeletal muscle mitochondria that inactivation requires the cleavage of the gamma phosphate group of ATP and that modification is covalent. The nonmetabolized ATP analog p[NH]ppA, can block the inhibition of BCKA dehydrogenase when added prior to ATP addition, but cannot reverse the inhibition of already inactivated enzyme (Odessey, 1980). Incorporation of ^{32}P from labeled ATP into electrophoretically separated bands corresponding to BCKA dehydrogenase activity isolated from heart, skeletal muscle (Hughes and Halestrap, 1980; Odessey, 1980), adipose tissue and liver (Hughes and Halestrap, 1980) have been observed. It should be noted that several investigators have failed to detect evidence for inhibition of BCKA dehydrogenase by phosphorylation in liver and kidney preparations (Pettit et al., 1978; Parker and Randle, 1980; Danner et al., 1981). Odessey (1980, 1981) agreed that BCKA dehydrogenase in liver mitochondria is less responsive to experimental manipulations involving ATP, but demonstrated that the kidney mitochondrial preparations utilized in his experiments were susceptible to ATP inhibition.

In general, three factors which regulate BCKA dehydrogenase have been identified (Danner et al., 1981). They are: (1) concentration of BCKA, (2) end product inhibition (branched chain acyl CoA/CoA and NADH/NAD^+), and (3) regulation by phosphorylation - dephosphorylation, which is apparently quite important in heart and skeletal muscle, but is not an important regulatory mechanism in liver. It also is interesting that stimulatory properties of BCKA on dehydrogenase activity were prevented by the addition of NaF (an inhibitor of phosphoprotein phosphatase) or removal of divalent cations, indicating substrate activation of BCKA dehydrogenase may be mediated by a phosphorylation - dephosphorylation cycle (Odessey, 1980). Finally, marked diurnal variation in oxidation of leucine have been observed with a ratio of peak to nadir of 4.4 (Wohlhueter and Harper, 1970; Buckley and Marquardt, 1980). Minimum activity was observed 2 h prior to beginning of the feeding period and maximum activity was reached 4 h later. Measurements made 2 and 6 h after the end of the 12 h feeding period represented mean daily rate of oxidation so the data obtained during this time period should be utilizable with a minimum of error caused by diurnal variation (Buckley and Marquardt, 1980).

Tissue Distribution of BCAA Oxidative Activity and Interorgan Relationships in the Degradation of BCAA

Location of BCAA Aminotransferase Activity

In rats, workers generally agree that BCAA aminotransferase activity expressed on a per gram of tissue basis is high in mammary gland, heart and kidney, intermediate in skeletal muscle and lowest in liver (Ichihara and Koyama, 1966; Adibi et al., 1975; Ichihara, 1975; Dohm et al., 1976; Kadowaki and Knox, 1982). Ichihara and Koyama (1966) reported that with leucine as substrate the ratio of aminotransferase activity between heart, kidney, skeletal muscle and liver was 3.5:2.0:1.0:.01. Kadowaki and Knox (1982) found the mammary gland:kidney:skeletal muscle:brain:liver ratio for BCAA aminotransferase was 5.5:1.4:1.0:.9:.05. Activity of BCAA aminotransferase in adipose tissue and gastrointestinal tract is more controversial. Ichihara and Koyama (1966) obtained BCAA aminotransferase activities in adipose tissue and gastrointestinal tract that were similar to the low activity they obtained in liver, while Tischler and Goldberg (1981) suggested adipose tissue was a very important site of BCAA aminotransferase activity, and Ichihara et al. (1981)

reported BCAA aminotransferase activity in stomach and pancreas, that was 10-fold greater than the activity in kidney.

On a total tissue basis skeletal muscle because of its bulk has the most BCAA aminotransferase activity in rats (Adibi et al., 1975; Chang and Goldberg, 1978a,b,c; Harper and Zapalowski, 1981). In fed rats skeletal muscle, liver and kidney constitute approximately 45, 4 and .9% of live body weight, respectively. During fasting, liver and kidney lost proportionately more weight than skeletal muscle and BCAA aminotransferase activity in muscle increases, so that in the fasted state skeletal muscles' relative contribution to BCAA aminotransferase activity is even more important (Adibi et al., 1975). There are few reports in which BCAA aminotransferase activity has been specifically measured and compared between organs in species other than the rat.

Location of BCKA Dehydrogenase Activity

Workers generally agree liver is the primary site of BCKA dehydrogenase activity in the rat (Connelly et al., 1968; Wohlhueter and Harper, 1970; Shinnick and Harper, 1976; Khatra et al., 1977b; Williamson et al., 1979; Hauschildt and Brand, 1980; Harper and Zapalowski, 1981).

Shinnick and Harper (1976) used crude homogenates or tissue slices and reported that the ratio of BCKA dehydrogenase activity in liver, kidney, brain and muscle was 30:9:1:.05, respectively.

Connelly et al.(1968) conducted a study of BCKA dehydrogenase in liver, kidney, heart, brain and skeletal muscle crude homogenates from rats, mice, guinea pigs, pigs, rabbits and beef cattle. In the species studied except for beef cattle, the liver exhibited the highest activity on a nmol x mg protein^{-1} x min^{-1} basis and in all of the species other than guinea pigs and beef cattle, kidney had the second highest activity. Activities of BCKA dehydrogenase (nmol x mg protein^{-1} x min^{-1}) in liver, kidney, heart, brain and skeletal muscle from beef cattle were 5.4, 6.4, 2.5, 0 and 3.8, respectively, while those activities in the rat were 3.2, 1.5, 0, 0 and 0, and in guinea pigs were 11.0, 1.5, 7.7, 0 and 0, respectively. Khatra et al. (1977b) conducted a similar study comparing BCKA dehydrogenase activity in liver, kidney and skeletal muscle crude homogenates from rats, monkeys and humans. BCKA dehydrogenase activities in rat, liver, kidney and skeletal muscle were 542, 433 and 7 nmol x g tissue^{-1} x min^{-1}, respectively. Activities of BCKA dehydrogenase in liver, kidney and skeletal muscle from monkeys were 183, 200 and 3, and in man were 31, 13 and 4, respectively. The

distribution of BCKA dehydrogenase in the rat body was reported to be 70% in liver, 12% in kidney and 10% in skeletal muscle and the remaining 8% was localized in heart, brain, lung, gastrointestinal tract and spleen (Khatra et al., 1977b). In comparison, human liver, kidney and skeletal muscle contributed 30, 2 and 60% of the total BCKA dehydrogenase activity in the eight tissues studied (Khatra et al., 1977b). It also is worthy of note that in rats BCKA dehydrogenase activity in diaphragm muscle was twofold greater than in gastrocnemius and soleus muscle (Paul and Adibi, 1978a).

Interorgan Relationships

It is generally agreed that the first step in the degradation of BCAA occurs primarily in skeletal muscle, however the site of BCKA degradation under physiological conditions is more equivocal. In studies with [1-^{14}C]-leucine or [U-^{14}C]- leucine as substrate Manchester (1965), Adibi et al.(1971, 1974), Odessey and Goldberg (1972), Goldberg and Odessey (1972) and Meikle and Klain (1972) concluded that skeletal muscle because of its bulk was the major site of BCAA oxidation to $^{14}CO_2$. However, later studies have indicated that while BCKA dehydrogenase

is rate limiting in extrahepatic tissues (Odessey and Goldberg, 1979), BCAA aminotransferase is the rate limiting enzyme in liver (Shinnick and Harper, 1976, 1977), which raises the question regarding the use of labeled leucine as a marker for overall BCAA degradation. Work of Noda and Ichihara (1976) illustrated this point. Using labeled leucine they found very low BCAA degradative activity in liver slices, but in vivo, ligation of circulation to liver decreased total body leucine decarboxylation by 60%. Studies measuring the release of BCKA from skeletal muscle and uptake in liver suggest a complex interaction between these tissues (Harper and Zapalowski, 1981). Goldberg and Odessey (1979) and Hutson and Harper (1981) showed that skeletal muscle is the major source of BCKA in blood, and liver removes an amount (.7 to 1.1 mmol/d) of BCKA similar to that released by skeletal muscle (.9 to 1.7 mmol/d, Livesey and Lund, 1980).

In his review, Adibi (1976) reported that transamination and some decarboxylation of BCAA occurs in extrahepatic tissue (primarily skeletal muscle); the remaining BCKA and the branched chain fatty acid products of BCKA dehydrogenase are then transported by blood to the liver for metabolism to ketone bodies, and the ketone bodies are subsequently metabolized in extrahepatic tissues.

In dogs (McMenamy et al., 1962, 1965) and humans (Felig, 1975; Wahren et al., 1976; Elia and Livesey, 1981), BCAA appear to be primarily metabolized in nonhepatic tissues. McMenamy et al. (1965) showed that BCAA in dogs are poorly taken up and utilized by liver, but BCKA concentrations in blood increased for the first hour after hepatectomy, indicating the liver may play a similar role in dogs as it does in rats.

Feeding steak or egg nog to normal human males caused a 20% increase in plasma amino acid concentration, but the concentration of BCAA rose 100 to 200% (Felig, 1975; Wahren et al., 1976). Elia and Livesey (1981) measured arterio-venous (A-V) differences across human forearm, and reported a release of BCKA (A-V equaled -3.6 μmol/l) that was less (P<.05) than the corresponding release across the rat hindlimb (A-V = -19.9 μmol/l). This difference in release of BCKA occurred while uptake of BCAA by human forearm and rat hindlimb was equal. The authors concluded these results indicated that BCKA are more readily oxidized in human muscle than in rat muscle, which is in accordance with the distribution of BCKA dehydrogenase activity in human (60%) and rat (10%) muscle reported by Khatra et al. (1977b).

Studies with ruminants have further illustrated that species differences in BCAA metabolism exist. Sheep have

relatively higher BCAA concentrations in free amino acid pools than those reported for rats and humans (Bergen, 1979). Branched chain amino acid output from human skeletal muscle is only 25 to 40% of the alanine output (Felig, 1975), but in sheep the combined output of BCAA is 145% of the alanine output from skeletal muscle (Ballard et al., 1976). In addition, nonruminants release less BCAA from muscle than expected (Odessey et al., 1974; Ruderman and Berger, 1974), but ruminants release an amount of BCAA equivalent to their muscle amino acid composition (Lindsay, 1980). Buttery (1979) reported that he failed to demonstrate marked oxidation of BCAA in ruminant skeletal muscle. These reports indicate that skeletal muscle has only limited capability to oxidize BCAA as was originally suggested by Ballard et al. (1976). Also of interest is the study of Heitmann and Bergman (1980) who concluded that unlike nonruminant liver, sheep liver removes BCAA.

Role of Adipose Tissue in BCAA Oxidation

While most studies have concentrated on skeletal muscle and liver, it is known that adipose tissue also actively metabolizes BCAA in mice (Feller and Feist, 1962) and rats (Goodman, 1963a,b, 1964, 1977; Leveille, 1966; Meikle and

Klain, 1972; Rosenthal et al., 1974; Tischler and Goldberg, 1980a, 1981). Rosenthal et al. (1974) suggested that adipose tissue is a major site of leucine degradation second only to skeletal muscle since on a tissue basis, in 200 g rats, adipose tissue utilized 3 times more leucine than liver and 50% as much leucine as skeletal muscle for the combined production of CO_2, protein and lipids. It has also been postulated that since 200 g rats contain only 7% dissectable fat (Caster et al., 1956), adipose tissue may be of even greater importance in older, more obese rats or in species that contain a higher percentage of adipose tissue such as humans (Tischler and Goldberg, 1980a, 1981; Goldberg and Tischler, 1981).

Effects of Oxidizable Substrate, Dietary Energy, Dietary Protein, Diabetes, Insulin and Age on BCAA Metabolism

Oxidizable Substrates

Effects of oxidizable substrates other than BCAA on BCAA oxidation is interesting and not completely understood. Glucose inhibits $^{14}CO_2$ production from BCAA in heart (Buse et al., 1972; Sans et al., 1980), but is stimulatory in adipose tissue (Goodman, 1964; Goodman, 1977;

Tischler and Goldberg, 1980a, 1981; Frick and Goodman, 1981). Odessey and Goldberg (1972) reported a 15% decrease in BCKA dehydrogenase activity in skeletal muscle when glucose was included in the media, but the reports of Manchester (1965) and Paul and Adibi (1976) suggest that the rate of BCAA oxidation in skeletal muscle is unresponsive to the presence or absence of glucose.

Pyruvate decreases BCKA dehydrogenase activity in heart (Buse et al., 1972; Buffington et al., 1979, 1981; Sans et al., 1980; Waymack et al., 1980) and skeletal muscle (Buse et al., 1972, 1975; Hedden and Buse, 1981). Buse et al.(1976) reported that pyruvate increased leucine oxidation in diaphragm muscles excised from diabetic rats while leucine degradation in control diaphragms was inhibited by pyruvate addition. In the work of Patel et al. (1981) KIC dehydrogenase activity in perfused rat liver was inhibited by the presence of pyruvate, but Danner et al.(1978) found that partially purified BCKA dehydrogenase from liver was unresponsive to pyruvate. BCKA dehydrogenase activity in epididymal fat pads was not affected by pyruvate concentration.

Octanoate apparently increases BCKA dehydrogenase activity in skeletal muscle (Buse and Buse, 1967; Buse et al., 1972, 1975; Spydevold, 1979; Bremer et al., 1981; Spydevold and Hokland, 1981, 1983) and decreases activity in

liver (Spydevold and Hokland, 1981). May et al.(1980) agreed that BCKA dehydrogenase activity in liver was decreased by octanoate but these workers found BCKA dehydrogenase activity in skeletal muscle was unaffected by octanoate. Buffington et al.(1979) and Waymack et al.(1980) reported an inhibitory effect of octanoate on perfused heart BCKA dehydrogenase activity, while Buse et al.(1972) showed that addition of this fatty acid to their heart perfusion media stimulated BCAA oxidation.

Effects of ketone bodies on BCAA oxidation also have been studied extensively. It is generally accepted that beta-hydroxybutyrate inhibits BCAA oxidation in rats (Buse and Buse, 1967; Buse et al., 1972; Palaiologos and Felig, 1976; Buffington et al., 1979; Sans et al., 1980), humans (Sherwin et al., 1975; Landaas, 1977; Robinson and Williamson, 1980) and ruminants (Lindsay and Buttery, 1980). The results were obtained in heart, skeletal muscle, liver, hindquarter and whole body preparations. Results with acetoacetate additions have been more ambiguous (Buffington et al., 1979; Zapalowski et al., 1981). Perfusion of hindquarters from diabetic rats with a combination of 2 mM beta-hydroxybutyrate and 1 mM acetoacetate caused a 40% decrease in KIC dehydrogenase activity (Zapalowski et al., 1981), but in studies in which only acetoacetate was added BCKA dehydrogenase activity in skeletal muscle was

stimulated (Paul and Adibi, 1978a, 1979, 1980). Lindsay and Buttery (1980) hypothesized that in sheep a high beta-hydroxybutyrate:acetoacetate ratio may inhibit BCAA oxidation and an increase in this ratio is related to a high $NADH:NAD^+$ ratio. The results of Buffington et al.(1979) indicate this generalization could apply to other species. In their heart perfusion study, acetoacetate inhibited BCKA dehydrogenase activity but this activity was inhibited several-fold more by beta-hydroxybutyrate. These workers also reported that beta-hydroxybutyrate increased $NADH/NAD^+$ to a much greater extent than acetoacetate. Buse et al.(1976, 1980) utilized methylene blue (an electron acceptor like NAD^+) to further illustrate the possible importance of the redox state in regulating the degradation of BCAA. Methylene blue stimulated leucine oxidation in diaphragms excised from normal or diabetic rats.

Dietary Energy

Undoubtedly more work has been done to ascertain the effect of fasting on BCAA metabolism than any other factor, but the results are far from conclusive. However, many of the inconsistencies can be accounted for by species, tissue and experimental technique differences.

Most workers have generally concluded that fasting increased plasma BCAA concentrations in rats (Goldberg and Odessey, 1972; Adibi, 1976; Goldberg and Tischler, 1981; Zapalowski et al., 1981), humans (Felig et al., 1969a; Felig, 1975; Adibi, 1976; Elia et al., 1980; Williamson, 1980; Elia and Livesey, 1981; Zapalowski et al., 1981), dogs (Nissen and Haymond, 1981) and sheep (Ballard et al., 1976; Bergen, 1979; Heitmann and Bergman, 1980; Bergman and Pell, 1983); however, the length of fast required to initiate this response has varied. Goldberg and Odessey (1972) reported that 24 h of fasting increased plasma valine and isoleucine concentrations by 25% and did not alter leucine concentration in 60 to 90 g rats, but after 2 and 3 d of fasting, concentrations of all three BCAA were decreased. Hutson and Harper (1981), on the other hand, obtained increased ($P<.002$) plasma BCAA concentrations after fasting 160 to 190 g rats 3 d. In humans plasma BCAA concentration increased for the first 1 or 2 wk of a fast and then returned to normal (Felig et al., 1969a). Adibi (1976) reported that 6 d of fasting were required to raise plasma BCAA concentrations in rats, but concentrations of BCAA are elevated after only 1 d of fasting in humans. It is apparent that the results with rats have been contradictory, but the varying animal weights utilized in the different

studies may be a partial explanation. Results with human subjects have been more consistent.

The metabolic response to starvation in humans has been described by Felig (1975) as being biphasic. Phase I involves maintaining hepatic gluconeogenesis, while the second response is directed at maintaining body protein reserves by minimizing protein catabolism. During phase I muscle protein and amino acid degradation is increased to supply substrates (primarily alanine and glutamine) for glucose synthesis in the liver. Later in starvation, glucose is replaced by ketone bodies as the predominant metabolic fuel, and body protein is spared at the expense of lipids. During short-term fasting, concentrations of BCAA in plasma could be increased by decreasing protein synthesis, increasing protein degradation and (or) decreasing BCAA degradation. Concentrations of BCAA are higher in muscle protein than in free amino acid pools, thus a shift towards net catabolism of muscle protein would rapidly increase concentrations of these amino acids in plasma (Bergen, 1979). The possible contribution of decreased BCAA degradation to increased concentrations of these amino acids after a fast also has been investigated.

Work with rats indicates that BCAA aminotransferase is only marginally responsive to fasting. Adibi et al.(1975) reported a 12 h fast decreased leucine aminotransferase

activity in skeletal muscle and kidney slightly, but by 24 h leucine aminotransferase activity was increased twofold over unfasted controls. Liver BCAA aminotransferase was unresponsive to fasting for up to 5 d. Other workers have found no effect of fasting on leucine aminotransferase in muscle (Goldberg and Chang, 1978), liver or kidney (Wohlhueter and Harper, 1970). Tischler and Goldberg (1980a) showed that a 2 d fast decreased BCAA aminotransferase activity in rat adipose tissue by 47%. Therefore only in adipose tissue could regulation of BCAA aminotransferase play a role in increasing plasma BCAA concentration during fasting.

In rats it is generally agreed that fasting increases BCKA dehydrogenase activity in heart (Buse et al., 1973; Tischler and Goldberg, 1980b; Goldberg and Tischler, 1981) and skeletal muscle (Adibi et al., 1971, 1974; Goldberg and Odessey, 1972; Meikle and Klain, 1972; Odessey and Goldberg, 1972, 1979; Paul and Adibi, 1976, 1978a,b, 1979, 1980, 1981; Goldberg and Chang, 1978; Goldberg and Tischler, 1981), while decreasing activity in adipose tissue (Meikle and Klain, 1972; Frick and Goodman, 1979; Tischler and Goldberg, 1980a, 1981; Goldberg and Tischler, 1981). The only contradictory report is that of Sketcher et al.(1974) who found starvation of rats decreased BCAA oxidation in muscle. Their methodology involved the freezing of the

whole muscle prior to homogenization which may account for the apparently aberrant results. Goldberg and Odessey (1972) and Goldberg and Tischler (1981) reported that a 3 d fast increased KIC dehydrogenase activity in kidney, but 1 d of food deprivation had no effect on kidney BCKA dehydrogenase activity (Wohlhueter and Harper, 1970). Most studies indicate that liver BCKA dehydrogenase is unresponsive to fasting (Goldberg and Odessey, 1972; Adibi et al., 1974; Adibi, 1976; Goldberg and Chang, 1978; Frick and Goodman, 1979; Goldberg and Tischler, 1981), but others report a slight increase in liver BCKA dehydrogenase activity due to fasting (Wohlhueter and Harper, 1970; Sketcher et al., 1974; Paul and Adibi, 1981). The small to nonexistent response of BCKA dehydrogenase in liver and kidney to fasting indicate that these organs are unimportant in regulating BCAA degradation, however, the consistent increase (two- to fivefold) and decrease (50 to 70%) in activity observed in rat skeletal muscle and adipose tissue, respectively, due to fasting may be important.

In vivo infusions of uniformly labeled BCAA into fasted rats have shown increased oxidation of BCAA to $^{14}CO_2$ compared to infusions into unfasted controls (Sketcher et al., 1974; Tischler and Goldberg, 1980a; Zapalowski et al., 1981). However, Tischler and Goldberg (1980a) demonstrated that overall rates of leucine catabolism were quite similar

in fed and fasted rats when L-[1-^{14}C]-leucine was infused as substrate. Uniformly labeled leucine, when degraded in skeletal muscle as an energy source, should yield $^{14}CO_2$. When degraded in adipose tissue, the end products should be primarily [^{14}C]- triglycerides. These results indicate fasting increased oxidation of leucine by skeletal muscle, but decreased the conversion of leucine to triglycerides in adipose tissue (Tischler and Goldberg, 1980a, 1981; Goldberg and Tischler, 1981). Therefore at least in rats the rise in plasma BCAA appears to be due mainly to a shift toward protein catabolism since the rate of leucine catabolism is unchanged.

In humans there are no studies that have been specifically designed to measure the effect of fasting on BCAA aminotransferase or BCKA dehydrogenase activities. Sherwin (1978) and Elia et al.(1980) obtained a two- to threefold greater increase in plasma leucine concentration upon leucine infusion in fasted than in fed human subjects. The latter authors along with Williamson (1980) concluded from these studies that leucine is conserved during fasting. However, another possible interpretation may be the effect of insulin concentration associated with fasting which could have caused the decreased leucine uptake and incorporation into protein. Alternatively, leucine degradation could be depressed during fasting. Certainly,

humans possess a greater percentage of adipose tissue than normal rats, so if adipose tissue BCKA dehydrogenase is decreased in humans to a similar extent as in rats by fasting, this could account for the decreased leucine removal observed by Sherwin (1978). It is also possible that human muscle, like rabbit muscle (Ryan et al., 1974), exhibits decreased BCKA dehydrogenase activity during fasting. However, Felig (1975) noted that liver and skeletal muscle output is unchanged during 3 d of fasting so a major contribution of adipose tissue to increasing plasma BCAA concentration seems plausible.

Nissen and Haymond (1981) and Tessari et al.(1982) utilized a double label technique to analyze the effect of fasting 14 or 96 h on 7 to 15 mo old dogs. Plasma concentrations of leucine were increased while KIC concentrations were unchanged by either a 14 or 96 h fast. In addition, metabolic clearance rates of both leucine and KIC were decreased by fasting, while the clearance rate of KIC was fivefold greater than that of leucine for both 14 and 96 h fasted dogs. Nissen and Haymond (1981) suggest that transamination is a major regulator of leucine metabolism in fasting dogs. In dogs fasted for 14 h, the infusion of intralipid decreased plasma leucine concentration as well as KIC oxidation without altering insulin or glucagon concentrations, indicating that plasma

free fatty acid availability may cause nitrogen sparing in fasted dogs by decreasing both proteolysis and essential amino acid oxidation (Tessari et al., 1982).

In sheep, plasma concentrations of BCAA are increased 50% by a 3 d fast (Bergman and Pell, 1983). Hepatic removal of BCAA is continual and relatively constant even after a 3 d fast (Heitmann and Bergman, 1980), but extrahepatic tissue changes from a net utilization of BCAA in the fed state to production during fasting (Ballard et al., 1976; Heitmann and Bergman, 1980; Bergman and Pell, 1983). Interestingly, Lindsay (1982) reported that direct studies of BCAA oxidation in his laboratory have failed to show that fasting increases BCAA oxidation in skeletal muscle.

Overall, fasting appears to increase plasma BCAA concentrations in all of the species studied, but the cause and timing of this increase varies between species. Free BCAA pools are controlled by net proteolysis or protein accretion and by BCAA degradation. It is well established that the increased proteolysis associated with a short term fast contributes to the increased concentration of free BCAA (Bergen, 1979), but the effect of fasting on BCAA degradation in different tissues and in different species is highly variable. In rats, degradation of BCAA by liver and kidney is relatively unaltered, while BCAA oxidation is increased in skeletal muscle by fasting and decreased in

adipose tissue (Goldberg and Tischler, 1981). Sensitivity of skeletal muscle and adipose tissue to food deprivation in species other than the rat has not been thoroughly investigated, but there is no evidence indicating that BCAA oxidation is enhanced in skeletal muscle by fasting in species other than the rat (Ryan et al., 1974; Lindsay, 1982; Nissen and Haymond, 1981). Finally, the conversion of BCAA to lipid is impaired during starvation (Frick and Goodman, 1979) and this could be a possible mechanism for the accumulation of BCAA during fasting (Adibi, 1976; Goldberg and Tischler, 1981).

Dietary Protein

Protein deprivation, unlike energy deprivation, decreases plasma BCAA concentrations, and conversely, consumption of a high protein diet increases plasma BCAA concentrations in rats (Elia et al., 1979; Hutson and Harper, 1981), humans (James, 1972; Felig, 1975; Adibi, 1976) and sheep (Bergen et al., 1973). Hutson and Harper (1981) fed diets to 160 to 190 g rats that contained 8%, 24% or 60% crude protein. Rats fed the 60% crude protein diet had two- to threefold higher plasma concentrations of BCAA than controls (24% crude protein). Interestingly, BCAA

concentrations in the rats fed the 8% crude protein diet were not different than those of controls, indicating that either rat plasma BCAA concentrations are unresponsive to a low protein diet or that an extremely low protein diet (lower than 8% crude protein) is required to produce the decrease in BCAA concentration observed in other species (Bergen et al., 1973; Adibi, 1976).

Excess intake of leucine decreased the plasma concentrations of valine, isoleucine, KIV and KMV in rats fed a deficient or adequate protein diet, but feeding excess valine or isoleucine did not depress the concentrations of any of the BCAA (Shinnick and Harper, 1977; Walser, 1984). Excess dietary leucine did not alter $^{14}CO_2$ production from infused [1-^{14}C]-valine so the authors postulated that leucine decreased valine and isoleucine concentrations by decreasing protein degradation or increasing protein synthesis while valine or isoleucine did not alter protein turnover. Shinnick and Harper (1977) noted that leucine's effect on protein turnover might be mediated by insulin since leucine is a potent stimulator of insulin secretion. Harper and Benjamin (1984) reported that as dietary leucine was increased from 0 to 2.4%, plasma concentrations of leucine and KIC were increased, while concentrations of valine, isoleucine, KIV and KMV were decreased. The rate of leucine oxidation was low until dietary leucine content

exceeded that needed for maximum weight gain, and thereafter leucine oxidation increased linearly with increasing dietary leucine. In the same study rats were fed diets devoid of leucine to which graded amounts (0 to 2.05%) of KIC were added. KIC oxidation increased as dietary KIC was increased over the entire range of dietary levels tested. Leucine and KIC concentrations in plasma of rats did not increase in proportion to increasing dietary KIC, but concentrations of valine, isoleucine, KIV and KMV were decreased as dietary KIC was increased (Harper and Benjamin, 1984).

Effects of dietary protein on BCAA aminotransferase have been studied in rats. Adibi et al.(1975) demonstrated that feeding a protein deficient diet inhibited leucine transamination in skeletal muscle but leucine aminotransferase activity was unaffected in liver and kidney. Wohlhueter and Harper (1970) and Shinnick and Harper (1977) also reported BCAA aminotransferase in liver and kidney was unresponsive to dietary protein manipulations, but others (Ichihara et al., 1967; McFarlane and von Holt, 1969a; Krebs, 1972) have found that the liver was responsive. Ichihara et al.(1967) reported that feeding a 50% casein diet increased leucine aminotransferase activity in the supernatant fraction of liver several fold while leucine aminotransferase activity in liver mitochondrial or either kidney fraction was unchanged.

Valine and isoleucine transamination was not altered in liver or kidney by feeding the 50% casein diet. Stimulation of leucine transamination by feeding the high protein diet was completely blocked by puromycin or actinomycin, indicating cytosolic isoenzyme II (which is specific for leucine) is inducible. McFarlane and von Holt (1969a) also observed that leucine aminotransferase activity in liver was induced by feeding a high protein diet, but this inducible activity was localized in the mitochondrial fraction. Using crude homogenates of liver, Krebs (1972) stimulated leucine aminotransferase activity by feeding rats a high protein diet. Rats fed a protein deficient diet exhibited markedly reduced leucine aminotransferase activity in epididymal fat pads (Tischler and Goldberg, 1980a).

In vivo studies of overall BCAA oxidation (McFarlane and von Holt, 1969b; Sketcher and James, 1974) and enzyme assays of skeletal muscle (Sketcher et al., 1974; Sketcher and James, 1974, 1976; Shinnick and Harper, 1977), liver (McFarlane and von Holt, 1969a; Wohlhueter and Harper, 1970; Adibi, 1976; Shinnick and Harper, 1977; Frick and Goodman, 1979) and adipose tissue (Frick and Goodman, 1979; Tischler and Goldberg, 1981) indicate that BCKA dehydrogenase is stimulated or inhibited by feeding a diet high or low in protein, respectively. However, Wohlhueter and Harper

(1970) reported that BCKA dehydrogenase activity in rat kidney was unaltered by changes in dietary protein content.

Diabetes and Insulin

Plasma concentrations of BCAA are increased several fold by diabetes in rats (Schaarf and Wool, 1966; Chua and Morgan, 1978; Goldberg and Chang, 1978; Hutson and Harper, 1981; Goldberg and Tischler, 1981; Zapalowski et al., 1981), humans, (Carlsten et al., 1966; Felig, 1975; Elia and Livesey, 1981) and sheep (Prior and Smith, 1981). Hutson and Harper (1981) showed that diabetes increased BCAA concentration two- to threefold and BCKA concentration twofold in rats. In humans (Carlsten et al., 1966) Felig et al., 1969b; Felig and Wahren, 1974) and sheep (Prior and Smith, 1981) insulin infusions decreased the elevated BCAA concentrations observed in diabetic individuals back to normal levels.

Effects of diabetes on BCAA aminotransferase have been studied in liver, kidney and skeletal muscle of rats. Shinnick and Harper (1977) observed that leucine aminotransferase activity expressed per gram of tissue was increased ($P<.05$) in liver, but liver weights were greatly reduced so this apparent change could have been due to an

increase in the concentration of enzyme protein. In the study of Ichihara et al.(1967) BCAA aminotransferase in rat liver was unaffected by diabetes but actinomycin-inhibited induction of BCAA aminotransferase by diabetes was observed in enzyme preparations from rat kidney. Activity of BCAA aminotransferase in rat hindquarters was inhibited ($P<.05$) by the inclusion of insulin in the perfusion medium.

In rats diabetes has been shown to stimulate the decarboxylation of BCAA in skeletal muscle (Odessey and Goldberg, 1972; Herlong et al., 1974; Buse et al., 1974, 1976, 1980; Goldberg and Chang, 1978; Paul and Adibi, 1978a, b, 1979, 1980, 1981; Zapalowski et al., 1981), heart (Chua and Morgan, 1978), liver (Paul and Adibi, 1978a, 1981) and kidney (Paul and Adibi, 1978a), but the ability of insulin to reverse this effect is more equivocal (Manchester, 1965; Buse and Buse, 1967; Meikle and Klain, 1972; Buse et al., 1974, 1975; Paul and Adibi, 1976; Zapalowski et al., 1981). Paul and Adibi (1978a) obtained a twofold greater KIC dehydrogenase activity in skeletal muscle, liver and kidney mitochondrial preparations from diabetic rats than in the preparations from normal rats. In another study these authors measured mitochondrial BCKA dehydrogenase activity with or without a preincubation period in skeletal muscle and liver preparations from normal or diabetic rats (Paul and Adibi, 1981). Without preincubation, liver and skeletal

muscle preparations from diabetic rats had twofold greater activity than preparations from controls, while after a 30 to 60 min preincubation period the treatment groups were both elevated but not different (P>.1) from one another. Adding ATP reversed the effect of preincubation on BCKA dehydrogenase activity from controls but did not affect BCKA dehydrogenase activity from diabetics, indicating the role of ATP in regulating BCKA dehydrogenase is greatly reduced or abolished by diabetes (Paul and Adibi, 1981).

Buse et al.(1974) reported that diabetes increased leucine oxidation of rat diaphragms by 50% and addition of insulin (with but not without glucose) to the medium decreased leucine oxidation to control levels, but diaphragms excised from control animals were unaffected by insulin. On the other hand, in the rat hindquarter perfusion study of Zapalowski et al.(1981) the greatly elevated BCAA degradation observed in hindlimbs from diabetic animals was unaffected by insulin, even though glucose was included in the perfusion medium. In fact, insulin addition increased the rate of BCAA oxidation in starved rat hindquarters. The studies analyzing the effect of insulin on BCKA dehydrogenase in skeletal muscle have shown increased (Manchester, 1965), decreased (Buse et al., 1974; Herlong et al., 1974; Hutson et al., 1978) or unchanged (Buse and Buse, 1967; Meikle and Klain, 1972; Buse

et al., 1975; Paul and Adibi, 1976) enzyme activities due to insulin. In adipose tissue, the oxidation of leucine was stimulated by the presence of insulin (Goodman, 1963a, b, 1964, 1977; Smith and Biegelman, 1968; Rosenthal et al., 1974; Frick and Goodman, 1980, 1981; Tischler and Goldberg, 1980a, 1981). Goodman (1977) reported that insulin stimulated $^{14}CO_2$ release from L-[1-^{14}C]-leucine or [1-^{14}C]-KIC by epididymal fat segments, but a 20 min lag time was required for the effect. This stimulatory effect was observed with or without glucose and with leucine concentrations ranging from .1 to 10 mM. Glucose also stimulated KIC dehydrogenase activity and this effect was additive with the insulin stimulation. The lag time for the effect of insulin is difficult to explain since glucose acceleration of leucine oxidation required no delay. Also insulin increased glucose utilization without a lag time, and neither cycloheximide nor puromycin blocked the increase in KIC dehydrogenase activity caused by insulin (Goodman, 1977).

Observations of Lindsay and Buttery (1980) indicate that the ability of sheep skeletal muscle to oxidize BCAA is enhanced by diabetes. In diabetic sheep the output of all amino acids from muscle increased with the exception of the BCAA, the output of which fell markedly (Lindsay and Buttery, 1980). These results in sheep may be of particular

interest, since unlike the rat, the capacity of sheep muscle to metabolize BCAA is probably not enhanced by starvation (Lindsay, 1982).

Age. Effects of age on BCAA oxidation have not been extensively studied in rats and have not been analyzed in other species. Odessey and Goldberg (1979) in citing Odessey (1974) reported there was no difference in skeletal muscle BCKA dehydrogenase activity between 60 to 100 g rats and mature rats. Sketcher and James (1974) also studied BCAA oxidation at different stages of development. L-[1-^{14}C]-leucine was infused into 35, 85 or 200 g rats fed a normal or protein-free diet. Production of $^{14}CO_2$ per gram of body weight was not different for all three weight groups fed the normal diet. In addition, rats from all three groups fed the protein-free diet exhibited reduced BCKA dehydrogenase activity, but 35 g rats had the greatest reduction in activity.

Ballard and Francis (1983) incubated L6 myoblasts with L-[4,5-^{3}H]-leucine for 18 h. At the end of this period leucine accounted for 98 and 95% of the radioactivity in the cells and medium, respectively, demonstrating L6 myoblasts have very limited ability to degrade leucine.

Effects of BCAA and BCKA on Muscle Protein Turnover

In Vitro Studies

Whether muscle undergoes growth, remains constant or atrophies depends on the relative rates of protein synthesis and protein degradation. Therefore from a clinical perspective the most important aspects of BCAA metabolism are their proposed effects on muscle protein turnover. Numerous studies from various laboratories have demonstrated that BCAA or their respective BCKA enhance protein synthesis (Fulks et al., 1975; Buse and Reid, 1975; Atwell et al., 1977; Goldberg and Chang, 1978; Li and Jefferson, 1978; Hedden and Buse, 1979; Goldberg, 1980a, b) and inhibit protein degradation (Buse and Weigand, 1977; Chua and Morgan, 1978; Chua et al., 1979; Tischler, 1980; Goldberg and Tischler, 1981; Odessey and Parr, 1982; Tischler et al., 1982) in skeletal and cardiac muscle isolated from rats.

In diaphragm preparations, Fulks et al.(1975) initially reported that addition of BCAA or leucine alone to the medium stimulated protein synthesis and decreased protein breakdown to the same extent as did all plasma amino acids when supplied together. Buse and Reid (1975) concluded from their work with rat diaphragm that leucine alone, but no

other amino acid significantly promoted protein synthesis and inhibited protein degradation. Subsequently, Atwell et al.(1977) and Hedden and Buse (1979) showed that in incubations of rat diaphragm, BCAA stimulated the incorporation of uniformly labeled tyrosine into myofibrillar, soluble and total protein by 50 to 60%. Tyrosine incorporation into individual proteins within myofibrillar and soluble protein fractions was stimulated without preferential synthesis of a particular protein. The lack of selectivity of stimulation is compatible with an effect on translation (Hedden and Buse, 1979). Boyd and Jefferson (1979) utilized polysome to ribosomal subunit ratios to show that the block in peptide chain initiation that normally develops in isolated gastrocnemius muscle preparations is prevented by the addition of leucine at one to fifteen times the normal plasma concentration. That leucine's stimulatory effect on muscle protein synthesis involves enhancement of the peptide chain initiation step of translation has been substantiated (Li and Jefferson, 1978; Buse et al., 1979; Tischler et al., 1982). It was suggested that the effect of leucine on peptide initiation could be accomplished by increasing the availability of leucyl-tRNA (Goldberg and Chang, 1978), but more recent studies indicate that this is not the case (Morgan et al., 1981; Goldberg and Tischler, 1981). Formation of leucyl-tRNA showed a K_m of 6

μM or less. Therefore since intracellular concentrations of leucine vary between .1 and .5 mM, under physiological conditions, leucyl-tRNA must be fully charged (Goldberg and Tischler, 1981).

Tischler et al.(1982) clarified many points relating to the influence of BCAA on protein turnover. L-cycloserine (an inhibitor of leucine transamination) was used to determine if leucine degradation is essential for its effects on protein turnover in skeletal muscle. Cycloserine prevented the inhibition of protein degradation by leucine, but not that induced by insulin and glucose. However, cycloserine did not diminish the stimulatory effect of leucine on protein synthesis. Addition of .5 mM KIC in the presence of cycloserine reduced proteolysis by 25%, but had no effect on synthesis. Therefore, KIC or some product of KIC metabolism inhibits protein degradation, but leucine itself probably is responsible for the stimulation of protein synthesis. On the other hand, Buse and Weigand (1977) reported that leucine, but not KIC, inhibited protein degradation.

Chua et al.(1979) reported that leucine or insulin alone increased latency of cathepsin D in heart muscle, and leucine and insulin together increased latency more than either agent alone.

Goldberg et al.(1980a) pointed out that isolated

skeletal muscles incubated in unsupplemented Krebs Ringer bicarbonate buffer exhibited net proteolysis; but factors can be added to the buffer system to help achieve neutral nitrogen balance. Insulin increases protein synthesis and decreases protein degradation, and glucose also inhibits protein breakdown. Addition of leucine further increases protein synthesis and decreases protein degradation, but these factors still do not prevent net protein catabolism. However, inclusion of these factors (insulin, glucose and leucine) in the medium with the application of repetitive stimulation of or passive tension on isolated muscles can produce neutral or even positive nitrogen balance (Goldberg et al., 1980a). In any case, this study (Goldberg et al., 1980a) as well as others (Fulks et al., 1975; Atwell et al., 1977; Frayn and Maycock, 1979) indicate that the effect of leucine or its metabolites on inhibition of net proteolysis is additive with the effects of glucose and insulin. Li and Jefferson (1978) disagreed with these studies. In their study they used the hemicorpus of 80 g rats and reported that the effect of BCAA on fasted muscle was not as great as that of insulin, and that the presence of BCAA did not augment the effect of insulin.

One report (Shangrow and Turinsky, 1980) indicated that the addition of leucine did not decrease protein degradation. Since most workers have used thin diaphragm or

soleus muscles from small rats (<100g) and Shangrow and Turinsky (1980) used unstretched soleus muscle from 200 g rats diffusion may have been a problem in the larger muscles. In addition, Li and Jefferson (1978) demonstrated that leucine infusion into 80 g rat hemicorpus preparations increased protein synthesis 25 to 50% and decreased protein degradation by 30% in gastrocnemius muscles, but skeletal muscle of 200 g rats was unresponsive to perfusion with leucine.

Cardiac muscle protein turnover is also responsive to BCAA concentrations but differences do exist between cardiac and skeletal muscle responsiveness. Leucine, but not valine or isoleucine, stimulated protein synthesis (50%) and inhibited degradation (Chua and Morgan, 1978; Morgan et al., 1981) in heart muscle, but unlike skeletal muscle, other oxidizable (30%) substrates such as fatty acids, ketone bodies, pyruvate, acetate (Rannels et al., 1974), KIC, KIV and KMV (Chua et al., 1979) exerted effects similar to those of leucine on heart protein turnover. Insulin, also increased protein synthesis and decreased protein degradation in cardiac muscle, and in the presence of this hormone the addition of leucine had no further effect on protein turnover (Chua and Morgan, 1978; Chua et al., 1979). Therefore the concentration of leucine may alter cardiac muscle protein turnover only in physiological states when

insulin concentrations are low. It is interesting that during states when insulin concentration is low, such as fasting and diabetes, concentrations of fatty acids, BCAA and ketone bodies are elevated, indicating that these substrates may be of physiological significance in keeping cardiac protein turnover in check during these states (Rannels et al., 1974).

Acceleration of protein synthesis in both cardiac and skeletal muscle involved faster rates of peptide chain initiation, and in heart as well as skeletal muscle this effect was not accounted for by increased tissue concentrations of leucyl-tRNA (Morgan et al., 1981). In isolated working hearts supplied with physiological concentrations of glucose, lactate, insulin and glucagon, an increase in concentration of leucine in the perfusion medium from .2 to 1 mM converted nitrogen balance from slightly negative to positive. Cardiac work inhibited protein degradation, while stimulating protein synthesis, and positive nitrogen balance. Effects of leucine and cardiac work were additive for protein degradation and nitrogen balance but not for protein synthesis (Chua et al., 1980; Morgan et al., 1981). Overall, results with isolated tissues suggest that leucine or its metabolites may play a significant physiological role in regulating protein turnover in both skeletal and cardiac muscle.

In Vivo Studies

The roles of BCAA and BCKA in regulating protein turnover also have been investigated in intact animals and man, but with varying results. Improvements in nitrogen balance in response to treatment with BCAA or BCKA in fasted (Sapir and Walser, 1977; Sherwin, 1978; Mitch et al., 1979, 1981) or post-operative (Freund et al., 1979) humans and septic fractured (Blackburn et al., 1979) or laparotomized (Freund et al., 1980) rats have been observed. Mitch et al.(1979) infused 11 mmol of leucine, KMV, KIV or KIC daily into fasting obese humans for the first week of a 2 wk fast. Leucine, KIV and KMV did not alter urinary nitrogen but KIC decreased ($P<.05$) urinary nitrogen compared to control fasts in the same subjects in both the first and second week of the fast, suggesting that KIC possessed all the nitrogen sparing effects of BCKA. Sherwin (1978), however, obtained a 25 to 30% decrease in negative nitrogen balance when leucine was infused into humans fasted for 3 d or 4 wk. Despite the improved nitrogen balance, 3-methylhistidine excretion was unchanged, so Sherwin (1978) suggested, leucine affected nitrogen balance by promoting protein synthesis. Giordano (1980) stated that 1) the nitrogen sparing effects of BCKA were not confirmed, 2) BCKA

could inhibit gluconeogenesis in kidney (starving nephrons) and 3) that BCKA should not be used in clinical practice until their usefulness is proven.

In vivo measurements of protein turnover have also been inconclusive. Blackburn et al.(1979) reported that administration of BCAA intragastrically to fasted septic fractured or fasted normal rats spared nitrogen by increasing fractional synthesis rate in rectus abdominus muscle and this effect was not explained by BCAA nitrogen content alone. Buse et al.(1979) obtained an increase in the proportion of aggregated polysomes isolated from psoas muscles of rats fasted for 48 to 96 h that had been infused with all three BCAA or leucine as the sole amino acid along with insulin and glucose 1 or 2 h prior to sacrifice compared to controls infused with only insulin and glucose. However, in laparotomized rats valine, but not leucine, infusion stimulated fractional synthesis rate in skeletal muscle (Freund et al., 1981).

Buse (1981) used a 6 h constant infusion of [^{14}C]-tyrosine (.5 ml/h) in .9% saline or 2.8 M glucose or 2.5 M glucose with .1 M each of leucine, valine and isoleucine into 80 to 100 g rats fasted 2 d to estimate protein synthesis in skeletal muscle. Heart, soleus and diaphragm muscles incorporated more tyrosine into proteins than psoas and gastrocnemius muscles following all three

infusions. Infusion of glucose alone did not alter tyrosine incorporation ($P>.08$) in any of the muscles studied, but infusion of BCAA with glucose increased tyrosine incorporation into diaphragm proteins twofold and into soleus and psoas muscles to a lesser extent (1.5-fold). Tyrosine incorporation into proteins in heart and gastrocnemius muscle was unaffected by all infusion treatments.

McNurlan et al.(1982) obtained no stimulatory effect of leucine on protein synthesis in gastrocnemius muscle, heart, jejunal serosa, jejunal mucosa or liver in fed, 2 d starved or protein-deprived rats (90 to 135g). A flooding dose of [^{3}H] phenylalanine was used to measure fractional synthesis rate, and 100 μmol of leucine was infused either with the labeled phenylalanine or 1 h prior to phenylalanine infusion. Both methods of leucine administration were ineffective in stimulating protein synthesis, even though leucine did transiently increase plasma insulin concentration by about twofold in all three dietary treatment groups (McNurlan et al., 1982). Buse (1981) concluded that their in vivo data supported previous observation in isolated muscles, that BCAA are rate limiting for muscle protein synthesis, while McNurlan et al.(1982) suggested that in fed, starved or protein-deprived rats, leucine does not play an important part in the regulation of

tissue protein synthesis. But, interestingly their results do not conflict with each other, as in both studies leucine failed to stimulate protein synthesis in heart and gastrocnemius muscle, which were the only tissues common to both studies (Buse, 1981; McNurlan et al., 1982).

While results in isolated muscles indicate that leucine or its metabolites have a positive regulatory effect on protein balance, the in vivo results are inconclusive (Walser, 1984). It appears that leucine may have some regulatory effect on protein synthesis and degradation, at least in some muscles. But without more definitive evidence of their beneficial effects and the assurance that leucine or KIC administration does not provide undesirable side effects, their usage should not be indiscriminately advocated.

CHAPTER I

EFFECTS OF AGE ON LEUCINE AMINOTRANSFERASE AND ALPHA-KETOISOCAPROATE DEHYDROGENASE ACTIVITY IN SELECTED TISSUES FROM RAM LAMBS

Introduction

The major pathways in the degradation of BCAA have been identified (Meister, 1965) and reviewed (Dancis and Levitz, 1972). The first two steps in BCAA degradation are the reversible transamination (catalyzed by BCAA aminotransferase) followed by the irreversible decarboxylation (catalyzed by BCKA dehydrogenase) of the resulting BCKA to a branched chain acyl CoA (Meister, 1965). Distribution of these two enzyme activities among tissues is not uniform (Harper and Zapalowski, 1981). In rats, the BCAA are unique among essential amino acids, in that they are primarily catabolized by extrahepatic tissues (Miller, 1962; Odessey and Goldberg, 1972). Rat skeletal muscle, because of its bulk is probably the major site of BCAA degradation (Adibi, 1976; Chang and Goldberg, 1978a,b), but kidney (Kadowaki and Knox, 1982) and adipose tissue (Goodman, 1963a,b; Rosenthal et al., 1974; Tischler and Goldberg, 1980a) also actively degrade BCAA. It is interesting, however, that while rat liver is low in BCAA aminotransferase activity, liver could be important in the degradation of BCKA.

Buttery (1979) reported that he failed to demonstrate marked oxidation of BCAA in skeletal muscle of ruminants. In addition, the release of BCAA into the circulation of ruminants parallels their muscle amino acid composition (Ballard et al., 1976; Lindsay, 1980), but nonruminants release less BCAA than expected from their muscle protein composition (presumably because of oxidation). Therefore ruminant skeletal muscle may have a more limited capability to oxidize BCAA (Teleni et al., 1983).

The effects of age on BCAA oxidation have not been extensively studied in rats and have not been determined at all in other species. Odessey and Goldberg (1979), in citing Odessey (1974) reported that no difference in skeletal muscle BCKA dehydrogenase activity existed between 60 to 100g rats and mature rats. Sketcher and James (1974) measured in vivo BCAA oxidation in 35, 85 and 200g rats fed a conventional laboratory chow or protein-free diet. The stage of development had no effect on BCAA oxidation in rats fed the conventional diet. Even though BCAA oxidation was reduced in all rats fed the protein-free diet, the reduction was most pronounced in the 35g rats. Ballard and Francis (1983) showed that L 6 myoblasts have limited ability to degrade leucine and thus the effect of developmental changes on the ability to degrade leucine is of interest. The developmental effects of age on BCAA metabolism in ruminants

may be of particular interest since they develop from a functional nonruminant at birth to a functioning ruminant by 50 to 100 d of age (Oh et al., 1972; Noble, 1980).

Therefore this study was designed to ascertain the effect of age on BCAA degradation in ram lambs and also to study the relative proportion of total activity contributed by skeletal muscle, liver, kidney and adipose tissue to BCAA aminotransferase and BCKA dehydrogenase activity. Leucine transamination and decarboxylation were measured on crude homogenates of skeletal muscle, liver, kidney and adipose tissues excised from ram lambs at various ages up to 1 year.

Experimental Procedure

Materials

The sodium salts of alpha-ketoglutarate and thiamine pyrophosphate, dithiothreitol, NAD^+, EGTA and L-leucine were obtained from Sigma Chemical Co., (St. Louis, MO). L-[1-^{14}C]-leucine was purchased from Research Products International Corp., (Mt. Prospect, IL) and Aqueous Counting Scintillant was obtained from Amersham Corp., (Arlington Heights, IL).

Experimental Design

Forty-nine Suffolk x Targhee cross ram lambs reared at Michigan State University were slaughtered at 1, 28, 56, 84, 112, 140, 168, 196, 224 or 365 d of age. The experimental design is shown in table I-1. Four lambs were slaughtered at 365 d of age and five lambs were slaughtered at each of the other ages. The 84 d through 365 d old lambs were born in March of 1982, and the 1 d through 56 d old lambs were born in March of 1983. Both lamb crops were from the same Targhee ewe flock and were sired by Suffolk rams of similar breeding. The lambs were fed a creep diet until weaning at 60 d of age. Lambs were then adjusted to ad libitum feeding of the experimental diet (table I-2), which they received until 12 h before slaughter.

Tissue Preparation

The lambs scheduled for slaughter on 28 d through 365 d of age were fasted overnight prior to electrical stunning and exsanguination. Day 1 lambs were removed from their dams for approximately 4 h and then slaughtered by decapitation. Longissimus and trapezius muscle, liver and kidney samples were rapidly excised and placed in ice cold 15 mM potassium phosphate homogenization buffer (pH 7.5) containing .25 M sucrose, 3 mM $MgCl_2$ and 1 mM EGTA (see flow

TABLE I-1. EXPERIMENTAL DESIGN

Item	Age at slaughter, d									
	1	28	56	84	112	140	168	196	224	365
No. of Lambs	5	5	5	5	5	5	5	5	5	4

TABLE I-2. EXPERIMENTAL DIET

Ingredient	%
Alfalfa, dehy.	38.00
Corn	29.50
Wheat	10.00
Oats	8.00
Soybean meal	6.00
Molasses (wet)	7.00
Dicalcium phosphate	.5
Limestone	.5
Trace mineral salt	.5

diagram, figure I-1). The tissues were then minced with scissors, passed through a tissue press (Harvard Apparatus Co. Inc., Dover, MA) and 1.25 g of the tissue preparation were suspended in 10 ml of the homogenization buffer (pH 7.5) containing 50% glycerol. The suspensions were homogenized in Potter-Elvehjem ground glass homogenizing tubes with eight passes of a motor driven Teflon pestle. Homogenates were then strained through a single layer of cheese cloth, sealed in screw cap test tubes and cryogenically frozen in Dry Ice and acetone (-70 C) for assay later the same day. Perirenal adipose tissue samples removed from the d 1 lambs and subcutaneous adipose tissue samples excised from the dorsal surface of the 6th through 12th thoracic vertebrae region of the lambs at 28, 56 and 365 d of age were handled identically to the muscle, liver and kidney samples except that they were placed and homogenized in room temperature buffer. Room temperature buffer was used for more efficient homogenization of adipose tissues because at ice cold temperature homogenization was very difficult to accomplish. Perirenal adipose tissue was removed from the d 1 lambs because an inadequate quantity of separable subcutaneous fat was present on these young lambs.

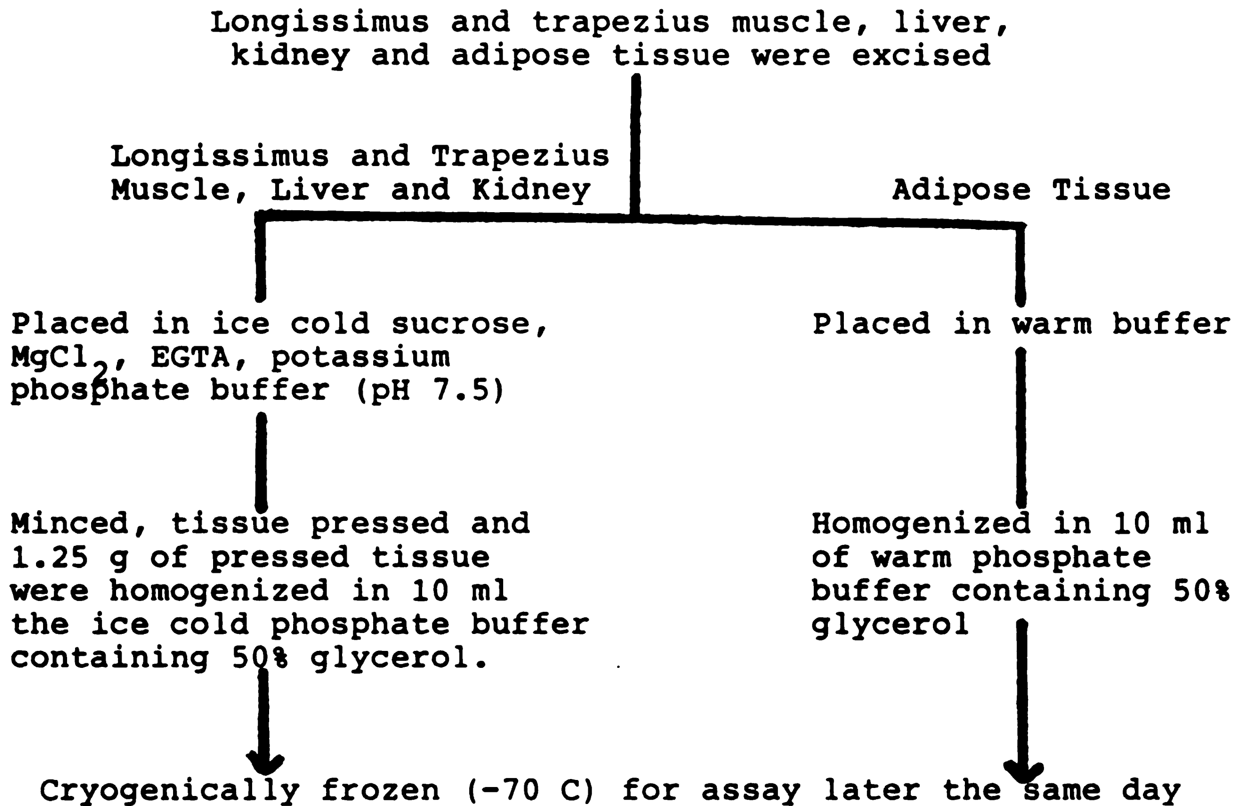


Figure I-1. Flow diagram of tissue preparation.

Enzyme Assays

L-[1-^{14}C]-leucine was used as substrate to measure BCAA aminotransferase activity and BCKA dehydrogenase activity. These enzymes will be referred to hereafter as leucine aminotransferase (LAT) and alpha-ketoisocaproate dehydrogenase (KICDH). The frozen homogenates contained in screw cap test tubes were thawed for 2 min in a water bath at 37 C. Subsequently, .5 ml of homogenate was added to 1.9 ml of assay medium contained in side-arm reaction flasks (Kontes, Vineland, NJ). The homogenates were preincubated in triplicate at 37 C for 15 min and then a 20 min reaction period was initiated by the addition of .2 μCi of L-[1-^{14}C]-leucine (40 mCi/mol) in 100 μl of buffer. The final concentration of the reaction buffer, cofactors and substrate has .2 mM thiamine pyrophosphate, 2.5 mM $MgCl_2$, .5 mM NAD^+, 2 mM alpha-ketoglutarate, 1 mM dithiothreitol, 50 mM sucrose, .2 mM EGTA, 2 mM leucine and 15 mM potassium phosphate (pH 7.5) in a total volume of 2.5 ml. Blanks containing the entire reaction mixture, with the exception of the homogenized tissues, were run simultaneously. Following addition of the isotope, the flasks were immediately capped and shaken at 60 rpm. At the end of 20 min, .5 ml of 2N H_2SO_4 was injected through the sidearm stopper to stop the reaction, and release all of the cleaved $^{14}CO_2$ from the media. The $^{14}CO_2$ was subsequently collected

for 1 h with continuous shaking in .3 ml of ethylene glycol monomethyl ether and ethanolamine (12:1, v/v) which was suspended in a center-well trap (Kontes). The traps were then removed, placed in scintillation vials containing 10 ml of aqueous counting fluid, and radioactivity was counted by liquid scintillation. Following the removal of the traps, the reaction flasks were recapped and the carboxyl carbons on the KIC in the media were nonenzymatically cleaved by the addition of 3 ml of 4N H_2SO_4 saturated with $CeSO_4$. The $^{14}CO_2$ thus cleaved was again trapped in ethanolamine and counted as described previously for the 2N H_2SO_4-released $^{14}CO_2$. The initial $^{14}CO_2$ collection represents the total enzymatic decarboxylation of leucine, i.e., the KICDH activity. The sum of the two $^{14}CO_2$ collections is a measure of leucine transamination or the LAT activity.

Homogenate protein was determined by the method of Lowry et al. (1951) and data were analyzed by one-way analysis of variance. Significant differences ($P<.05$) among means were separated by Duncan's New Multiple Range Test as outlined by Steel and Torrie (1960).

Results and Discussion

Preliminary Results

LAT and KICDH activities were linear for at least 30 min for kidney and skeletal muscle homogenates (figure I-2). Therefore in all subsequent experiments a 20 min reaction period was utilized. In another experiment LAT and KICDH activities were measured on sheep kidney crude homogenates with various concentrations of leucine (figure I-3). LAT activity increased sharply as leucine concentration was increased from .1 mM to .5 mM, but as leucine concentration was increased from .5 to 2 mM, the rate of increase in LAT activity was reduced. Even at 2 mM leucine concentrations, LAT was not saturated with substrate. However, the most active tissues degraded less than 2.5% of the leucine at 2 mM concentration during the 20 min reaction period. This slight decrease in substrate concentration during the reaction should have little effect on LAT activity. These observations are supported by the linearity of enzyme activity throughout the incubation period shown in figure I-2. Thus, in all subsequent experiments 2 mM leucine was used.

Figure I-3 shows that the effects of leucine concentration on KICDH activity are similar to those found for LAT activity. But it is important to realize that when

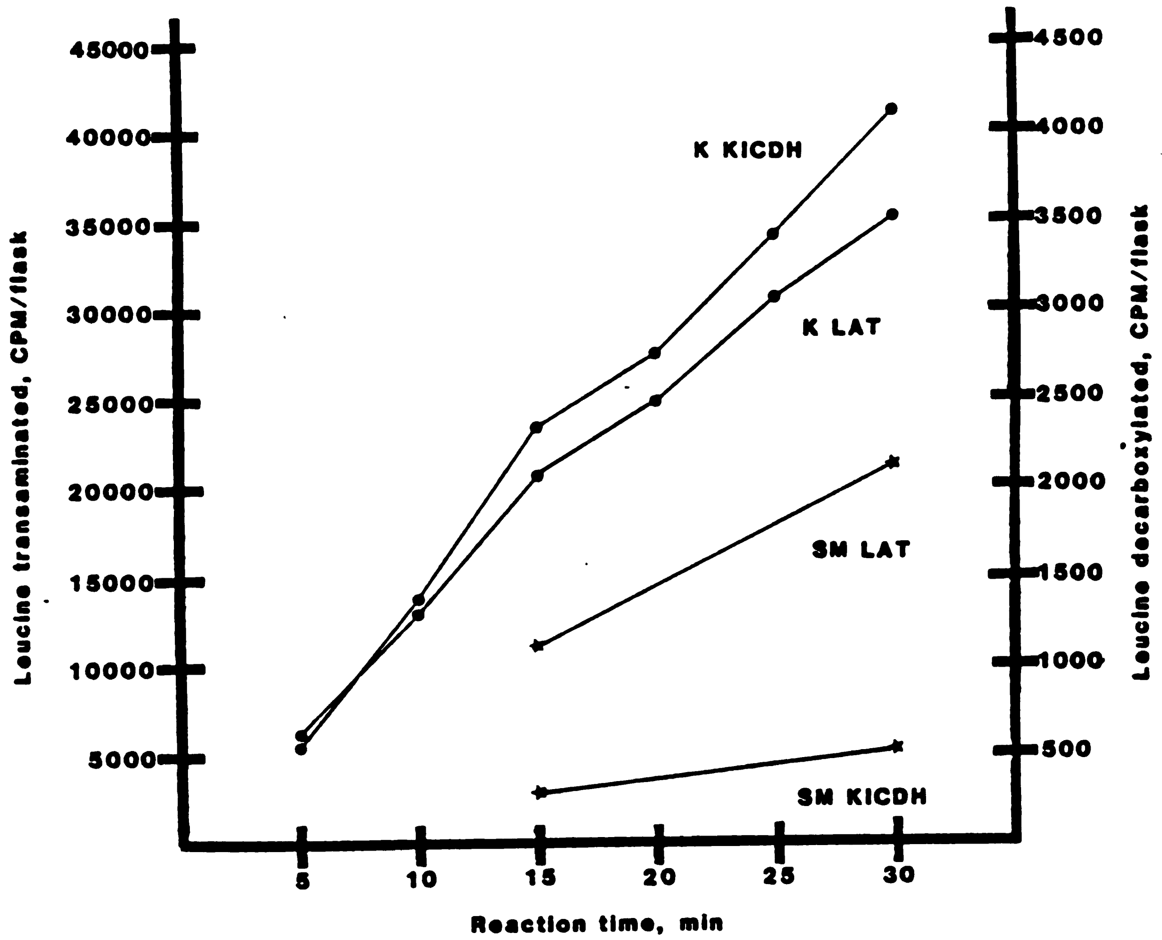


Figure I-2. The effects of reaction time on the transamination (LAT) and decarboxylation (KICDH) of L-[1-^{14}C]-leucine by rat kidney (K) and semimembranosus muscle (SM) crude homogenates. Points represent means of from four to 16 observations.

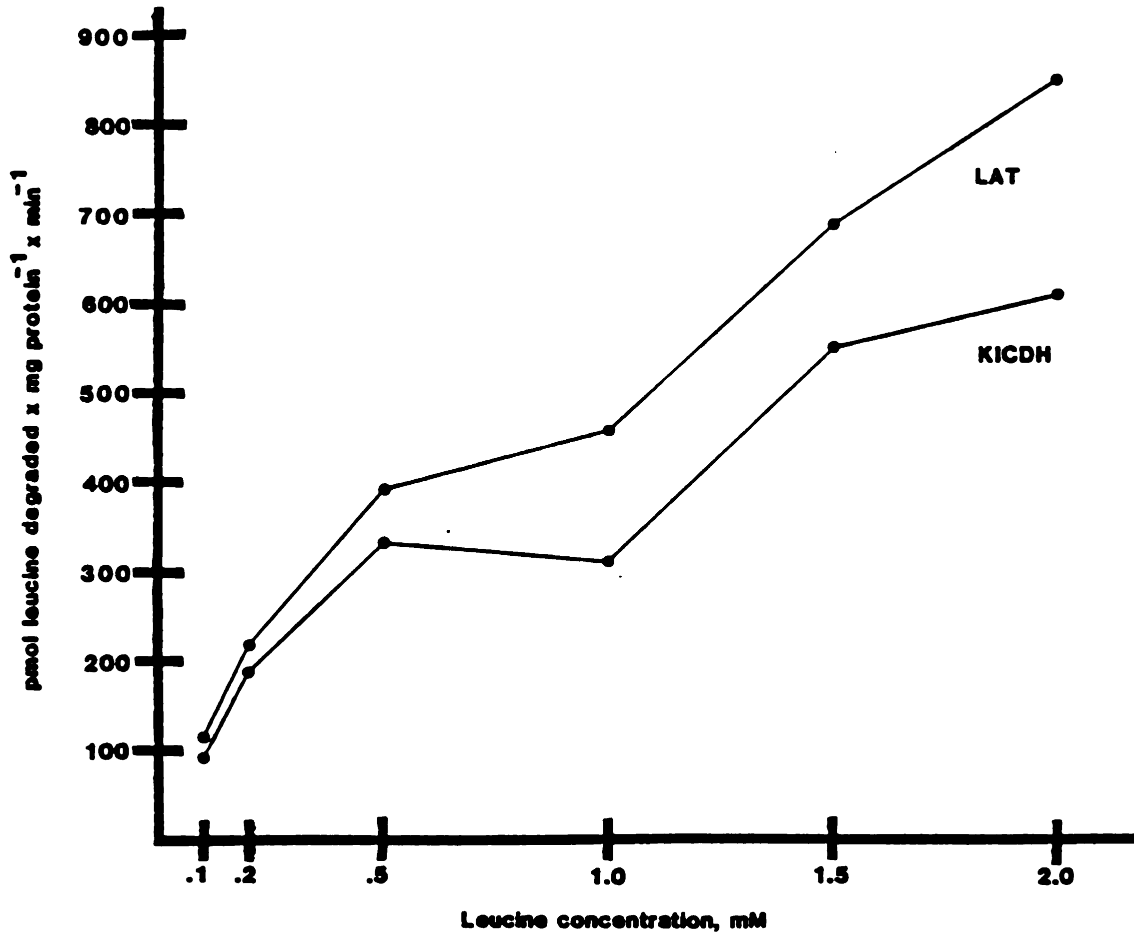


Figure I-3. The effect of leucine concentration on leucine aminotransferase (LAT) and alpha-ketoisocaproate dehydrogenase (KICDH) activities. Homogenates were prepared fresh and incubated with various concentrations of L-[1-^{14}C]-leucine (specific activity; 40 mCi/mol). Points represent the means of four observations.

leucine is used as the substrate, the dehydrogenase enzyme must rely on the aminotransferase enzyme to supply its substrate (KIC). This is particularly important in a tissue, such as liver, in which LAT is rate limiting (Harper and Zapalowski, 1981). In these cases KICDH activity may merely reflect LAT activity.

Freezing the skeletal muscle 10,000 x g pellets for 1 d has been reported to destroy up to 90% of the KICDH activity. Suspending the pellets in glycerol failed to preserve this activity (Odessey and Goldberg, 1979). On the other hand, freezing for up to 1 mo had no significant effect on aminotransferase activity (Odessey and Goldberg, 1979). In the present study, however, cryogenic freezing (-70 C) of kidney and skeletal muscle homogenates in 50% glycerol had no apparent effect on the activity of either enzyme compared to fresh homogenates (figure I-4). Therefore, because of the flexibility freezing allows, the crude homogenates were frozen for 2 to 8 h in all experiments. The preservation of KICDH activity in homogenates frozen in 50% glycerol could be due to the use of crude homogenates while Odessey and Goldberg (1979) studied the effects of freezing on KICDH activity in 10,000 x g pellets. The difference in freezing procedure could also be a possible explanation.

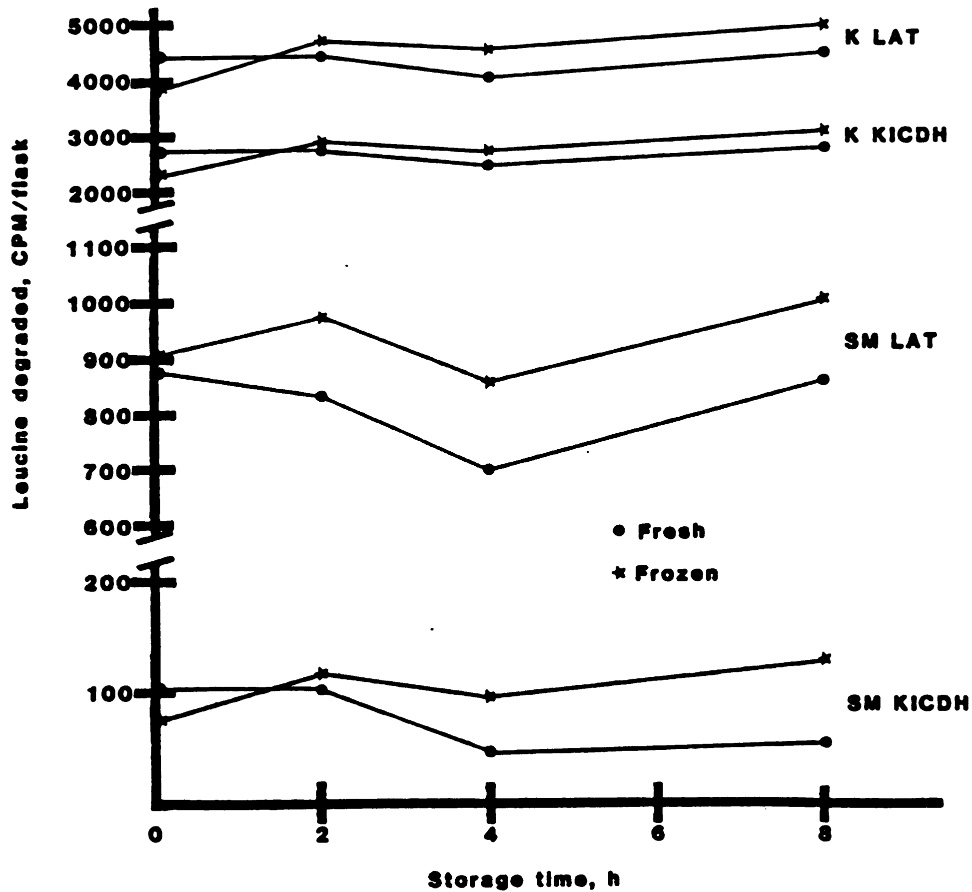


Figure I-4. The effects of cryogenic freezing and length of storage on the transamination (LAT) and decarboxylation (KICDH) of L-[1-^{14}C]-leucine by sheep kidney (K) and semimembranosus muscle (SM) crude homogenates. Points represent means of four observations.

Figure I-5 shows the effect of freezing rat kidney and skeletal muscle homogenates with or without glycerol, on LAT and KICDH activities. It is interesting that LAT activity was higher in homogenates frozen without glycerol than in homogenates frozen with glycerol, especially in kidney. However freezing without glycerol caused KICDH activity to decrease with frozen storage time. Also, muscle and kidney homogenates frozen without glycerol exhibited increased LAT and decreased KICDH activity as storage time increased from 6 to 24 h, while LAT and KICDH activities in homogenates frozen in 50% glycerol were not affected by length of frozen storage up to 24 h.

Enzyme Activity Per Milligram of Homogenate Protein

LAT and KICDH activities in longissimus and trapezius muscles are presented in table I-3. In longissimus muscle homogenates LAT activity was highest in d 1 lambs, drastically reduced by d 28 and tended to increase again ($P<.05$) at d 365. KICDH activity in longissimus muscle exhibited the same general trend. Dehydrogenase activity was highest in the neonatal lambs, significantly reduced by d 28 and then there was a trend for activity to increase as the lambs matured. The effects of age on enzyme activities in the trapezius are similar to those of the longissimus muscle except for the extremely high LAT activity in

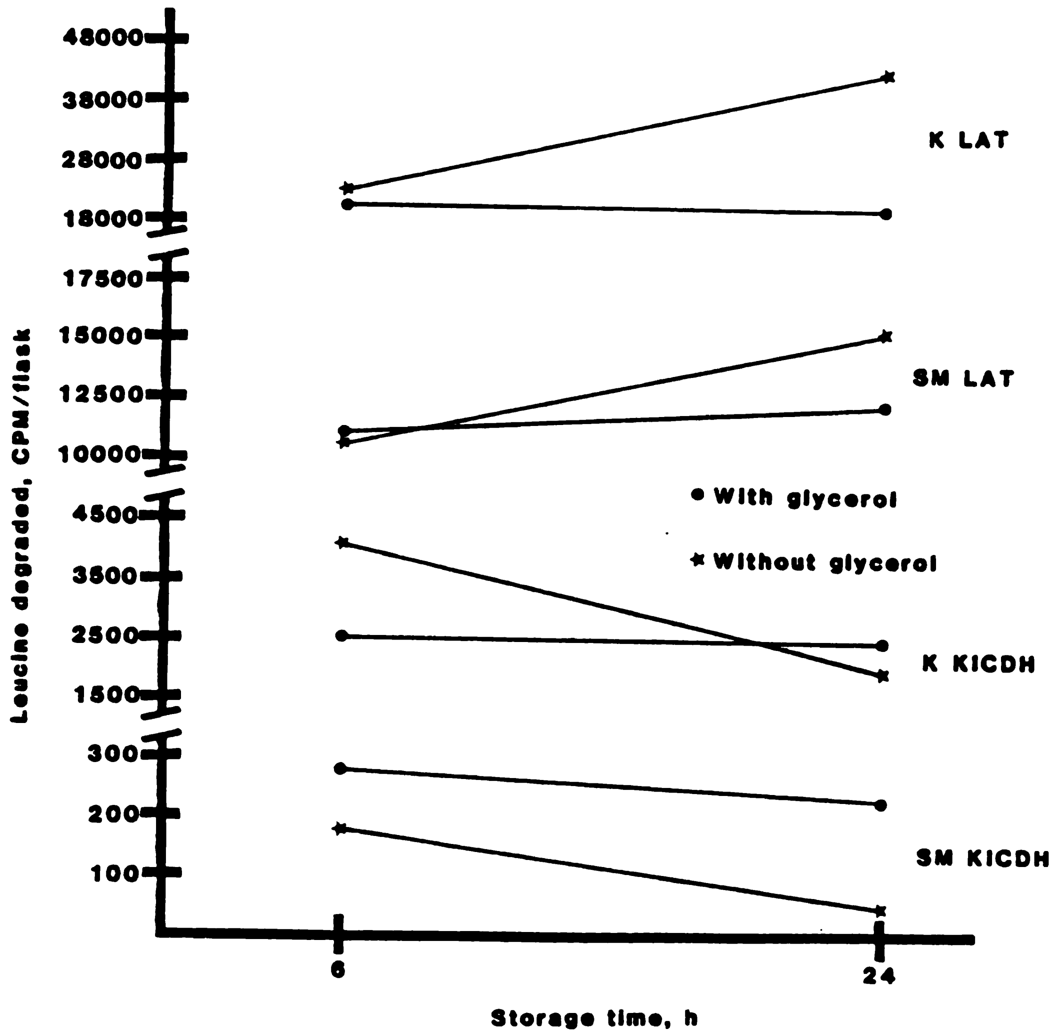


Figure I-5. The effects of cryogenic freezing, with or without glycerol on the transamination (LAT) and decarboxylation (KICDH) of L-[1-^{14}C]-leucine by rat kidney (K) and semimembranosus muscle (SM) crude homogenates. Points represent the means of four observations.

TABLE I-3. MEANS AND STANDARD ERRORS FOR LEUCINE AMINOTRANSFERASE AND ALPHA-KETOISOCAPROATE DEHYGROGENASE ACTIVITIES OF CRUDE HOMOGENATES OF SKELETAL MUSCLES EXCISED FROM LAMBS AT VARIOUS AGES

	Leucine aminotransferase		Alpha-ketoisocaproate dehydrogenase	
Age, d	Longissimus	Trapezius	Longissimus	Trapezius
	----pmol leucine degraded x mg protein^{-1}x min^{-1}----			
1	246[c]	173[a]	27.7[b]	25.4[b]
28	28[a]	70[a]	2.4[a]	6.3[a]
56	26[a]	64[a]	1.2[a]	2.8[a]
84	63[ab]	105[a]	0 [a]	0 [a]
112	16[a]	33[a]	.8[a]	1.4[a]
140	17[a]	66[a]	0 [a]	.9[a]
168	22[a]	124[a]	1.1[a]	2.0[a]
196	49[ab]	113[a]	5.9[a]	10.7[a]
224	24[a]	122[a]	2.1[a]	2.0[a]
365	82[b]	506[b]	6.4[a]	9.2[a]
Standard error	16.8	65.3	2.3	3.4

[abc]Means in the same column with no superscripts in common differ (P<.05).

trapezius muscle at d 365. Ignoring this value (506 pmol x mg protein $^{-1}$ x min^{-1}), activities of both enzymes decreased after d 1 and then tended to increase as the lambs matured. Age had no effect on LAT and KICDH activities in rat skeletal muscles (Odessey, 1974; Sketcher and James, 1974). In both longissimus and trapezius muscle homogenates, LAT activity was 7- to over 100-fold higher than KICDH activity, suggesting that in muscle, KICDH is the rate limiting enzyme. This is in agreement with the work of others in studies with rat skeletal muscle (Shinnick and Harper,]976; Odessey and Goldberg, 1979).

In liver homogenates, LAT and KICDH activities (p mol x mg protein^{-1} x min^{-1}) decreased (P<.05) after d 1 and then remained fairly constant from d 28 to d 365, although KICDH activity was higher (P<.05) in the 84 d old lambs than at 28, 56 and 140 d of age (table I-4). When L-[1-^{14}C]-leucine is used as substrate, measured KICDH activity cannot exceed LAT activity. In liver homogenates KICDH activity ranged from 33 to 70% of the LAT activity, thus these results suggest that LAT is probably rate limiting in sheep liver. In subsequent work (Chapter IV), with labeled KIC as substrate, this rate limiting observation was substantiated. LAT has been shown to be the rate limiting enzyme in rat liver BCAA metabolism (Shinnick and Harper, 1976; Harper and Zapalowski, 1981).

TABLE I-4. MEANS AND STANDARD ERRORS FOR LEUCINE AMINOTRANSFERASE AND ALPHA-KETOISOCAPROATE DEHYDROGENASE ACTIVITIES IN HOMOGENATES OF LIVER AND KIDNEY EXCISED FROM LAMBS AT VARIOUS AGES

	Leucine aminotransferase		Alpha-ketoisocaproate dehydrogenase	
Age, d	Liver	Kidney	Liver	Kidney
	-----pmol leucine degraded x mg protein^{-1}x min^{-1}-----			
1	181[b]	279[a]	64.6[d]	82.3[a]
28	46[a]	252[a]	22.4[ab]	116.1[a]
56	37[a]	246[a]	26.0[ab]	101.5[a]
84	93[a]	610[cd]	45.7[c]	305.8[de]
112	62[a]	448[bc]	34.0[abc]	297.6[cde]
140	38[a]	362[ab]	16.9[a]	209.3[b]
168	76[a]	467[bc]	36.4[abc]	293.7[cde]
196	68[a]	654[d]	27.1[abc]	340.0[e]
224	107[a]	568[cd]	35.2[abc]	244.9[bcd]
365	77[a]	495[bcd]	38.2[bc]	234.0[bc]
Standard error	22	54	6.0	22.6

[abcde]Means in the same column with no superscripts in common differ (P<.05).

Unlike muscle and liver, LAT and KICDH activities in the kidney were lower ($P<.05$) in the younger lambs (d 1, 28 and 56) than in the older age groups (table I-4). In addition, as in the liver, LAT activities were less than threefold greater than the means of KICDH activities in the same age group, indicating that LAT might be rate limiting in the kidney. In a subsequent study (Chapter IV), KICDH activity was found to be less than that of LAT in sheep kidney when KIC was used as a substrate. KICDH also has been shown to be rate limiting in rat kidney (Harper and Zapalowski, 1981).

In other experiments conducted during the course of this longitudinal study, high LAT and KICDH activities were observed in adipose tissue. Therefore, the LAT and KICDH activities were determined in adipose tissue from the final four age groups (d 1, 28, 56 and 365) of this study (table I-5). In addition, results from three ram lambs from the same breeding as this group of lambs, fed the same diet and fasted for 12 h prior to slaughter at 84 d of age are included in table I-5. But these data were not analyzed statistically with the data from the d 1, 28, 56 and 365 lambs. Also perirenal adipose tissue was studied in the neonatal group, while subcutaneous adipose tissue homogenates were studied from the 28, 56, 84 and 365 d old groups. The perirenal adipose tissue from the d 1 lambs had

TABLE I-5. MEANS AND STANDARD ERRORS FOR LEUCINE AMINOTRANSFERASE AND ALPHA-KETOISOCAPROATE DEHYDROGENASE ACTIVITIES IN CRUDE HOMOGENATES OF ADIPOSE TISSUE EXCISED FROM LAMBS AT AT VARIOUS AGES[a,b]

Age, d	Leucine aminotransferase	Alpha-ketoisocaproate dehydrogenase
	---pmol leucine degraded x mg protein^{-1}x min^{-1}---	
1	929[c]	261.0[e]
28	423[c]	7.1[c]
56	417[c]	8.5[c]
84	1223	29.7
365	4612[d]	26.8[d]
Standard error	444	6.3

[a]Perirenal adipose tissue samples were homogenized and assayed from the 1 d old lambs. Subcutaneous adipose tissue samples were homogenized and assayed from the 28, 56, 84 and 365 d old lambs.

[b]Means for the 84 d old lambs were obtained from three lambs that were part of another study. They are included for the sake of discussion and were not analyzed statistically with the data from the 1, 28, 56 and 365 d old lambs.

[c,d,e]Means in the same column with no superscripts in common differ ($P<.05$).

the visual appearance of brown adipose tissue, which is in agreement with Noble (1980) and Vernon (1980) who reported that nearly 98% of the adipose tissue present in neonatal lambs had the cytological characteristics of brown adipose tissue.

Sheep adipose tissue LAT activity decreased nonsignificantly from d 1 to d 28 but the 365 d rams had significantly greater LAT activity than any of the other ages studied. KICDH activities in adipose tissue homogenates were 261.0, 7.1, 8.5, 29.7 and 26.8 for the d 1, 28, 56, 84 and 365 lambs, respectively. Thus, LAT activity was fivefold higher in the 365 d rams than in the d 1 lambs, while the d 1 lambs had approximately 10-fold higher KICDH activity than the 365 d rams. The high KICDH activity observed in the brown adipose tissue homogenates from the d 1 lambs may be explained by the large number of mitochondria found in brown adipose tissue (Allen et al., 1976). Additionally, in rats BCKA dehydrogenase has been found to be localized primarily on the inner surface of the inner mitochondrial membrane (Hinsbergh et al., 1978). Adipose tissue LAT activity was 3.5- to 180-fold higher than KICDH activity, indicating that KICDH was rate limiting in this tissue especially in the older rams. KICDH also has been shown to be rate limiting in rat adipose tissue (Frick and Goodman, 1979).

When comparing tissues, adipose tissue generally had high, kidney intermediate, and liver and skeletal muscle low LAT activities. The relative LAT activities in liver, kidney and adipose tissue were .9-, 1.3- and 4.4-fold higher, respectively, than the LAT activity in skeletal muscle of neonatal lambs. When the means of d 28 through 365 were averaged for LAT activities in liver, kidney and adipose tissue, the relative activities were .8-, 5.7- and 22.8-fold higher, respectively, than the activity in muscle. Ichihara and Koyama (1966) reported that the relative LAT activities of rat adipose tissue and liver, and kidney were .01- and 2-fold, respectively, the activity of skeletal muscle.

It is of interest that the relative activities in the d 1 lambs (which are functional nonruminants) more closely resembled those of the rat than did the relative activities of older lambs. Ichihara and Koyama (1966) found little LAT activity in adipose tissue but Rosenthal et al.(1974) suggested that in rats, adipose tissue is a major site of leucine degradation, second only to skeletal muscle. The results of the present study indicated that ovine adipose tissue and liver were higher in LAT activity while ovine skeletal muscle had lower activity relative to the other tissues studied when compared to the rat (Ichihara and Koyama, 1966; Odessey and Goldberg, 1972; Shinnick and

Harper, 1976; Goldberg and Tischler, 1981). Buttery (1979) also found that ruminant skeletal muscle was not as active in the degradation of BCAA as nonruminant skeletal muscle. Additionally, Heitmann and Bergman (1980) reported that, unlike rat liver, ovine hepatic tissue actively removed BCAA from the circulatory system.

The distribution of KICDH activity between ovine tissues approached the distribution found in the rat to a greater extent than did LAT activity. Although liver KICDH activity was probably limited by substrate availability (Chapter IV), it was higher than KICDH in longissimus and trapezius muscle homogenates at all ages studied. Also, at all ages, except at d 1, KICDH activity in kidney homogenates was higher than in all of the other tissues studied. Other studies have shown similar results in rat tissues when leucine was used as the substrate (Noda and Ichihara, 1976), but when KIC was supplied as the substrate, liver preparations had the greatest KICDH activity per milligram of protein. It also is worthy of note that KICDH activity in adipose tissue homogenates was greater than that observed in either of the skeletal muscle homogenates at all of the ages studied.

With the exception of d 1, trapezius muscle had greater LAT activity than longissimus muscle, and with the exception of d 1 and 224, trapezius muscle also had greater KICDH

activity than longissimus muscle. This observation could be due to the fact that trapezius muscle had a higher content of intramuscular lipid than longissimus muscle (table I-6), especially since adipose tissue had greater activities of these enzymes than skeletal muscle. Additionally, the enzyme activities in muscle generally tended to follow the changes in intramuscular lipid with age.

Enzyme Activity Per Gram of Tissue

Activities expressed on a per gram of tissue basis for LAT and KICDH are shown in tables I-7 and I-8, respectively. While the relative differences in activity due to age are similar whether expressed per gram of tissue or per milligram of protein, those tissues with low protein concentrations (such as adipose tissue) had lower activity when expressed on a per gram of tissue basis than when expressed on a protein basis (tables 7 and 8). For example, on a per gram of tissue basis, LAT activity in adipose tissue homogenates from d 1 lambs was significantly greater than the corresponding activity in the 28 and 56 d old lambs, but did not differ (P>.05) from the activity of the d 365 lambs (table I-7). However, on a milligram of protein basis, adipose tissue LAT activities in the 1, 28 and 56 d old lambs were not different (P>.05) from each other and activity in the 365 d old rams was fivefold greater

TABLE I-6. MEANS AND STANDARD ERRORS FOR ETHER EXTRACTABLE LIPID AND MOISTURE CONTENT OF TISSUES EXCISED FROM LAMBS AT VARIOUS AGES[a]

	Tissue					
	Muscle				Adipose	
Age, d	Longissimus	Trapezius	Liver	Kidney	Subcutaneous	Perirenal
	Ether extractable lipid, %					
1	1.6^{b}	7.7^{bc}	4.8^{c}	4.2	--	41.1^{b}
28	4.1^{cd}	15.7^{defg}	2.2^{b}	4.2	55.8^{b}	82.8^{cd}
56	3.0^{bc}	11.6^{cde}	2.4^{b}	4.7	68.1^{c}	82.3^{c}
84	3.0^{bc}	9.9^{bcd}	--	--	68.4^{c}	83.1^{cd}
112	3.2^{bc}	13.5^{cdef}	--	--	75.6^{cd}	83.6^{cd}
140	6.1^{e}	16.9^{efg}	--	--	75.6^{cd}	86.6^{cde}
168	4.5^{cde}	19.8^{g}	--	--	78.1^{d}	87.3^{de}
196	8.0^{f}	15.5^{defg}	--	--	81.7^{de}	88.8^{e}
224	5.2^{de}	5.5^{b}	--	--	87.1^{e}	93.3^{f}
365	9.7^{f}	19.4^{fg}	4.8^{c}	4.6	87.6^{e}	94.9^{f}
Standard error	.6	1.9	.7	.3	3.0	1.5
	Moisture, %					
1	79.6^{e}	74.4^{e}	74.1^{d}	82.2^{e}	--	47.5^{g}
28	75.7^{d}	67.8^{bcd}	71.6^{c}	80.5^{d}	37.2^{f}	14.8^{ef}
56	76.5^{d}	71.9^{de}	70.0^{bc}	79.4^{c}	27.8^{e}	16.1^{f}
84	76.0^{d}	72.2^{de}	--	--	24.5^{de}	14.8^{ef}
112	75.4^{d}	69.3^{cd}	--	--	20.5^{cde}	14.9^{ef}
140	73.2^{c}	65.8^{bc}	--	--	20.6^{cde}	12.2^{def}
168	73.3^{c}	63.8^{b}	--	--	17.3^{bcd}	11.1^{de}
196	70.9^{b}	66.5^{bc}	--	--	15.4^{bc}	9.8^{cd}
224	72.7^{c}	73.0^{de}	--	--	10.7^{b}	6.5^{bc}
365	69.5^{b}	63.2^{b}	69.3^{b}	76.7^{b}	10.0^{b}	4.2^{b}
Standard error	.5	1.7	.5	.4	2.6	1.3

[a] Liver and kidney from the 84, 112, 140, 168, 196 and 224 d old lambs and subcutaneous adipose tissue from the 1 d old lambs were not analyzed for moisture and lipid content.

[b,c,d,e,f,g] Means in the same column, representing the same variable, with no superscripts in common differ ($P<.05$).

TABLE I-7. MEANS AND STANDARD ERRORS FOR LEUCINE AMINOTRANSFERASE ACTIVITY FOR SEVERAL TISSUES EXCISED FROM LAMBS AT VARIOUS AGES

	Tissue				
	Muscle				
Age, d	Longissimus	Trapezius	Liver	Kidney	Adipose[ab]
	--------nmol leucine degraded x g tissue^{-1}x min^{-1}--------				
1	12.9[d]	8.5[c]	26.1[d]	27.2[c]	49.4[d]
28	2.4[c]	3.0[c]	6.8[c]	25.1[c]	3.3[c]
56	3.7[c]	4.5[c]	8.0[c]	37.0[cd]	10.7[c]
84	2.9[c]	3.4[c]	11.8[c]	45.4[de]	36.8
112	1.5[c]	1.6[c]	9.2[c]	48.0[de]	--
140	2.0[c]	4.9[c]	8.5[c]	61.9[f]	--
168	2.2[c]	8.9[c]	14.0[c]	68.8[f]	--
196	2.9[c]	4.4[c]	9.8[c]	55.6[ef]	--
224	1.8[c]	4.3[c]	15.2[c]	63.2[f]	--
365	3.8[c]	18.7[d]	10.8[c]	48.1[de]	78.0[d]
Standard error	.9	3.2	3.1	4.5	10.2

[a]Perirenal adipose tissue samples were homogenized and assayed from the 1 d old lambs. Subcutaneous adipose tissue samples were homogenized and assayed from the 28, 56, 84 and 365 d old lambs.

[b]The adipose tissue mean for the 84 d old lambs was obtained from three lambs that were part of another study. It is included for the sake of discussion and was not analyzed statistically with the data from the 1, 28, 56 and 365 d old lambs.

[c,d,e,f]Means in the same column with no superscripts in common differ ($P<.05$).

TABLE I-8. MEANS AND STANDARD ERRORS FOR ALPHA-KETOISOCAPROATE DEHYDROGENASE ACTIVITY FOR SEVERAL TISSUES EXCISED FROM LAMBS AT VARIOUS AGES

	Tissue				
	Muscle				
Age, d	Longissimus	Trapezius	Liver	Kidney	Adipose[ab]
	--------nmol leucine degraded x g tissue^{-1}x min^{-1}-------				
1	1.50[d]	1.31[d]	9.4[d]	8.0[c]	13.91[d]
28	.20[c]	.27[c]	3.3[c]	11.6[c]	.06[c]
56	.16[c]	.20[c]	5.6[c]	15.1[c]	.42[c]
84	.00[c]	.00[c]	5.8[c]	23.0[d]	.86
112	.07[c]	.09[c]	5.1[c]	31.9[ef]	--
140	.00[c]	.08[c]	3.8[c]	35.7[f]	--
168	.12[c]	.19[c]	6.1[c]	43.4[g]	--
196	.41[c]	.38[c]	3.9[c]	28.3[de]	--
224	.14[c]	.07[c]	4.9[c]	27.1[de]	--
365	.30[c]	.30[c]	5.3[c]	22.8[d]	.43[c]
Standard error	.44	.15	.83	2.17	.16

[a]Perirenal adipose tissue samples were homogenized and assayed from the 1 d old lambs. Subcutaneous adipose tissue samples were homogenized and assayed from the 28, 56, a84 and 365 d old lambs.

[b]The adipose tissue mean for the 84 d old lambs was obtained from three lambs that were part of another study. It is included for the sake of discussion and was not analyzed statistically with the data from the 1, 28, 56 and 365 d old lambs.

[c,d,e,f,g]Means in the same column with no superscripts in common differ ($P<.05$).

(P<.05)than activity in the d 1 lambs (table I-5). As the rams matured, the lipid content of the adipose tissue increased and protein content decreased (table I-6). Even though the enzyme activities in adipose tissue were lower in magnitude when expressed on a per gram of tissue basis compared to a milligram of protein basis, at d 1 and d 365 adipose tissue LAT activities per gram of tissue were the highest of all the tissues studied (table I-7).

Because of the high LAT and KICDH activities observed in adipose tissue preparations, the contribution of intramuscular adipose tissue to the enzyme activity observed in skeletal muscle homogenates is of interest. The values in table I-9 represent this contribution, but were arrived at by the assumption that intramuscular adipose tissue had the same activity as that observed in the perirenal adipose tissue for neonatal lambs, and that the lambs at d 28, 56 and 365 had the same activity in their intramuscular fat as that found in subcutaneous fat. This assumption is strengthened further by the observation that LAT and KICDH activities in the subcutaneous and perirenal adipose tissues excised from the d 28 lambs did not differ significantly (table I-10). In fact, LAT and KICDH activities, expressed per gram of tissue, may be even higher in intramuscular fat than in perirenal or subcutaneous fat, since intramuscular fat has a higher protein concentration (lower lipid content)

TABLE I-9. MEANS AND STANDARD ERRORS FOR CALCULATED PERCENTAGE CONTRIBUTION OF INTRAMUSCULAR ADIPOSE TISSUE TO LEUCINE AMINOTRANSFERASE AND ALPHA-KETOISOCAPROATE DEHYDROGENASE ACTIVITY IN LONGISSIMUS AND TRAPEZIUS MUSCLES[a]

	Leucine aminotransferase		Alpha-ketoisocaproate dehydrogenase	
Age, d	Longissimus	Trapezius	Longissimus	Trapezius
1	5.8[b]	46.3[b]	17.2	98.2[c]
28	6.7[b]	19.4[b]	1.4	3.7[b]
56	8.6[b]	27.5[b]	17.2	37.8[bc]
365	245.2[c]	126.8[c]	16.4	42.5[bc]
Standard error	40.55	24.96	7.63	18.05

[a]Potential contribution of intramuscular adipose tissue was calculated as:

$$\frac{\text{Adipose tissue activity x muscle lipid content}}{\text{Skeletal muscle activity}} \times 100$$

See Appendix II-A.

[b,c]Means within the same column with no superscripts in common differ (P<.05).

TABLE I-10. MEANS AND STANDARD DEVIATIONS FOR ENZYME ACTIVITY IN CRUDE HOMOGENATES OF SUBCUTANEOUS AND PERIRENAL ADIPOSE TISSUE EXCISED FROM 28 DAY OLD RAM LAMBS

Adipose depot	Leucine aminotransferase	SD	Alpha-ketoisocaproate dehydrogenase	SD
	----pmol leucine degraded x mg protein^{-1}x min^{-1}----			
Subcutaneous	423	224	7.1	3.6
Perirenal	682	393	19.1	18.2
Probability level	P>.2		P>.1	
	-----nmol leucine degraded x g tissue^{-1}x min^{-1}-----			
Subcutaneous	3.25	.86	.06	.02
Perirenal	3.62	1.51	.09	.05
Probability level	P>.5		P>.2	

than perirenal and subcutaneous fat (Allen et al., 1976). From table I-9 it is apparent that intramuscular adipose tissue could account for a substantial portion of the LAT and KICDH activity attributed to skeletal muscle, particularly in the 365 d old rams.

Activity Expressed On a Total Tissue Basis

The means and standard errors of LAT and KICDH activity expressed on a tissue basis are presented in tables I-11 and I-12. The equations used to calculate the activity on a tissue basis are shown in Appendix II-B. Briefly, the activity per gram of tissue was multiplied by the weight of the tissue to calculate activity per tissue. Tissue weights are shown in appendix table 1. The means under the heading, total, represent the sum of the activities in skeletal muscle, liver, kidney and the adipose tissue depot assayed.

The contribution of adipose tissue in the d 1 lambs is represented by perirenal adipose tissue only, and the adipose tissue contribution in the d 28, 56 and 365 lambs is represented by subcutaneous adipose tissue only. The sums of LAT activities (table I-11) in the four tissues studied (skeletal muscle, liver, kidney and adipose tissue) were from five-(d 1) to 52-fold (d 365) higher than the corresponding KICDH activities (table I-12). These four tissues are the most important sites of BCAA degradative

TABLE I-11. MEANS AND STANDARD ERRORS OF LEUCINE AMINOTRANSFERASE ACTIVITY IN SEVERAL TISSUES EXCISED FROM LAMBS AT VARIOUS AGES

	Tissue				
Day	Skeletal muscle	Liver	Kidney	Adipose[a]	Total[b]
	----nmol leucine degraded x tissue^{-1} x min^{-1}----				
1	10008[d]	2383[d]	755[d]	1235	14381
28	10927[d]	1868[d]	1799[de]	990	15584
56	30999[de]	4261[de]	3942[ef]	12543[d]	51746[d]
84	31617[de]	9110[ef]	6381[fg]		
112	20106[de]	9207[ef]	8042[g]		
140	56663[def]	9919[ef]	11885[h]		
168	101663[f]	17725[gh]	13611[h]		
196	70844[ef]	12892[fg]	11336[h]		
224	64445[def]	21469[h]	13134[h]		
365	375919[g]	16003[gh]	10833[h]	1095858[e]	1498613[e]
Standard error	17500	1999	996	163020[c]	156212[c]

[a]Perirenal adipose tissue was assayed from the 1 d old lambs and subcutaneous adipose tissue was assayed from the 28, 56 and 365 d old lambs.

[b]The sum of activity in skeletal muscle, liver, kidney, and the adipose tissue depot that was assayed. See Appendix II-B for equations.

[c]Standard error applies only to 56 and 365 d old lambs.

[d,e,f,g,h]Means in the same column with no superscripts in common are different ($P<.05$).

TABLE I-12. MEANS AND STANDARD ERRORS OF ALPHA-KETOISOCAPROATE DEHYDROGENASE ACTIVITY IN SEVERAL TISSUES EXCISED FROM LAMBS AT VARIOUS AGES

	Tissue				
Day	Skeletal muscle	Liver	Kidney	Adipose[a]	Total[b]
	----nmol leucine degraded x tissue^{-1}x min^{-1}----				
1	1387[d]	853[d]	221[d]	348	2809
28	944[d]	914[d]	834[d]	18	2710
56	1354[d]	3023[e]	1606[d]	547[d]	6530[d]
84	99[d]	4520[e]	3269[e]		
112	1002[d]	5045[ef]	5333[f]		
140	800[d]	4418[e]	6832[g]		
168	2897[de]	7710[g]	8613[h]		
196	7469[ef]	5027[ef]	5692[fg]		
224	2264[de]	6997[fg]	5655[fg]		
365	9745[f]	7866[g]	5143[f]	6063[e]	28817[e]
Standard error	1848	655	476	655[c]	2373[c]

[a]Perirenal adipose tissue was assayed from the 1 d old lambs and subcutaneous adipose tissue was assayed from the 28, 56 and 365 d old lambs.

[b]The sum of the activities in skeletal muscle, liver, kidney and the adipose tissue depot that was assayed. See Appendix II-B for equations.

[c]Standard error applies only to 56 and 365 d old lambs.

[d,e,f,g,h]Means in the same column with no superscript in common differ ($P<.05$).

enzymes in rats (Harper and Zapalowski, 1981), thus in the sheep, as is the case in rats (Goldberg and Tischler, 1981), KICDH is probably the rate limiting enzyme of whole body leucine degradation.

The means from tables I-11 and I-12 were used to calculate the contribution of the individual tissues to the LAT and KICDH activities, expressed as a percentage of the sum of activities found in all four tissues. These percentages are shown in tables I-13 and I-14. Results from the three additional 84 d old lambs are also included in these tables. It is of interest that in the youngest lambs (d 1, 28 and 56), which probably are not yet fully functional ruminants (Oh et al., 1972), skeletal muscle is the primary site of LAT activity, while in the more mature 365 d old rams, most of the LAT activity was found in adipose tissues (table I-13). Tischler and Goldberg (1980a) reported that while adipose tissue also actively degraded leucine, skeletal muscle, because of its bulk was the primary site of leucine degradation in rats. In addition, Tischler and Goldberg (1980a) suggested that in animals containing a high percentage of fat, adipose tissue probably plays a significant role in BCAA degradation. Certainly the d 365 rams contained a high percentage of fat and this in part may explain the high contribution of adipose tissue to total LAT activity, but as noted earlier, LAT activity

TABLE I-13. RELATIVE CONTRIBUTION OF VARIOUS TISSUES TO LEUCINE AMINOTRANSFERASE ACTIVITY AS AFFECTED BY AGE[a]

	Tissue				
Age, d	Skeletal muscle	Liver	Kidney	Adipose[b]	Total
	---------------Contribution, %---------------				
1	69.6	16.6	5.2	8.6	100.0
28	70.1	12.0	11.5	6.4	100.0
56	59.9	8.2	7.6	24.3	100.0
84	30.1	6.6	5.9	57.4	100.0
365	25.1	1.1	.7	73.1	100.0

[a]See Appendix II-B for equations used for calculating the percentage contribution.

[b]Perirenal adipose tissue was assayed from the day 1 lambs and subcutaneous adipose tissue was assayed from 28, 56, 84 and 365 d old lambs.

[c]Values for the 84 d old were obtained from three lambs that were a part of another study. They are included for the sake of discussion.

TABLE I-14. RELATIVE CONTRIBUTION OF VARIOUS TISSUES TO ALPHA-KETOISOCAPROATE DEHYDROGENASE ACTIVITY AS AFFECTED BY AGE[a]

Age, d	Tissue				
	Skeletal muscle	Liver	Kidney	Adipose[b]	Total
	Contribution, %				
1	49.4	30.4	7.9	12.3	100.0
28	34.8	33.7	30.8	.7	100.0
56	20.7	46.3	24.6	8.4	100.0
84	12.9	40.2	27.9	19.0	100.0
365	33.8	27.3	17.9	21.0	100.0

[a]See Appendix II-B for equations used for calculating the percentage contribution.

[b]Perirenal adipose tissue was assayed from the day 1 lambs and subcutaneous adipose tissue was assayed from 28, 56, 84 and 365 d old lambs.

[c]Values for the 84 d old were obtained from three lambs that were a part of another study. They are included for the sake of discussion.

expressed per gram of tissue was also elevated in these 365 d old rams compared to the younger age groups. Even though the LAT activity of kidney was second only to that of adipose tissue on a milligram of protein basis, kidney appeared to be relatively insignificant when activity was expressed on a total tissue basis.

In the d 1 lambs, muscle was also an important site of KICDH activity, and at d 365 all four tissues appeared to make significant contribution to the total activity (table I-14). The percentage contribution of muscle decreased from 49.4 to 12.9% from d 1 to d 84 but then increased to 33.8% by d 365. The concomitant increase in intramuscular adipose tissue that occurred between d 84 and d 365 may be an explanation for the increase in skeletal muscle activity. The contribution of liver to KICDH activity ranged from 27.3 to 46.6% and if L-[1-^{14}C]-KIC had been supplied as substrate the contribution of liver would have been even greater, as shown later in Chapter IV. This significant contribution by ovine liver to KICDH is consistent with observations by Heitmann and Bergman (1980) and Bergman and Pell (1983), who reported that in adult sheep, unlike in nonruminants the BCAA were always removed by the liver and were released by kidneys and hindlimbs during fasting. The relative contribution of kidney to KICDH activity ranged from 7.9 to 30.8%, indicating that in sheep, kidney could play a more

important role in the degradation of BCKA than the degradation of BCAA (tables I-13 and I-14). In the d 1, 28, 56, 84 and 365 lambs the relative contribution of adipose tissue to KICDH activity in all four tissues combined were 12.3, .7, 8.4, 19.0 and 21.0, respectively. The d 1 and 28 lambs were in effect nonruminants (Oh et al., 1972) and the d 28 lambs had a greater quantity of adipose tissue, so the question is raised as to why the adipose tissue in the d 1 lambs makes a much larger contribution to KICDH activity than in the d 28 lambs. A possible explanation, however, is the high mitochondrial content (Allen et al., 1976) of the brown adipose tissue in the d 1 lambs and in rats KICDH activity is found primarily in the mitochondria (Hinsbergh et al., 1978; May et al., 1980). By d 28 the brown adipose tissue would have been converted to white adipose tissue (Noble, 1980) and visual observations in the present study indicated that this was the case. The gradual increase in the importance of adipose tissue as a site of KICDH activity as the lambs matured from 28 to 365 d of age could be attributed to at least two factors. First the rumen function gradually became fully developed between 50 and 100 d of age (Oh et al., 1972; Noble, 1980), thus the increase in KICDH activity in adipose tissue may be associated with this transformation from a nonruminant to a ruminant.

Secondly, the increased contribution of adipose tissue to KICDH activity was related to increased deposition of fat.

It is of interest to note that the values for adipose tissue in the d 1 lambs reflect only the contribution of perirenal adipose tissue, which constitutes only 25 to 35% of the brown adipose tissue present in lambs at birth (Noble, 1980). Adipose tissue values for the 28, 56, 84 and 365 d old lambs reflects only the contribution of subcutaneous adipose tissue, which constitutes approximately 44% of the total adipose tissue in growing lambs (Kauffman et al., 1963). Thus, if all adipose tissue deposits were included, the contribution of adipose tissue to LAT and KICDH activities would be greater than is indicated by the data in tables I-13 and I-14. The physiological significance of the high LAT and KICDH activities in adipose tissue from the more mature ram lambs has yet to be ascertained. Lindsay (1982) stated that if leucine is catabolized in ruminant adipose tissue (and the present results show that is the case), it may be possible that leucine or some metabolite of leucine may play a part in the partitioning of nutrients. This suggestion is particularly interesting in light of the present findings that adipose tissue LAT and KICDH activities were highest in those lambs which were partitioning a large portion of their nutrients towards fat. KICDH activity in brown adipose tissue from the lambs

at d 1 was an apparent exception to the rule, but it has recently been shown that brown adipose tissue synthesized fatty acids more actively than white adipose tissue (McCormack, 1982; Pearce, 1983). Thus, in the d 1 lambs the enzymes responsible for the degradation of leucine may be involved in the partitioning of nutrients towards brown adipose for thermogenesis, while in the older lambs nutrients are partitioned towards white adipose tissue for storage.

Summary and Conclusions

Forty-nine crossbred ram lambs at 1, 28, 56, 84, 112, 140, 168, 196, 224 or 365 d of age were used to study the effect of age on BCAA degradation in longissimus and trapezius muscle, liver, kidney and adipose tissue homogenates. L-[1-^{14}C]-leucine was utilized as substrate to measure LAT and KICDH activities. LAT activity for both skeletal muscles was high in the d 1 lambs, decreased by d 28 and increased again by d 365. LAT activity in liver was highest in the d 1 lambs, while older lambs displayed the greatest LAT activity in kidney and adipose tissues. In longissimus and trapezius muscles, liver and adipose tissue, d 1 lambs displayed the highest KICDH activities of all the ages studied, but in kidney homogenates, the d 1 lambs had the lowest KICDH activity. The activity of LAT expressed on

a protein basis in liver relative to LAT activity in the other tissues studied was higher in these sheep than has been reported for rat liver. The relationship of this relatively higher LAT activity in sheep liver to the constant gluconeogenesis that occurs in ruminant liver merits further study. As in the rat, LAT appeared to be the rate limiting enzyme in liver while KICDH was probably rate limiting in muscle and adipose tissues. In the d 1, 28 and 56 lambs, which are in effect nonruminants, skeletal muscle was the predominant location of LAT activity and an important site of KICDH activity. On the other hand, adipose tissue was the predominant location of LAT activity and an important site of KICDH activity in the older lambs. Liver and kidney were relatively insignificant sites of LAT activity at all of the ages studied, but both of these tissues and especially liver made significant contributions to KICDH activity. Since KICDH relies on LAT to supply the ketoacid as substrate when L-[1-^{14}C]-leucine is provided, further studies with labeled KIC supplied as substrate should be undertaken with sheep to more specifically measure dehydrogenase activity. This is particularly important in tissues such as liver in which the aminotransferase enzyme is probably rate limiting.

CHAPTER II

EFFECTS OF DIETARY PROTEIN CONTENT AND FASTING ON TISSUE LEUCINE AMINOTRANSFERASE AND ALPHA-KETOISOCAPROATE DEHYDROGENASE ACTIVITY IN WETHER LAMBS

Introduction

Amino acids primarily serve as building blocks for protein synthesis. However, they can undergo oxidative degradation in at least three metabolic circumstances: 1) during normal protein turnover if they are not reincorporated during the synthesis of new proteins; 2) when fed in excess of the body's need for protein synthesis; and 3) during fasting when carbohydrates are unavailable or limiting, proteins are degraded and the amino acids can serve as a source of energy (Lehninger, 1982). The effects of dietary protein content and fasting on the metabolism of the BCAA are of particular interest (Adibi, 1976; Snell, 1980). Of the BCAA leucine has been the one most extensively studied. The first two steps in the degradation of leucine are reversible transamination (by LAT), followed by irreversible decarboxylation (by KICDH) of the resulting KIC to form isovaleryl CoA.

Effects of dietary protein on BCAA aminotransferase and BCKA dehydrogenase activities have been studied in rats. Adibi et al. (1975) demonstrated that feeding a protein deficient diet inhibitied leucine transamination in skeletal muscle but LAT activity was unaffected in liver and kidney.

muscle but LAT activity was unaffected in liver and kidney. Wohlhueter and Harper (1970) and Shinnick and Harper (1977) also reported that LAT activity in liver and kidney was unresponsive to dietary protein manipulations, but others (Ichihara et al., 1967; McFarlane and von Holt, 1969a; Krebs, 1972) have found that LAT activity in liver was stimulated by feeding rats a high protein diet. LAT activity in epididymal fat pads was markedly reduced in rats fed a protein deficient diet (Tischler and Goldberg, 1980a). In vivo studies of overall BCAA oxidation (McFarlane and von Holt, 1969b; Sketcher and James, 1974) and enzyme assays of skeletal muscle (Sketcher and James, 1974, 1976; Shinnick and Harper, 1977), liver (Wohlhueter and Harper, 1970; Adibi, 1976; Shinnick and Harper, 1977) and adipose tissue (Frick and Goodman, 1979; Tischler and Goldberg, 1981) indicate that BCKA dehydrogenase is stimulated by a high, and inhibited by a low protein diet. The effect of dietary protein on LAT and KICDH has not been extensively studied in sheep.

Fasting, unlike protein deprivation appears to increase plasma BCAA concentrations in rats (Adibi, 1976; Zapalowski et al., 1981), humans (Felig, 1975; Elia and Livesey, 1981), dogs (Nissen and Haymond, 1981) and sheep (Ballard et al., 1976; Bergen, 1979; Heitmann and Bergman, 1980; Bergman and Pell, 1983), but the cause and timing of this increase

varies between species. Free BCAA pools are controlled by net proteolysis or protein accretion and by BCAA degradation. It is well established that the increased proteolysis associated with a short term fast contributes to the increased concentration of free BCAA (Bergen, 1979), but the effect of fasting on BCAA degradation in different tissues and in different species is highly variable. In rats, degradation of BCAA by liver and kidney is relatively unaltered by fasting, while BCAA oxidation is increased in skeletal muscle by fasting and decreased in adipose tissue (Goldberg and Tischler, 1981). Sensitivity of skeletal muscle and adipose tissue to food deprivation in species other than the rat has not been thoroughly investigated. But there is no evidence indicating that BCAA oxidation is enhanced in skeletal muscle in species other than the rat, including rabbits (Ryan et al., 1974), dogs (Nissen and Haymond, 1981) and sheep (Lindsay, 1982). Also, the conversion of BCAA to lipid is impaired during starvation (Frick and Goodman, 1979), and this could be a possible mechanism for the accumulation of BCAA during fasting, especially in animals with a high adipose tissue content such as humans (Adibi, 1976), obese rats (Goldberg and Tischler, 1981) and domesticated livestock.

Therefore this study was designed to ascertain the effects of dietary protein content and fasting length on LAT

and KICDH activities in growing wethers, and also to study the relative contribution of skeletal muscle, liver, kidney and subcutaneous adipose tissue to BCAA degradative activities in lambs subjected to various dietary regimens. Accordingly, leucine transamination and decarboxylation were measured on crude homogenates of skeletal muscle, liver, kidney and subcutaneous adipose tissue excised from unfasted lambs fed diets containing 8, 12 or 18% crude protein (CP) and, in lambs fed a 12% CP diet and fasted for either 48 or 96 h.

Experimental Procedures

Materials

The materials used in these experiments were as described previously (Chapter I), except in this study L-[1-^{14}C]-leucine was obtained from Amersham Corp., (Arlington Heights, IL, U.S.A.).

Experimental Design

Twenty-five, 5 mo old, crossbred wether lambs were obtained from a single commercial source and gradually adjusted to ad libitum feeding of a 12% CP diet. Five lambs were assigned to each of the following five treatments: unfasted lambs fed 8, 12 or 18% CP diets or lambs fed the

12% CP diet fasted either 48 or 96 h prior to slaughter. The experimental design is shown in table II-1, and the contents of the three experimental diets are listed in table II-2. In Experiment 1, the lambs fed the 12% CP diet were compared with the lambs fed 8 and 18% CP to ascertain the effects of dietary protein content on LAT and KICDH activities. In Experiment 2, the effects of fasting on enzyme activities were analyzed by comparing three groups of five lambs fed 12% CP which were fasted for either 0, 48 or 96 h. In Experiment 1 after assignment to a treatment group, the lambs fed the 8 and 18% CP diets were adjusted to their diets over a 1 wk period. The lambs from both experiments were weighed when the experiments began. A 4 wk experimental feeding period was then initiated. Body weights were obtained when fasts were initiated and at the time of slaughter for all lambs including those after the 48 and 96 h fast.

Tissue Preparation and Enzyme Assay

One lamb from each treatment group was sacrificed on each of five consecutive slaughter days to minimize possible daily variation in the enzyme assays. Tissue preparation and the enzyme assays were described previously (Chapter I). Briefly, longissimus and trapezius muscle, liver and kidney samples were rapidly excised, minced and then gently

TABLE II-1. EXPERIMENTAL DESIGN

	Dietary protein, %				
	8	18	12	12	12
	Fast, h				
Item	0	0	0	48	96
Number of wether lambs	5	5	5	5	5

TABLE II-2. EXPERIMENTAL DIETS[a]

	Crude protein percentage		
Ingredient	8	12	18
	---------Composition, %[b]---------		
Corn, ground	67	64	52
Oats, rolled	20	20	18
Molasses	5	5	5
Trace mineral salt	2	2	2
Limestone	1	1	1
Glucose monohydrate	4.5	--	--
Soybean meal	--	7.5	21.5
Ammonium chloride	.5	.5	.5

[a]Adequate in calcium, phosphorus and trace minerals; no fat soluble vitamins were added.
[b]Percentage composition was calculated on an air dry basis.

homogenized in ice cold 15 mM potassium phosphate buffer (pH 7.5) containing .25 M sucrose, 3 mM $MgCl_2$ and 1 mM EGTA, made up to contain 50% glycerol. Subcutaneous adipose tissue samples were also excised from each lamb and handled identically to the muscle, liver and kidney samples except room temperature buffer was used. All homogenates were strained through cheese cloth and cryogenically frozen (-70 C) for assay later the same day. LAT and KICDH activities were assayed simultaneously by measuring $^{14}CO_2$ release from L-[1-^{14}C]-leucine. The assay conditions are shown in figure II-1. The data are expressed on a per milligram of protein, per gram of tissue, and total tissue basis.

Homogenate protein was determined by the method of Lowry et al. (1951). The data were analyzed by one way analysis of variance and significant differences ($P<.05$) among means were separated by Duncan's New Multiple Range Test as outlined by Steel and Torrie (1960).

Results

Experiment 1: Effect of Dietary Protein Content on LAT and KICDH Activities

Activity Per Milligram of Protein. The effect of dietary protein content in Experiment 1 on LAT activity is presented in figure II-2. There were no differences ($P>.05$)

Reaction medium: 2.5 ml containing .2 mM-thiamin pyrophosphate, 2.5 mM-$MgCl_2$, .5 mM-NAD^+, 2 mM-alpha-ketogluterate, 1 mM-dithiothreitol, 50 mM-sucrose, .2 mM-EGTA 2 mM-L-leucine and 15 mM-potassium phosphate buffer (pH 7.5).

L-[1-^{14}C]-leucine specific activity - 40 mCi/mol

Temperature: 37 C

Preincubation period: 15 min

Reaction period: 20 min

Figure II-1. Enzyme assay conditions including the reaction medium contents, reaction temperature, isotope specific activity preincubation period and reaction period.

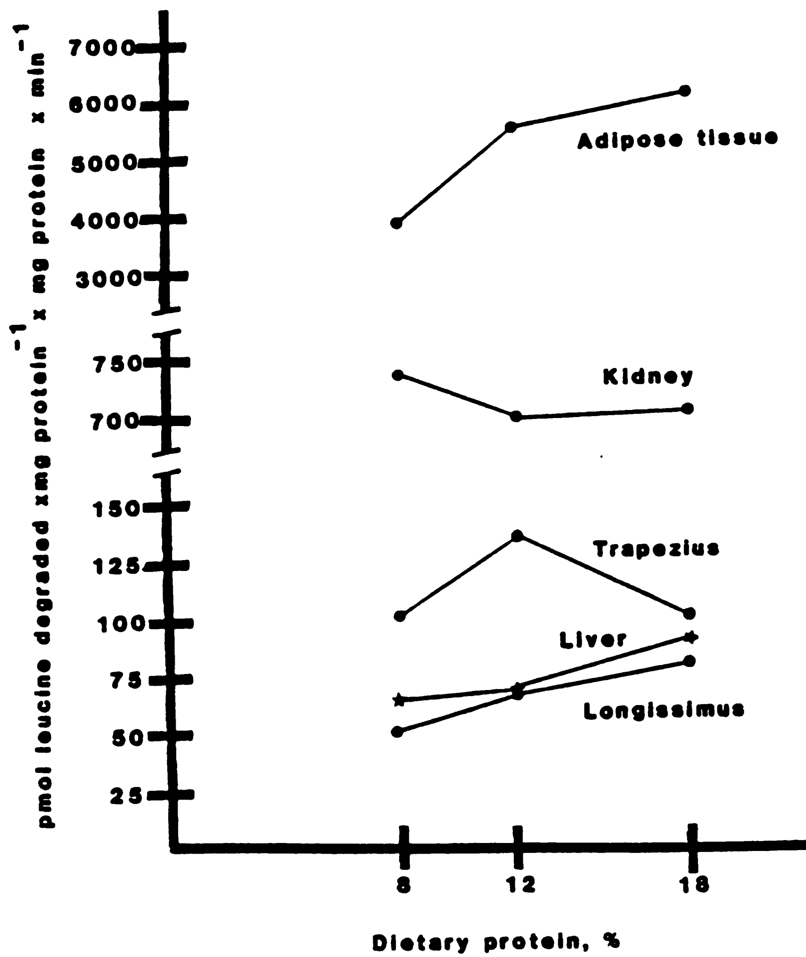


Figure II-2. The effect of dietary protein content on leucine aminotransferase activity. The standard errors for longissimus and trapezius muscles, liver, kidney and subcutaneous adipose tissue were 8.4, 45.1, 8.3, 76.9 and 1451.0, respectively.

in LAT activity for any of the tissues studied due to dietary protein content, but activity in longissimus muscle, liver and adipose tissue tended to increase as CP content of the diet increased from 8 to 18%. In all dietary treatment groups adipose tissue had high, kidney intermediate and liver and skeletal muscle low LAT activities. Also, in all cases trapezius muscle had greater LAT activity than longissimus muscle (Figure II-2), and this higher activity corresponded with the ether extractable lipid content (table II-3). With the exception of kidney, KICDH activity tended to increase with percentage protein fed, and the KICDH activity of longissimus muscle and liver differed significantly between the lambs fed the 8 and 18% CP diets (figure II-3). On a per milligram of protein basis, kidney homogenates had the highest KICDH activity followed by adipose tissue, liver, trapezius and longissimus muscles in all dietary treatment groups. However, in liver, LAT is rate limiting, and therefore KICDH activity in liver is probably greater than indicated by these results.

Enzyme Activity Per Gram of Tissue. Activities expressed on a per gram of tissue basis for LAT and KICDH are shown in table II-4. As would be expected, tissues with low protein concentrations (such as, adipose tissue) appear to have lower activity when activity is expressed per gram of tissue rather than per milligram of protein. But the

TABLE II-3. MEANS AND STANDARD ERRORS FOR MOISTURE AND ETHER EXTRACTABLE LIPID CONTENT OF TISSUES EXCISED FROM LAMBS FED DIETS CONTAINING 8, 12 OR 18% CRUDE PROTEIN

	Dietary protein content,%			
Tissue	8	12	18	Standard error
	-------Moisture content, %-------			
Muscle				
Longissimus	71.7	72.2	71.4	0.6
Trapezius	68.6	71.7	70.7	1.1
Liver	70.2[b]	70.1[b]	69.0[a]	.2
Kidney	77.4	77.5	78.3	.5
Adipose				
Subcutaneous	18.8[a]	25.5[b]	23.2[b]	1.3
Perirenal	6.6	8.2	7.8	.6
	---Ether extractable lipids, %---			
Muscle				
Longissimus	7.1	6.3	7.3	.7
Trapezius	13.0	8.3	10.8	1.2
Liver	1.9	2.3	2.4	.4
Kidney	4.9	4.7	4.9	.5
Adipose				
Subcutaneous	78.0	71.7	73.1	1.9
Perirenal	92.4	91.2	90.8	.7

[a,b]Means in the same row with no superscripts in common differ ($P<.05$).

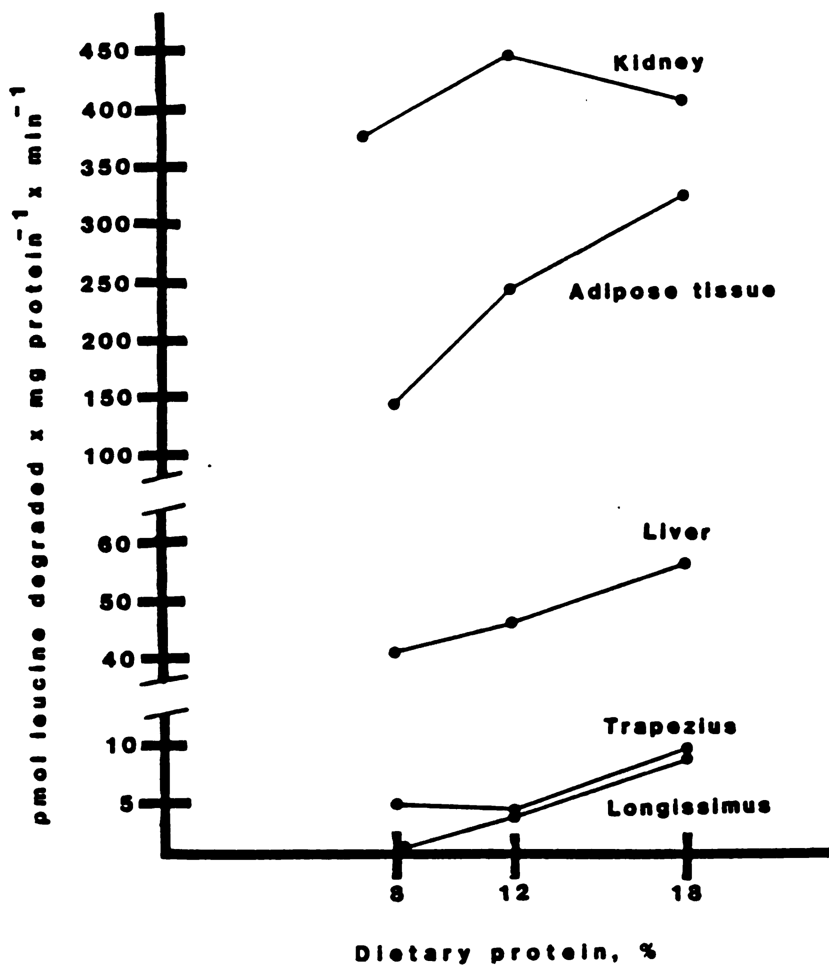


Figure II-3. The effect of dietary protein content on alpha-ketoisocaproate dehydrogenase activity. The standard errors for longissimus and trapezius muscles, liver, kidney and subcutaneous adipose tissue were 1.6, 1.6, 3.1, 32.4 and 78.4, respectively.

TABLE II-4. MEANS AND STANDARD ERRORS OF ENZYME ACTIVITY IN SEVERAL TISSUES EXCISED FROM FASTED WETHER LAMBS FED DIETS CONTAINING 8, 12 OR 18% CRUDE PROTEIN

Enzyme activity and tissue	Dietary protein content,% 8	12	18	Standard error
Leucine aminotransferase	----nmolx g tissue^{-1}x min^{-1}----			
Longissimus muscle	3.68	4.60	5.34	.78
Trapezius muscle	4.36	5.10	4.74	1.28
Liver	8.54	8.84	11.68	1.30
Kidney	67.86	62.12	59.28	6.89
Subcutaneous adipose	29.46	81.70	78.74	22.93
Alpha-ketoisocaproate dehydrogenase				
Longissimus muscle	.07[a]	.25[ab]	.50[b]	.1
Trapezius muscle	.30	.21	.49	.11
Liver	5.45[a]	6.06[ab]	7.65[b]	.59
Kidney	35.48	39.31	35.28	3.85
Subcutaneous adipose	1.36	3.93	4.44	1.56

[a,b]Means in the same row with no superscripts in common differ ($P<.05$).

relative differences in LAT and KICDH activities between dietary protein treatment groups were similar, whether expressed on a per gram of tissue or on a protein basis. The only real variations were those for LAT activity in subcutaneous adipose tissue. On a per milligram of protein basis subcutaneous adipose tissue homogenates from the lambs fed the 18% CP diet had 12% greater LAT activity than the lambs fed 12% CP while on a per gram of tissue basis adipose tissue from the lambs fed 18% CP had 4% lower LAT activity than adipose issue from lambs fed the 12% CP diet (table II-4). This discrepancy can be explained, at least in part, by the higher lipid content (lower protein content) of the subcutaneous adipose tissue from the lambs fed 18% CP compared to subcutaneous adipose tissue from the lambs fed 12% CP (table II-3).

Because of the high LAT and KICDH activities observed in adipose tissue, the contributions of intramuscular adipose tissue to the enzyme activities observed in skeletal muscle homogenates were calculated as described in Chapter I and Appendix II-A. These values shown in table II-5, were arrived at with the assumption that intramuscular adipose tissue has the same activity (expressed per gram of tissue) as the subcutaneous adipose tissue analyzed in this study. These values probably represent maximums. In all treatment groups it is apparent that intramuscular adipose tissue

TABLE II-5. MEANS AND STANDARD ERRORS FOR CALCULATED PERCENTAGE CONTRIBUTION OF INTRAMUSCULAR ADIPOSE TISSUE TO ENZYME ACTIVITY IN SKELETAL MUSCLES FROM WETHER LAMBS FED DIETS CONTAINING 8, 12 OR 18% CRUDE PROTEIN[a]

Enzyme activity and tissue	Dietary protein content,%			Standard error
	8	12	18	
Leucine aminotransferase,	%			
Longissimus muscle	48.9	109.7	140.1	45.8
Trapezius muscle	84.2	180.8	183.1	55.0
Alpha-ketoisocaproate dehydrogenase				
Longissimus muscle	222.3	261.3	295.6	189.0
Trapezius muscle	580.4	222.0	111.0	290.6

[1]Potential contribution of intramuscular adipose tissue was calculated as:

$$\frac{\text{Subcutaneous adipose tissue activity} \times \text{muscle lipid content}}{\text{Skeletal muscle activity}} \times 100$$

See Appendix II-A.

could account for a substantial portion of the LAT and KICDH activity attributed to skeletal muscle.

Activity Expressed On a Total Tissue Basis. The means and standard errors of LAT and KICDH activity expressed on a total tissue basis are presented in table II-6. The equations used to calculate activity on the tissue basis are shown in Appendix II-B and tissue weights are presented in appendix table 2. On a tissue basis LAT activity was not affected ($P>.05$) by dietary protein in any of the tissues studied. On the other hand, skeletal muscle KICDH activity on a tissue basis was higher ($P<.05$) for lambs fed 18% CP than for lambs fed either 8 or 12% CP, and liver KICDH activity was higher ($P<.05$) for the lambs fed 18% CP than those fed the 8% CP diet. The sums of LAT activities in the four tissues studied (skeletal muscle, liver, kidney and adipose tissue) were from 11- (in the lambs fed 18% CP) to 15-fold higher (in the lambs fed 12% CP) than the corresponding KICDH activities.

The contributions of individual tissues to LAT and KICDH activities, expressed as a percentage of the total activity in all four tissues are shown in figure II-4. In all three treatment groups most of the LAT activity was found in subcutaneous adipose tissue, skeletal muscle contributed a moderate amount and liver and kidney relatively little to total activity. There were no

TABLE II-6. MEANS AND STANDARD ERRORS OF ENZYMATIC ACTIVITY IN SEVERAL TISSUES EXCISED FROM FASTED WETHER LAMBS FED DIETS CONTAINING 8, 12 OR 18% CRUDE PROTEIN[a]

Enzyme activity and tissue	Dietary protein content,% 8	12	18	Standard error
Leucine aminotransferase	-----nmolx g tissue^{-1}x min^{-1}-----			
Skeletal muscle	52377	70613	72704	12833
Liver	7785	9704	12258	1289
Kidney	8328	8434	8930	2617
Subcutaneous adipose	159176	459378	397893	114245
Total[b]	227666	548129	491785	117792
Alpha-ketoisocaproate dehydrogenase				
Skeletal muscle	2582[c]	3250[c]	6964[d]	1247
Liver	4922[c]	6803[cd]	8044[d]	738
Kidney	4356	5379	5361	542
Subcutaneous adipose	7047	20838	22637	7469
Total[b]	18907	36270	43006	7699

[a]Equations used for calculations are shown in Appendix II-B.

[b]Total represents the sum of activities in all four tissues.

[c,d]Means in the same row with no superscripts in common differ (P<.05).

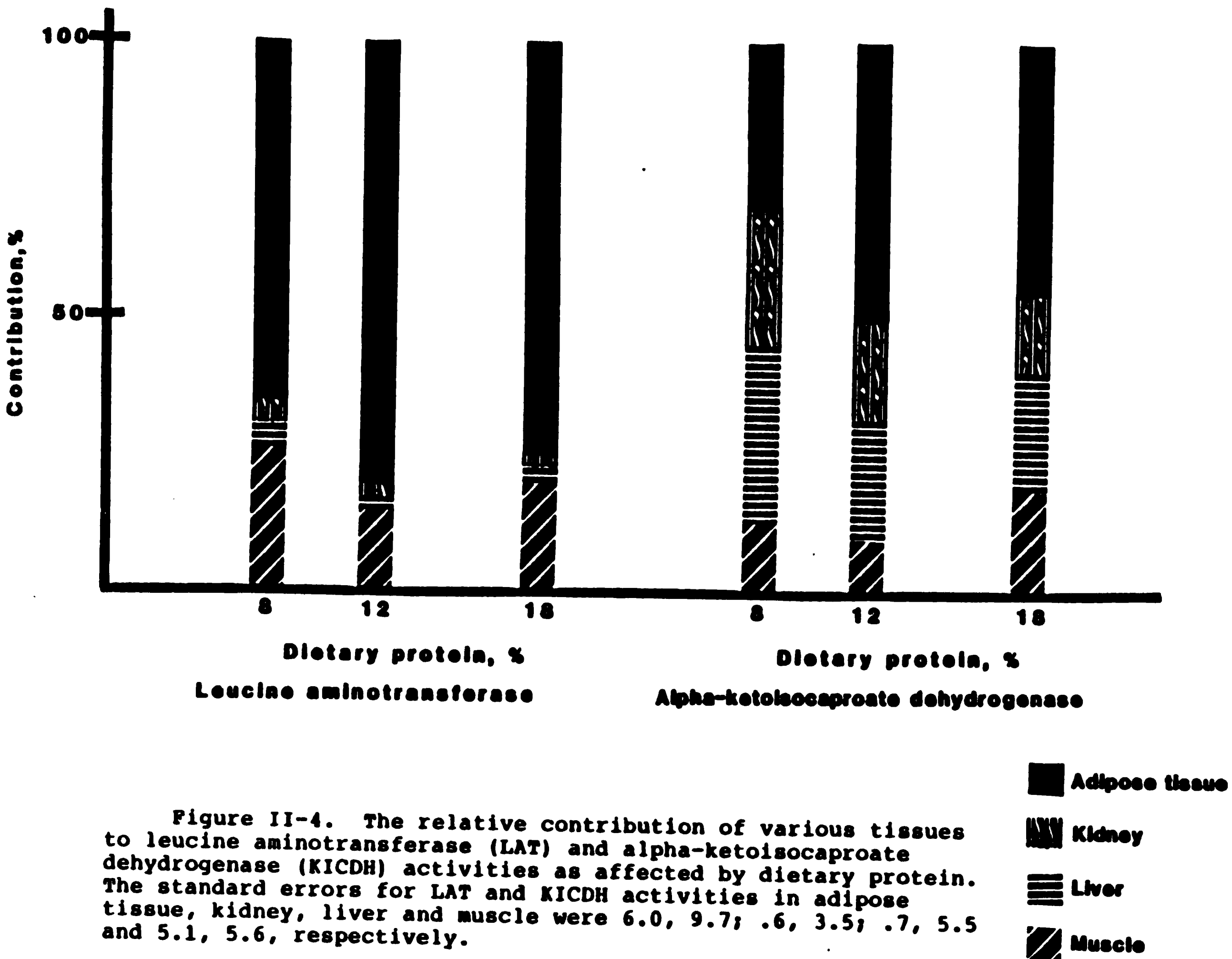


Figure II-4. The relative contribution of various tissues to leucine aminotransferase (LAT) and alpha-ketoisocaproate dehydrogenase (KICDH) activities as affected by dietary protein. The standard errors for LAT and KICDH activities in adipose tissue, kidney, liver and muscle were 6.0, 9.7; .6, 3.5; .7, 5.5 and 5.1, 5.6, respectively.

differences (P>.05) in the percentage contribution of any of the four tissues to LAT activity due to dietary protein content. Likewise, dietary protein did not alter (P>.05) the percentage contribution of any of the tissues to KICDH activity (figure II-4). The percentage contribution of adipose tissue and liver to KICDH activity were 29.7, 31.2; 50.0, 22.5 and 45.7, 21.2 for the lambs fed the 8, 12 and 18% CP diets, respectively. The percentage contribution of skeletal muscle and kidney to KICDH activity was 13.7, 25.4; 9.6, 17.9 and 18.5, 14.6 for the lambs fed 8, 12 and 18% CP, respectively.

Experiment 2: Effects of Length of Fasting on LAT and KICDH Activities

Enzyme Activity Expressed Per Milligram of Protein. Figure II-5 shows the effects of fasting on LAT and KICDH activities in longissimus and trapezius muscles. While LAT and KICDH activities in longissimus and trapezius muscles were not significantly altered by fasting, there was a tendency for activities of both enzymes to be increased by 48 h of fasting and to be decreased by 96 h of fasting when compared to activities for unfasted lambs. In these skeletal muscle homogenates, LAT activities ranged from 20- to 34-fold greater than KICDH activities.

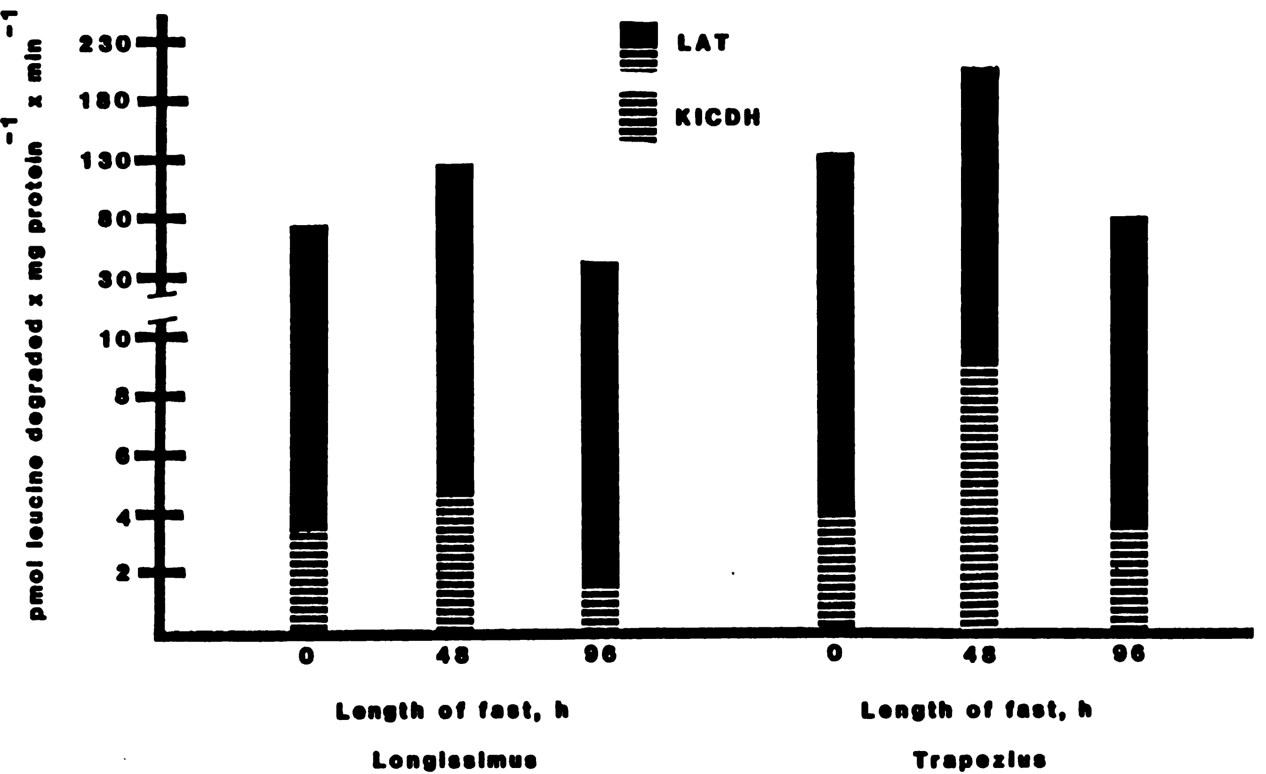


Figure II-5. The effects of fasting length on leucine aminotransferase (LAT) and alpha-ketoisocaproate dehydrogenase (KICDH) activities in homogenates of longissimus and trapezius muscles. The standard errors for longissimus LAT and KICDH activities and trapezius LAT and KICDH activities were 33.9, 1.3, 61.8 and 2.1, respectively.

As in muscle the activities of both enzymes in liver tended to increase (nonsignificantly) after the 48 h fast when compared to activities for lambs that were not fasted (figure II-6). Liver LAT activity of lambs fasted 96 h was less ($P<.05$) than lambs fasted 48 h, and liver KICDH activity of those fasted for 96 h was reduced ($P<.05$) when compared to either the 0 or 48 h fasted groups.

Kidney homogenates exhibited the same general pattern for LAT and KICDH activities as liver homogenates. However, the nonsignificant increase in activities of both enzymes, between 0 and 48 h of fasting was less than in liver and only the KICDH activity differed significantly between the lambs fasted for 0 or 48 h, and those fasted for 96 h (figure II-6). LAT activities in liver and kidney homogenates ranged from 1.4- to 2.5-fold greater than the respective KICDH activities (figure II-6).

In adipose tissue, neither LAT nor KICDH activities were significantly affected by the 48 h fast when compared to the activities for unfasted lambs (figure II-7). However, only in this tissue was the KICDH activity lower (nonsignificantly) for the 48 h fasted lambs than for the unfasted lambs. Adipose tissue LAT and KICDH activities were lower after fasting for 96 h when compared to enzyme activities in the 0 or 48 h fasted lambs, but only the reduction in KICDH activity was significant. LAT activities

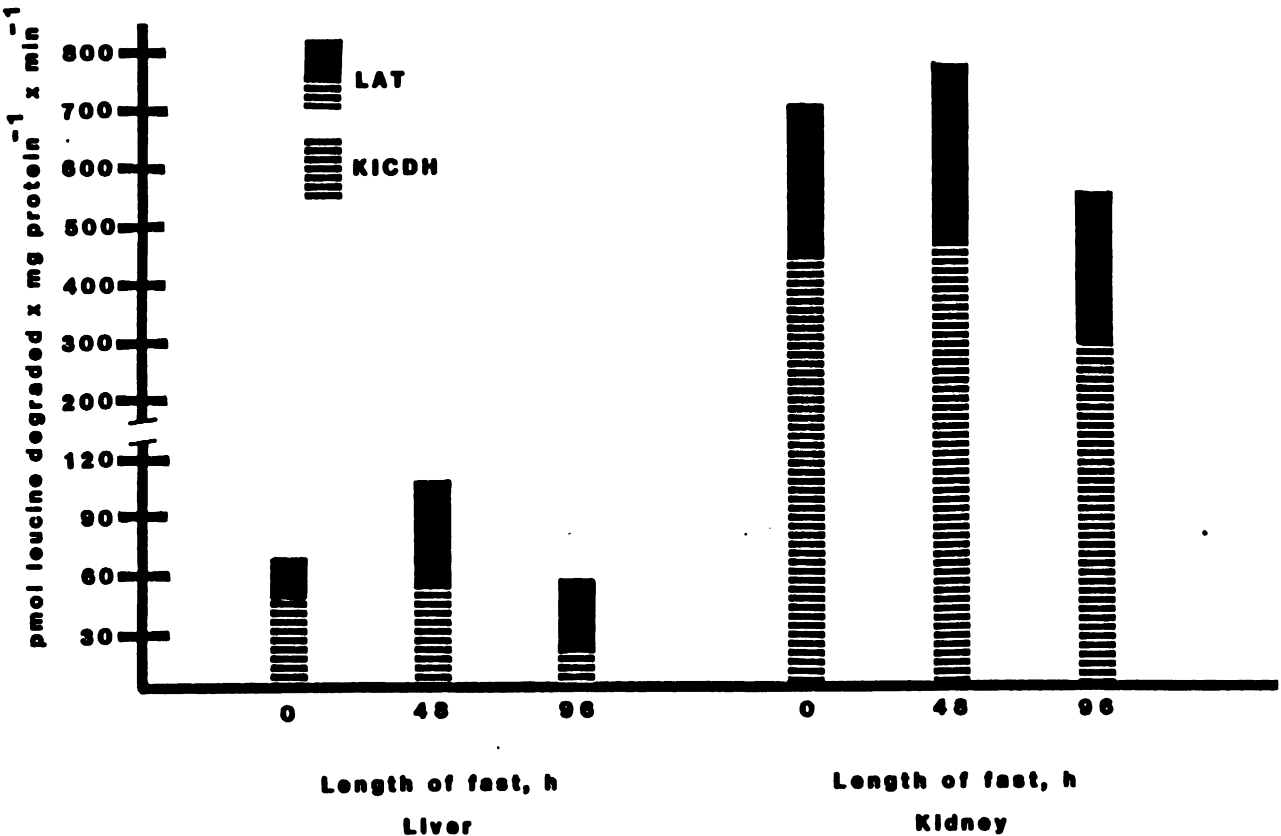


Figure II-6. The effects of fasting length on leucine aminotransferase (LAT) and alpha-ketoisocaproate dehydrogenase (KICDH) activities in homogenates of liver and kidney. The standard errors for liver LAT and KICDH activities and kidney LAT and KICDH activities were 15.6, 7.5, 85.3 and 51.2, respectively.

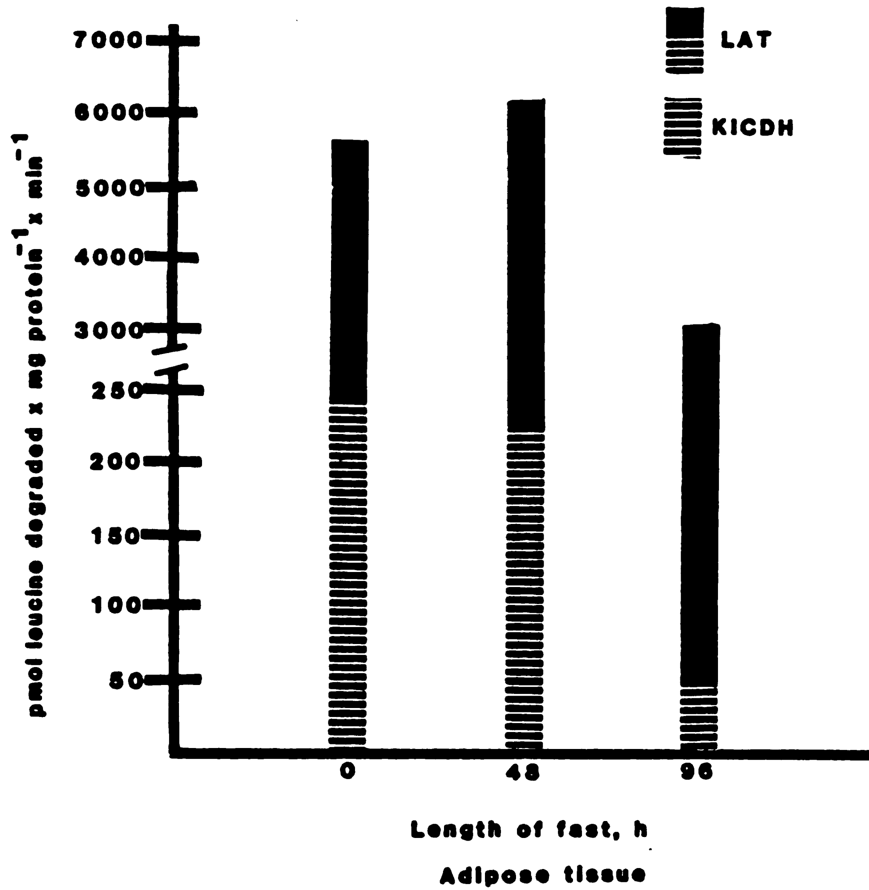


Figure II-7. The effects of fasting length on leucine aminotransferase (LAT) and alpha-ketoisocaproate dehydrogenase (KICDH) activities in homogenates of subcutaneous adipose tissue standard errors for adipose tissue LAT and KICDH activities were 1307.1 and 43.1, respectively.

in adipose tissue ranged from 23- to 62-times greater than the respective KICDH activities.

Activity Per Gram of Tissue. Activities expressed on a per gram of tissue basis for LAT and KICDH are shown in table II-7. Tissues with low protein concentrations had lower enzyme activities, relative to other tissues, when the activities were expressed per gram of tissue rather than on a protein basis. On a protein basis the subcutaneous adipose tissue:kidney:liver: trapezius muscle:longissimus muscle ratios of LAT activities averaged across fasting treatment groups, were 65:9:1:2:1, while those ratios for activities expressed per gram of tissue were 11:13:2:1:1. The subcutaneous adipose tissue:kidney:liver: trapezius muscle:longissimus muscle ratios of KICDH activities, averaged across fasting treatment groups, were 53:124:13:2:1, while those ratios for KICDH activities expressed per gram of tissue were 10:167:26:1:1.

With the exception of LAT activity in subcutaneous adipose tissue, the relative differences in LAT and KICDH activities between fasted treatment groups were similar, whether expressed per gram of tissue or on a protein basis. On a protein basis LAT activity in subcutaneous adipose tissue was slightly higher in the lambs fasted 48 h than in those that were not fasted, while on a tissue basis,

TABLE II-7. MEANS AND STANDARD ERRORS OF ENZYME ACTIVITY IN SEVERAL TISSUES EXCISED FROM FASTED WETHER LAMBS

Enzyme activity and tissue	Fast, h			Standard error
	0	48	96	
Leucine aminotransferase	-----nmolx g tissue^{-1}x min^{-1}-----			
Longissimus	4.60	6.74	2.70	1.63
Trapezius	5.10	8.86	3.82	1.58
Liver	8.84^{ab}	14.30^{b}	7.44^{a}	1.87
Kidney	62.12	63.60	53.16	7.38
Subcutaneous adipose	81.70^{b}	49.78^{ab}	19.76^{a}	15.21
Alpha-ketoisocaproate dehydrogenase				
Longissimus	.25	.26	.12	.09
Trapezius	.21	.47	.15	.12
Liver	6.06^{b}	7.34^{b}	2.94^{a}	.91
Kidney	39.31	38.13	27.45	4.62
Subcutaneous adipose	3.93	1.86	.32	1.14

^{a,b}Means in the same row with no superscripts in common differ (P<.05).

subcutaneous adipose tissue LAT activity decreased steadily as length of fasting increased from 0 to 96 h.

Means of the percentage contributions of intramuscular adipose tissue to the enzyme activities observed in skeletal muscle homogenates are shown in table II-8. These values were arrived at with the assumption that intramuscular adipose tissue had the same activity (expressed per gram of tissue) as the subcutaneous adipose tissue in this study. In all treatment groups, and especially in the unfasted group, intramuscular adipose tissue could account for a substantial portion of the LAT and KICDH activity attributed to skeletal muscle.

Enzyme Activity Expressed on a Total Tissue Basis. Means and standard errors of LAT and KICDH activities expressed on a total tissue basis are contained in table II-9. Appendix II-B contains the equations used to calculate activity in each tissue, and tissue weights are displayed in appendix table 3. The total LAT activity of all four tissues combined for the lambs fasted 96 h was 69.6% less than total LAT activity for the fed lambs, primarily because of the 75.8% decrease in subcutaneous adipose tissue LAT activity over the same period of fasting. Total tissues combined and the total subcutaneous adipose tissue KICDH activities decreased 73.9 and 91.3%, respectively, as fasting length increased from 0 to 96 h.

TABLE II-8. MEANS AND STANDARD ERRORS FOR CALCULATED PERCENTAGE CONTRIBUTION OF INTRAMUSCULAR ADIPOSE TISSUE TO ENZYME ACTIVITY IN SKELETAL MUSCLES FROM FASTED WETHER LAMBS[a]

Enzyme activity and muscle	Fast, h 0	48	96	Standard error
Leucine aminotransferase		%		
Longissimus muscle	109.70	68.00	77.30	24.87
Trapezius muscle	180.80	59.60	83.30	37.58
Alpha-ketoisocaproate dehydrogenase				
Longissimus muscle	261.30	123.00	124.00	126.03
Trapezius muscle	222.00	63.70	76.20	47.80

[a]Potential contribution of intramuscular adipose tissue was calculated as:

$$\frac{\text{Subcutaneous adipose tissue activity} \times \text{muscle lipid content}}{\text{Skeletal muscle activity}} \times 100$$

See Appendix II-A.

TABLE II-9. MEANS AND STANDARD ERRORS OF ENZYME ACTIVITY IN SEVERAL TISSUES EXCISED FROM FASTED WETHER LAMBS[a]

Item	Fast, h			Standard error
	0	48	96	
Leucine aminotransferase	-----nmolx tissue^{-1}x min^{-1}-----			
Skeletal muscle	70613[cd]	107864[d]	42667[c]	18985
Liver	9704[cd]	12251[d]	5731[c]	1567
Kidney	8434	8003	6886	1073
Subcutaneous adipose	459378[d]	266377[cd]	111152[c]	88787
Total[b]	548129[d]	394495[cd]	166436[c]	103338
Alpha-ketoisocaproate dehydrogenase				
Skeletal muscle	3250	5058	1823	1222
Liver	6803[d]	6354[d]	2270[c]	873
Kidney	5379	4802	3568	677
Subcutaneous adipose	20838[d]	9713[cd]	1808[c]	5622
Total[b]	36270[d]	25927[cd]	9469[c]	6389

[a]Equations used for calculations are shown in Appendix II-B.

[b]Total represents the sum of activities in all four tissues.

[cd]Means in the same row with no superscripts in common differ (P<.05).

Figure II-8 shows the contributions of individual tissues to LAT and KICDH activities, when activity was expressed as a percentage of the total activity of all four tissues. As was the case for Experiment 1, in all three treatment groups, most of the total LAT activity was located in subcutaneous adipose tissue, skeletal muscle made a moderate contribution and the contributions of liver and kidney were relatively insignificant. The percentage contributions of subcutaneous adipose tissue to total KICDH activity in lambs fasted 0, 48 and 96 h were 50.0, 36.4 and 16.5%, respectively, while the contributions of kidney to KICDH activity in these three groups were 17.9, 18.1 and 48.8%, respectively. The decrease in adipose tissue and the increase in kidney percentage contribution to total KICDH activity due to fasting was significant ($P<.05$). Skeletal muscle contributed 9.6, 20.9 and 12.0%, while liver contributed 22.5, 24.6 and 22.7% to total KICDH activity for lambs fasted 0, 48 and 96 h, respectively.

Discussion

Dietary Protein Content. While dietary protein content did not significantly alter LAT activity in the tissues studied, except for kidney, there was a trend for activity to increase as protein content increased. The increase in LAT activity with increasing protein content was expected,

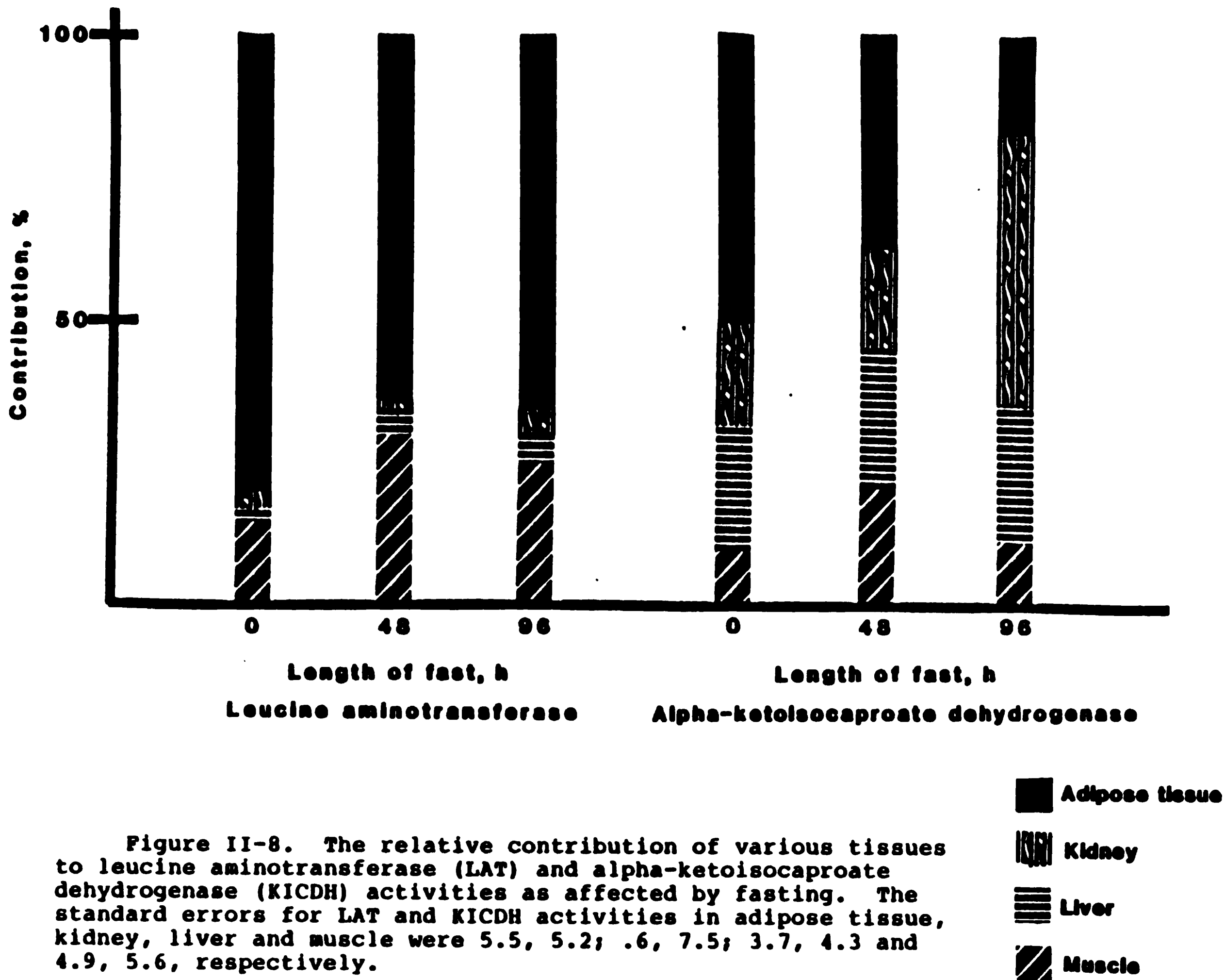


Figure II-8. The relative contribution of various tissues to leucine aminotransferase (LAT) and alpha-ketoisocaproate dehydrogenase (KICDH) activities as affected by fasting. The standard errors for LAT and KICDH activities in adipose tissue, kidney, liver and muscle were 5.5, 5.2; .6, 7.5; 3.7, 4.3 and 4.9, 5.6, respectively.

since amino acids in general (Lehninger, 1982), and BCAA in particular (Krebs, 1972), are degraded when fed in excess of the body's needs for protein synthesis. In the rat, skeletal muscle (Adibi et al., 1975) and liver (Ichihara et al., 1967; Krebs, 1972) LAT activities were found to increase as dietary protein was increased, while kidney (Shinnick and Harper, 1977) LAT activity was unresponsive to changes in dietary protein.

Kidney KICDH activity was also unresponsive to dietary protein manipulations, while KICDH activity was significantly increased in skeletal muscle and liver, and substantially though nonsignificantly (P>.05) increased in adipose tissue when dietary protein content was increased from 8 to 18% (tables II-4 and 6). In vivo studies of overall BCAA oxidation in rats (McFarlane and von Holt, 1969a; Sketcher and James, 1974) and enzyme assays of rat skeletal muscle (Sketcher et al., 1974; Sketcher and James, 1974, 1976; Shinnick and Harper, 1977), liver (McFarlane and von Holt, 1969b; Wohlhueter and Harper, 1970; Adibi, 1976) and adipose tissue (Frick and Goodman, 1979; Tischler and Goldberg, 1981) have also shown that BCKA dehydrogenase was stimulated by feeding a high protein diet. Wohlhueter and Harper (1970) also reported that BCKA dehydrogenase activity in rat kidney was unaltered by changes in dietary protein content as was observed in this study with lambs.

Fasting Length. Whether expressed per milligram of protein, per gram of tissue or on a total tissue basis, LAT and KICDH activities in longissimus and trapezius muscles tended to increase with 48 h, and decrease with 96 h of fasting, when compared to unfasted controls. However, these enzyme activities tended to decrease in subcutaneous adipose tissue following the 48 h and 96 h fasts. The increase and decrease in BCAA degradative enzyme activity caused by 48 h of fasting in muscle and adipose tissue, respectively, was not totally surprising since BCAA degradation is believed to be a catabolic process in muscle and an anabolic process in adipose tissue (Goldberg and Chang, 1978). In rat muscle, BCAA spare glucose by serving as a substitute energy source during fasting, and they also supply amino groups for the synthesis of alanine and glutamine, which are important gluconeogenic precursors (Chang and Goldberg, 1978a,b,c). On the other hand, BCAA spare glucose in adipose tissue in the fed state by supplying substrates for fatty acid synthesis (Tischler and Goldberg, 1980a; Goldberg and Tischler, 1981).

It should be noted that the increase in skeletal muscle LAT and KICDH activities of the lambs fasted for 48 h were not significant; however, fasting rats for 3 d caused a three- to fivefold increase ($P<.05$) in the oxidation of leucine, valine and isoleucine in muscle (Goldberg and Odessey, 1972). The smaller change in BCAA degradative

activity due to fasting in ruminant skeletal muscle compared to rat skeletal muscle would be expected, since ruminants rely on gluconeogenesis for their glucose supply even in the fed state and amino acids provide the carbon sources for gluconeogenesis to a limited degree in ruminants (Lindsay, 1982).

The lower LAT and KICDH activities observed in skeletal muscle of lambs fasted for 96 h compared to lambs fasted 0 or 48 h in the present study is consistent with what would be expected in nonruminants. Snell (1980) has suggested that in a long term fast in which the plasma concentrations of ketone bodies rise and ketone body metabolism for energy becomes more efficient, the need to spare glucose and thus to degrade BCAA diminishes. This argument is strengthened by the finding that beta-hydroxybutyrate inhibits BCAA oxidation (Landaas, 1977; Lindsay and Buttery, 1980; Zapalowski et al., 1981), and by the findings of Robinson and Williamson (1980) that beta-hydroxybutyrate infusion inhibited skeletal muscle alanine production (perhaps by limiting BCAA degradation). However, in rats there are no reports suggesting that BCAA degradative activity in skeletal muscle diminishes when fasting extends beyond 2 d. In fact, Goldberg and Odessey (1972) reported that the rate of BCAA oxidation was twofold greater for muscle diaphragm from rats fasted for 3 d than for diaphragm muscles from

those fasted for 2 d. It is important to emphasize, however, that: 1) the rats utilized in these direct assays of BCAA degradative activities weighed 40 to 125 g and therefore had low adipose tissue contents; 2) Goodman and Ruderman (1980), Goodman et al.(1980) and Dunn et al.(1982) have shown that the ability of rats to conserve protein during prolonged starvation requires the continued availability of lipid for fuels; and 3) BCAA degradation in rat skeletal muscle is closely associated with the release of alanine and glutamine (Goldberg and Chang, 1978). Additionally, Goodman et al.(1980) reported that in young lean rats (8 wk of age), there was no evidence that alanine production from muscle was diminished or that protein was spared in a long term fast of 5 to 6 d (terminal), while obese rats or 16 wk old lean rats did exhibit protein sparing and decreased alanine output during a long term fast. Therefore, the conservation of BCAA predicted by Felig (1975) and Snell (1980) may occur in more mature normal rats, or in obese rats that would have adequate stores of adipose tissue to supply lipid as fuel during prolonged starvation.

In ruminants, measurements of BCAA release from hindquarter preparations have indicated that BCAA degradation in skeletal muscle was not enhanced by fasting (Heitmann and Bergman, 1980; Lindsay, 1980). However, in

induced diabetic sheep the output of all amino acids increased from muscle except for the BCAA which fell markedly, indicating that diabetes does increase BCAA oxidation in ovine skeletal muscle (Lindsay and Buttery, 1980). Those authors hypothesized that a high beta-hydroxybutyrate/acetoacetate ratio (which is dependent on a high NADH/NAD^+ ratio) may inhibit BCAA oxidation in sheep. In support of that hypothesis the authors stated that diabetic animals have elevated concentrations of both acetoacetate and beta-hydroxybutyrate, but the acetoacetate concentration is elevated to a much greater extent than the beta-hydroxybutyrate concentration. However, the beta-hydroxybutyrate/acetoacetate ratio is also reduced in fasted ruminants (Baird et al., 1979), indicating that the hypothesis of Lindsay and Buttery (1980) does not completely explain the apparently diverse effects of fasting and diabetes on BCAA degradation in ruminant skeletal muscle. In the present study, it is noteworthy that a portion of the changes in ovine skeletal muscle enzyme activities due to fasting, may be accounted for by a decrease in intramuscular adipose tissue LAT and KICDH activities.

As previously mentioned, the decrease in adipose tissue LAT and KICDH activities caused by both the 48 and 96 h fasts was not surprising because fasting is known to reduce the conversion of BCAA to lipids in rats (Frick and Goodman,

1979) and mice (Rous et al., 1980). This may be of particular importance in ruminant adipose tissue since that tissue is responsible for most of the lipogenesis in ruminants. It is also known that insulin stimulates both fatty acid synthesis and BCAA degradation in rat adipose tissues (Minemura et al., 1970; Goodman, 1977). While adipose tissue is generally less responsive to insulin in ruminants than in nonruminants (Hood, 1981, 1983), insulin does inhibit ruminant adipose tissue lipolysis. Thus, fatty acid synthesis would be stimulated by decreasing the concentration of long chain acyl CoA, which are potent inhibitors of fatty acid synthesis (Vernon, 1980). That, BCAA spare glucose in ruminant adipose tissue by supplying substrates for fatty acid synthesis, as was suggested in rat adipose tissue (Tischler and Goldberg, 1980a), is doubtful since glucose is not readily utilized for fatty acid synthesis in ruminants (Vernon, 1980). Nonetheless, leucine degradative enzymes could play a part in the partitioning of nutrients as was suggested by Lindsay (1982).

It is interesting that in liver, LAT activity expressed on a protein, per gram of tissue or per tissue basis was lower for the lambs fasted 96 h than for the lambs fasted 48 h. In addition, liver KICDH activity was lower in the lambs fasted for 96 h than for those fasted 0 or 48 h. However, most studies with rats have indicated that liver BCAA

aminotransferase (Wohlhueter and Harper, 1970; Adibi et al., 1975) and BCKA dehydrogenase (Goldberg and Odessey, 1972; Adibi, 1976; Frick and Goodman, 1979; Tischler and Goldberg, 1981) activities were unresponsive to fasting and a few studies have indicated that BCKA dehydrogenase activity was enhanced by fasting. Furthermore, Heitmann and Bergman (1980) and Bergman and Pell (1983) reported that in sheep, net hepatic removal of leucine from the circulation was unaltered by 3 d of fasting. Interestingly, those results with sheep are compatible with the results of the present study. In the present study both LAT and KICDH activities in ovine liver were nonsignificantly increased by 48 h of fasting compared to the unfasted controls, and then were reduced ($P<.05$) by 96 h of fasting. Thus, if enzyme activity decreases somewhat linearly between 48 and 96 h of fasting, the LAT and KICDH activities after 3 d of fasting in the present study may have been similar to the activity in the unfasted lambs. It should also be noted that the effects of fasting on liver KICDH activity cannot be accurately determined from this study since LAT is rate limiting in liver (Shinnick and Harper, 1976; Chapter IV) and L-[1-^{14}C]-leucine was supplied as substrate.

LAT and KICDH activities in kidney were the least responsive to changes in length of fasting. On a total tissue basis, LAT and KICDH activities in kidney were

decreased 18.4 and 33.7%, respectively, while LAT and KICDH activities in skeletal muscle, liver and adipose tissue were decreased 39.6, 43.9; 40.9, 66.6 and 75.8, 91.3%, respectively. Results from studies of rat tissues also indicate that LAT and KICDH activities in kidney are relatively unresponsive to food deprivation (Wohlhueter and Harper, 1970).

The decrease in combined total LAT and KICDH activities from all four tissues (table II-9) following 48 or 96 h of fasting could at least in part explain the increased plasma BCAA concentrations associated with fasting in ruminants (Ballard et al., 1976; Bergen, 1979; Heitmann and Bergman, 1980; Bergman and Pell, 1983). Undoubtedly, the net proteolysis associated with fasting also contributed to the increased concentration of free BCAA (Bergen, 1979).

Tissue Distribution of Enzyme Activities. For all five dietary treatment groups studied in Experiments 1 and 2, subcutaneous adipose tissue was the most important site of LAT activity, skeletal muscle was moderately important and liver and kidney were relatively unimportant (figures II-4 and II-8). As mentioned previously (chapter I), these results were not surprising. Even in the rat, a species with a relatively low lipid content, adipose tissue is a major site of leucine degradation second only to skeletal muscle (Rosenthal et al., 1974). Further, Goldberg and

Tischler (1981) suggested that in obese rats, or in species that contain a high percentage of fat, adipose issue may be of even greater importance in BCAA degradation.

Subcutaneous adipose tissue also had a large proportion of the total KICDH activity. Subcutaneous adipose tissue constitutes about 44% of total ovine adipose tissue (Kauffman et al., 1963), thus, if all adipose tissue deposits were included, only in the lambs fasted for 96 h would adipose tissue not account for most of the KICDH activity. That kidney appears to be the predominant tissue for KICDH activity in the lambs fasted 96 h is somewhat surprising, since Heitmann and Bergman (1980) reported that BCAA were released by the kidney in both fed sheep and those fasted for 3 d. While kidney KICDH activity was not actually increased by fasting, its relative contribution increased because of the drastic decrease in adipose tissue KICDH activity. Moreover, since LAT activity is rate limiting in liver, if [1-^{14}C]-alpha-ketoisocaproate was supplied as substrate instead of L-[1-^{14}C]-leucine, liver could possibly have made a much greater contribution than was obtained in the present study.

The total LAT activities in the four tissues studied (skeletal muscle, liver, kidney and adipose tissue) were from 11- (in the lambs fed the 18% CP diet) to 18-fold (in the lambs fasted 96 h) higher than the corresponding KICDH

activities (tables II-6 and II-9). A similar ratio was obtained in the previous study (Chapter I), and these results indicate that in sheep, as in the rat (Goldberg and Tischler, 1981), KICDH is probably the rate limiting enzyme of whole body leucine degradation. LAT and KICDH activities are not, however, equally distributed among tissues. In the rat, KICDH is rate limiting in skeletal muscle, kidney and adipose, but LAT is rate limiting in liver (Harper and Zapalowski, 1981). The present results (tables II-6 and II-9) indicate that the distribution is similar in sheep. The ratio of LAT to KICDH activity in skeletal muscle and adipose tissue for the various treatment groups ranged from 10:1 to 61:1, while that ratio for activities in liver and kidney ranged from only 1.4:1 to 2.5:1. These ratios are similar to those obtained previously (Chapter I) and they indicate that KICDH activity may be rate limiting in skeletal muscle and fat, while LAT activity could be rate limiting in liver and kidney. In a subsequent study, however, when labeled KIC was supplied as substrate, LAT activity in kidney was found to be higher than the KICDH activity (Chapter IV).

Summary and Conclusions

Twenty-five, 5 mo old, crossbred wether lambs were used to study the effects of dietary protein content and fasting on LAT and KICDH activities in longissimus and trapezius muscles, liver, kidney and adipose tissue crude homogenates. Five lambs were assigned to each of the following five treatments: unfasted lambs fed diets containing 8, 12 or 18% CP or lambs fasted 48 or 96 h that were fed the 12% CP diet. L-[1-^{14}C]-leucine was utilized as substrate to measure LAT and KICDH activities. In Experiment 1 unfasted lambs fed the 12% CP diet were compared with lambs fed the 8 and 18% CP diets. In this experiment there was a trend for enzyme activities in skeletal muscle, liver and adipose tissue to be altered so that BCAA would be conserved or be more rapidly degraded when lambs are fed a diet containing deficient or excess protein, respectively, while LAT and KICDH activities in kidney appeared to be unresponsive to dietary protein manipulation.

In Experiment 2, the effects of fasting on enzyme activities were analyzed by comparing the three groups of lambs fed the 12% CP diet that were fasted for 0, 48 or 96 h. LAT and KICDH activities in skeletal muscle and liver tended to increase following a 48 h fast while those

activities in all tissues were decreased by 96 h of fasting. Only in adipose tissue did LAT and KICDH activity decrease after only 48 h of fasting. In both Experiments 1 and 2, when activity was expressed on a total tissue basis, adipose tissue was the primary location of LAT activity for all treatment groups studied but this was especially true in the unfasted groups. Adipose tissue was also the most important tissue for KICDH activity in the fed groups but as fasting length was increased to 96 h the relative contribution of KICDH activity was similar among the four tissues. LAT appeared to be the rate limiting enzyme in liver, and KICDH was probably rate limiting in skeletal muscle and adipose tissue BCAA metabolism. Therefore, it would be interesting to study the effects of fasting on KICDH activity using labeled KIC as substrate to more accurately ascertain the contribution of liver to total activity. In addition, further study is needed to discern what the apparently important role of leucine, valine and isoleucine is in adipose tissue metabolism.

In rats, a single multienzyme complex is apparently responsible for the decarboxylation of all three BCKA (Randle et al., 1981; Morrison and Mullings, 1983). A single transaminase is also capable of transferring amino groups from all three BCAA, but liver also contains a significant quantity of a transaminase specific for leucine (Ichihara et

al., 1973; Ichihara, 1975; Kadowaki and Knox, 1982). Therefore, the present results obtained, using leucine as substrate, probably reflect the effects of dietary manipulation on the metabolism of all three BCAA. A study utilizing leucine and valine or isoleucine as substrate to ascertain whether the degradation of leucine and valine or isoleucine is regulated in concert is warranted.

CHAPTER III

EFFECT OF FASTING ON THE RELATIVE RATES OF LEUCINE AND VALINE DEGRADATION BY SHEEP TISSUE HOMOGENATES

Introduction

The first two steps in the degradation of BCAA, i.e., reversible transamination followed by irreversible decarboxylation of the resulting BCKA to a branched chain acyl CoA, have been extensively studied in rats (Dancis and Levitz, 1978). Ichihara (1975) summarized the findings of his laboratory for several studies relating to properties of three isozymes of BCAA aminotransferase. Isozymes I (which is fairly ubiquitous in rat tissues) and III (found in brain, ovary and placental tissues) are similar in substrate specificity and equal in activity for the three BCAA, while isozyme II (found only in liver) is specific for leucine. Isozymes I, II and III are primarily localized in the cytosolic fraction, however, mitochondrial enzymes similar to isozymes I (Kadowaki and Knox, 1982) and II (Ikeda et al., 1976; Ichihara et al., 1981) have also been reported. The mitochondrial enzymes appear to be more important in liver and kidney preparations than in preparations from most other tissues. Both the mitochondrial and cytosolic isozyme I have a much higher K_m for valine than for leucine or isoleucine (Ichihara, 1975; Kadowaki and Knox, 1982).

Currently, the data in the literature are not clear as to whether multiple enzymes are involved in the decarboxylation of BCKA (Danner and Bowden, 1966; Sullivan et al., 1976; Kean and Morrison, 1979) or if a single enzyme complex is responsible for the decarboxylation of all three BCKA (Wohlhueter and Harper, 1970; Danner et al., 1975, 1978, 1979, 1981; Khatra et al., 1977b; Parker and Randle, 1978a; Pettit et al., 1978; Frick and Goodman, 1980; Odessey, 1980; Morrison and Mullings, 1983). However, the bulk of the evidence indicates that a single mitochondrial enzyme complex (BCKA dehydrogenase) is primarily responsible for the degradation of all three BCKA, although the possibility exists for three separate branched chain decarboxylase subunits associated with a single BCKA dehydrogenase complex (Danner et al., 1979; Randle et al., 1981). In addition, there apparently is an oxidase in the soluble fraction of liver cells that decarboxylates KIC (Johnson and Connelly, 1972; Sabourin and Bieber, 1981).

In ruminants there have been few experiments designed to study isozymes of BCAA aminotransferase and BCKA dehydrogenase. Therefore this study was designed to examine the transamination and decarboxylation of leucine and valine in sheep muscle, liver, kidney and adipose tissue to gain a better understanding of isozymes involved in the degradation of BCAA in sheep tissues.

Experimental Procedure

Materials

The materials used in this study were identical to those described previously in Chapter I.

Experimental Design

The experimental design is shown in table III-1. Six Suffolk X Targhee crossed ram lambs were fed a creep diet until weaning at 60 d of age, and they were then adjusted to ad libitum feeding of the experimental diet shown in table III-2. Three of the lambs were removed from the experimental diet 84 h prior to slaughter and three were removed from feed 12 h prior to sacrifice at 84 d of age. The lambs were electrically stunned prior to exsanguination.

Tissue Preparation

Longissimus and trapezius muscle, liver, kidney and subcutaneous adipose tissue samples were excised rapidly and homogenized as described in Chapter I and shown in figure III-1.

TABLE III-1. EXPERIMENTAL DESIGN

Item	Length of fast, h	
	12	84
Number of animals	3	3
Number of homogenates from each tissue assayed with L-[1-^{14}C]-leucine used as substrate	3	3
Number of homogenates from each tissue assayed with L-[1-^{14}C]-valine used as substrate	3	3

TABLE III-2. EXPERIMENTAL DIET

Ingredient	%
Alfalfa, dehy.	38.00
Corn	29.50
Wheat	10.00
Oats	8.00
Soybean meal	6.00
Molasses (wet)	7.00
Dicalcium phosphate	.5
Limestone	.5
Trace mineral salt	.5

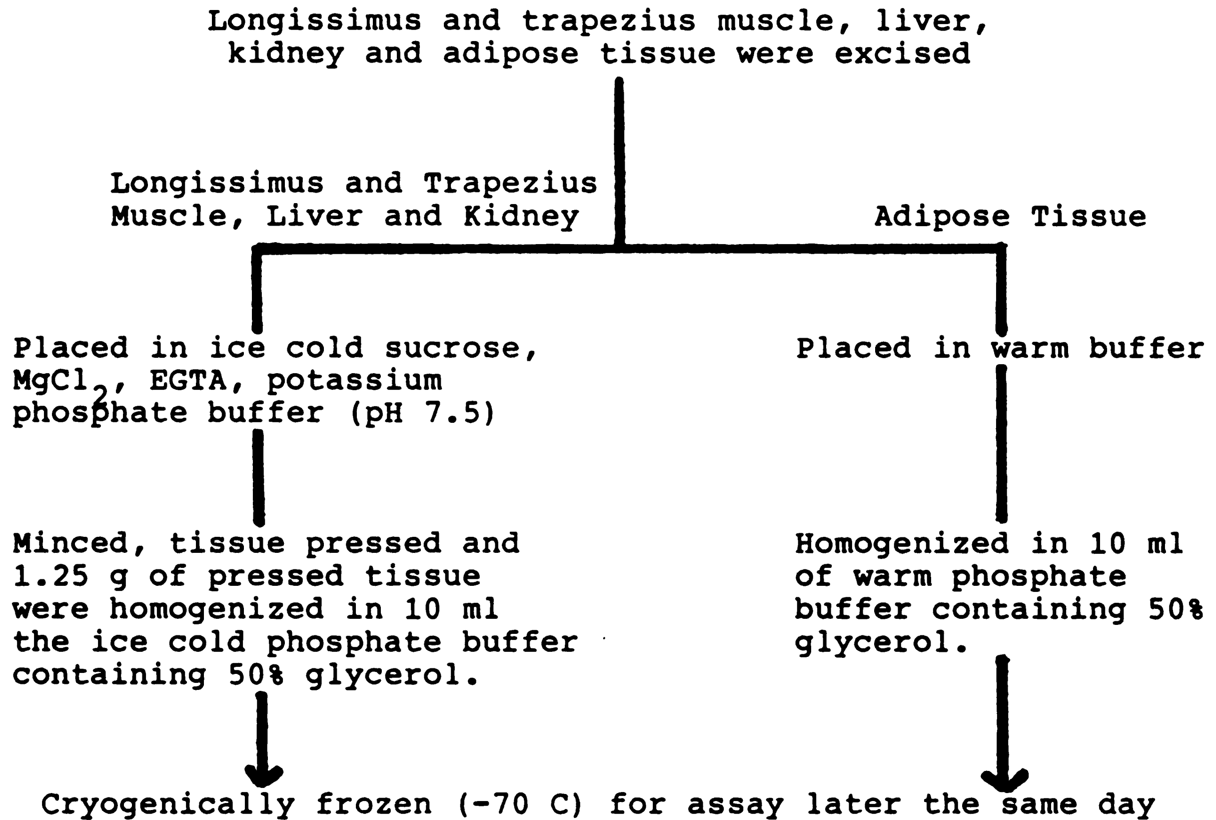


Figure III-1. Flow diagram of tissue preparation.

Enzyme Assays

L-[1-^{14}C]-leucine was used as substrate to measure LAT and KICDH activities as described in Chapter I. The same methodology was used to assay valine aminotransferase (VAT) and alpha-ketoisovalerate dehydrogenase (KIVDH) activities, except that L-[1-^{14}C]-valine was supplied as substrate instead of labeled leucine. The reaction conditions are outlined in figure III-2.

Homogenate protein was determined by the method of Lowry et al. (1951) and the data were analyzed by one-way analysis of variance to determine differences due to fasting length. Data were also analyzed by analysis of variance to determine differences between LAT and VAT activities, and between KICDH and KIVDH activities within dietary treatment groups.

Results and Discussion

Weights, Dressing Percentage, Tissue Moisture and Tissue Ether Extractable Lipid

Live weight at slaughter and hot carcass weight were not affected by length of fasting but as would be expected lambs fasted 84 h had a higher dressing percentage than the lambs fasted for 12 h (table III-3). Fasting length did not alter tissue weights or the percentage moisture of these

Reaction medium: 2.5 ml containing .2 mM-thiamin pyrophosphate 2.5 mM-$MgCl_2$, .5 mM-NAD^+, 2 mM-alpha-ketogluterate, 1 mM-dithiothreitol, 50 mM-sucrose, .2 mM-EGTA, 2 mM-L-leucine or L-valine, and 15 mM-potassium phosphate buffer (pH 7.5).

L-[1-^{14}C]-leucine specific activity: 40 mCi/mol

L-[1-^{14}C]-valine specific activity: 40 mCi/mol

Temperature: 37 C

Preincubation period: 15 min

Reaction period: 20 min

Figure III-2. Enzyme assay conditions.

TABLE III-3. MEANS AND SIGNIFICANCE PROBABILITIES OF THE F STATISTIC FOR LIVE AND CARCASS TRAITS OF FASTED RAM LAMBS

Item	Length of fast, h		
	12	84	(P⁻)
Weights, kg			
Live body	37.80	35.23	.358
Hot carcass	19.13	19.87	.631
Longissimus muscle	.94	.98	.640
Trapezius muscle	.08	.11	.328
Liver	.78	.73	.373
Kidneys	.14	.14	.833
Subcutaneous adipose[a]	2.59	2.81	.601
Dressing percentage	50.60	56.40	.016
Tissue moisture, %			
Longissimus muscle	75.40	74.80	.425
Trapezius muscle	72.30	70.50	.253
Liver	70.30	67.00	.177
Kidney	79.10	78.30	.256
Subcutaneous adipose	30.50	24.50	.123
Perirenal adipose	25.90	16.00	.193
Tissue ether extract, %			
Longissimus muscle	3.00	4.40	.049
Trapezius muscle	8.90	10.80	.325
Liver	7.10	11.50	.133
Kidney	4.50	4.90	.392
Subcutaneous adipose	64.10	71.00	.088
Perirenal adipose	71.40	81.20	.176

[a]The regression equation used to calculate subcutaneous adipose tissue weight is shown in Appendix II-B.

tissues (P>.05). That liver weight was not decreased after 84 h of fasting is somewhat surprising since in an earlier study with fasted lambs (Chapter II), liver weights were decreased by 25% after only 48 h of fasting. It is possible that in the present study, liver weights of the lambs before the 84 h fast may have been greater than those of the lambs fasted for 12 h, thus masking any effects on liver weight. The hot carcass weight of the lambs fasted for 84 h did not differ significantly from, but tended to be slightly greater than that of the lambs fasted for 12 h, which lends support to the previous suggestion. In addition, the percentage of ether extractable lipid in the liver was increased (p~.033) as fasting length increased from 12 to 84 h. This was in agreement with the results reported in Chapter II and also as expected, since during the first few days of a fast proteins are rapidly mobilized from liver, thus increasing lipid concentration (Goldberg and Odessey, 1972).

LAT and VAT Activities

The effect of fasting on LAT and VAT activities are shown in table III-4. Neither enzyme activity was altered (P>.05) by length of fasting in any of the tissues studied when data were expressed on a per milligram of homogenate protein or on a total tissue basis. When data were expressed on a per gram of tissue basis only enzyme

TABLE III-4. MEANS AND SIGNIFICANCE PROBABILITIES OF THE F STATISTIC FOR AMINOTRANSFERASE ACTIVITIES IN SEVERAL TISSUES EXCISED FROM FASTED RAM LAMBS.

	Labeled substrate					
	L-[1-^{14}C]-leucine			L-[1-^{14}C]-valine		
	Length of fast, h			Length of fast, h		
Tissue	12	84	(p⁻)	12	84	(p⁻)
	---pmol degraded x mg homogenate protein^{-1}x min^{-1}---					
Longissimus muscle	31	24	.413	10	11	.794
Trapezius muscle	71	61	.634	30	28	.853
Liver	54	25	.078	20	8	.134
Kidney	382	261	.079	191	134	.123
Subcutaneous adipose	1223	1492	.717	649	766	.753
	--------nmol degraded x g tissue^{-1}x min^{-1}--------					
Longissimus muscle	3.5	2.4	.270	1.1	1.1	1.000
Trapezius muscle	4.9	3.1	.086	2.1	1.5	.199
Liver	10.6	4.5	.072	4.0	1.4	.111
Kidney	53.9	35.4	.024	27.0	18.1	.050
Subcutaneous adipose	36.8	52.5	.596	19.7	27.3	.623
	--------nmol degraded x tissue^{-1}x min^{-1}[a]--------					
Skeletal muscle	41013	27811	.095	5080	13085	.394
Liver	8495	3301	.105	3270	1011	.138
Kidney	7767	4938	.116	3920	2526	.165
Subcutaneous adipose	86094	154110	.395	45457	80483	.399
Total[b]	143368	190161	.543	67728	97105	.479
	-------------Contribution of tissue, %[a]-------------					
Skeletal muscle	30.1	22.7	.623	23.8	20.5	.783
Liver	6.6	2.7	.268	6.0	2.0	.307
Kidney	5.9	3.7	.399	6.8	4.0	.423
Subcutaneous adipose	57.4	70.9	.508	63.5	73.5	.578

[a]Equations used to calculate activity per tissue and the percentage contribution of each tissue are shown in Appendix II-B.

[b]Total represents the sum of activities in skeletal muscle, liver, kidney and subcutaneous adipose tissue.

activities in kidney homogenates were altered ($P<.05$) by fasting. These results and the results presented in Chapter II are in agreement with the reports of Heitmann and Bergman (1980) and Lindsay (1980), who suggested that enzymes responsible for BCAA degradation in ruminant muscle are less responsive to fasting than those in rat tissues. In rat muscle, BCAA degradation is closely associated with the release of alanine and glutamine, which are important gluconeogenic precursors (Goldberg and Chang, 1978; Snell, 1980). Therefore in physiological states such as fasting, when gluconeogenesis is rapidly occurring, BCAA are being degraded rapidly by rat skeletal muscle. However in the ruminant, gluconeogenesis is important in the fed as well as the fasted state, so less dramatic changes in skeletal muscle BCAA degradation would be expected. Liver and kidney aminotransferase activities have been relatively unresponsive to fasting in studies with sheep (Chapter II; Bergman and Pell, 1983) or rats (Wohlhueter and Harper, 1970; Adibi et al., 1975). The slight nonsignificant increase in adipose tissue aminotransferase activity as fasting length increased from 12 to 84 h was not expected because lambs fasted for 96 h had lower adipose tissue LAT activity than unfasted controls (Chapter II). In addition, Tischler and Goldberg (1980a) reported that 2 d of food deprivation in rats caused a 47% decrease in adipose tissue

LAT activity. Therefore it seems likely that because of the length of time required to obtain a postabsorptive state, in ruminants, longer than an 84 h fast is required to cause the decrease in adipose tissue aminotransferase activity that has been observed in sheep and rats.

The percentage of aminotransferase activities in all four tissues accounted for by the individual tissues was not significantly altered by length of feed deprivation (table III-4). Adipose tissue was the most important site of LAT and VAT activity in both 12 and 84 h fasted ram lambs. Skeletal muscle also was an important location for leucine and valine aminotransferase activity, while kidney and liver provided only minor contributions to total activity. This is certainly in agreement with the results reported in Chapters I and II but the physiological significance of high aminotransferase activity in sheep adipose tissue is unclear and requires further study.

Means of the LAT activity compared to VAT activity in lambs fasted 12 or 84 h are presented in table III-5. For lambs fasted 12 or 84 h whether activity was expressed on a per milligram of protein, per gram of tissue or total tissue basis, leucine was more rapidly transaminated than valine by homogenates of all of the tissues studied, although the difference between leucine transamination and valine transamination was not significant in every case. In

TABLE III-5. MEANS AND SIGNIFICANCE PROBABILITIES OF THE F STATISTIC FOR LEUCINE AND VALINE AMINOTRANSFERASE ACTIVITIES IN SEVERAL TISSUES EXCISED FROM FASTED RAM LAMBS

	Length of fast, h					
	12			84		
	Labeled substrate[a]			Labeled substrate[a]		
Tissue	Leucine	Valine	(p^-)	Leucine	Valine	(p^-)
	---pmol degraded x mg homogenate protein^{-1}x min^{-1}---					
Longissimus muscle	31	10	.022	24	11	.081
Trapezius muscle	71	30	.057	61	28	.103
Liver	54	20	.071	25	8	.003
Kidney	382	191	.032	261	134	.001
Subcutaneous adipose	1223	649	.282	1492	766	.306
	--------nmol degraded x g tissue^{-1} x min^{-1}--------					
Longissimus muscle	3.5	1.1	.036	2.4	1.1	.081
Trapezius muscle	4.9	2.1	.029	3.1	1.5	.006
Liver	10.6	4.0	.077	4.5	1.4	.002
Kidney	53.9	27.0	.011	35.4	18.1	.001
Subcutaneous adipose	36.8	19.7	.404	52.5	27.3	.364
	--------nmol degraded x tissue^{-1}x min^{-1}[b]--------					
Skeletal muscle	41013	15080	.010	27811	13085	.009
Liver	8495	3270	.131	3301	1011	.001
Kidney	7767	3920	.071	4938	2526	.006
Subcutaneous adipose	86094	45457	.257	154110	80483	.379
Total[c]	143368	67728	.050	190161	97105	.283
	-------------Contribution of tissue, %[b]-------------					
Skeletal muscle	30.1	23.8	.477	22.7	20.5	.896
Liver	6.6	6.0	.985	2.7	2.0	.737
Kidney	5.9	6.8	.795	3.7	4.0	.915
Subcutaneous adipose	57.4	63.5	.708	70.9	73.5	.903

[a] L-[1-^{14}C]-leucine or L-[1-^{14}C]-valine were supplied as substrate.

[b] Equations used to calculate activity per tissue and percentage contribution of each tissue are shown in Appendix II-B.

[c] Total represents the sum of activities in skeletal muscle, liver, kidney and subcutaneous adipose tissue.

addition, the relative changes in LAT and VAT activities caused by length of fasting were similar. Furthermore, the relative contributions of the individual tissues to total aminotransferase activities of all four tissues were similar whether leucine or valine was supplied as the substrate. The parallel fluctuations of leucine and valine transamination indicate that a single enzyme could be primarily responsible for the degradation of both leucine and valine, and if that is the case, this enzyme would appear to have a higher K_m for valine than for leucine. In the rat a similar situation exists, as isozyme I, described by Ichihara (1975), readily transaminates all three BCAA with K_m values of 4.3, .8 and .8 mM for valine, leucine and isoleucine, respectively. Isozyme I (mitochondrial or cytosolic type) is the predominant isozyme in most rat tissues, but isozyme II, which is specific for leucine (K_m of 25 mM) is also prevalent in liver (Ichihara, 1975; Kadowaki and Knox, 1982). If an isozyme similar to isozyme II is prevalent in sheep liver, it was apparently affected by food deprivation similarly to isozyme I.

KICDH and KIVDH Activities

The means of KICDH and KIVDH activities in crude homogenates of several tissues excised from ram lambs fasted for 12 or 84 h are presented in table III-6. Only in liver

TABLE III-6. MEANS AND SIGNIFICANCE PROBABILITIES OF THE F STATISTIC FOR DEHYDROGENASE ACTIVITIES IN SEVERAL TISSUES EXCISED FROM FASTED RAM LAMBS

	Labeled substrate					
	L-[1-^{14}C]-leucine			L-[1-^{14}C]-valine		
	Length of fast, h			Length of fast, h		
Tissue	12	84	(P⁻)	12	84	(P⁻)
	---pmol degraded x mg homogenate protein^{-1}x min^{-1}---					
Longissimus muscle	1.0	2.7	.346	.4	1.3	.334
Trapezius muscle	2.7	1.9	.670	.9	1.0	.972
Liver	31.9	8.8	.008	14.7	4.1	.027
Kidney	171.3	108.7	.166	104.8	65.6	.139
Subcutaneous adipose	29.7	31.5	.821	42.8	32.9	.223
	--------nmol degraded x g tissue^{-1}x min^{-1}--------					
Longissimus muscle	.12	.27	.398	.05	.13	.394
Trapezius muscle	.19	.11	.506	.07	.04	.551
Liver	6.27	1.61	.009	2.88	.75	.025
Kidney	24.91	14.91	.118	14.79	9.03	.102
Subcutaneous adipose	.87	1.05	.654	1.21	1.12	.855
	--------nmol degraded x tissue^{-1}x min^{-1}[a]--------					
Skeletal muscle	1549	1862	.710	580	866	.512
Liver	4971	1171	.020	2305	538	.045
Kidney	3533	2101	.220	2162	1275	.203
Subcutaneous adipose	2202	3038	.513	3032	3280	.865
Total[b]	12256	8171	.063	8079	5959	.282
	-------------Contribution of tissue, %[a]-------------					
Skeletal muscle	12.9	23.8	.355	7.0	17.0	.224
Liver	40.2	14.9	.012	27.8	10.9	.056
Kidney	27.9	25.6	.683	26.0	21.7	.243
Subcutaneous adipose	19.0	35.8	.206	39.2	50.5	.414

[a]Equations used to calculate activity per tissue and percentage contribution of each tissue are shown in Appendix II-B.

[b]Total represents the sum of activities in skeletal muscle, liver, kidney and subcutaneous adipose tissue.

homogenates was LAT and (or) VAT activity altered ($P<.05$) by length of food deprivation whether expressed on a per milligram of protein, per gram of tissue or total tissue basis. Liver LAT and VAT activities expressed on a protein basis were both decreased by 72% after the 84 h fast compared to the 12 h fast. Previously it was shown that lambs fasted for 96 h had 66% lower liver KICDH activity than unfasted lambs while muscle KICDH activity was unresponsive to 96 h of food deprivation (Chapter II). The nonsignificant decrease in adipose tissue KIVDH activity as length of fasting increased from 12 to 84 h was expected, but the slight increase in adipose tissue KICDH activity was surprising (table III-6). The adipose tissue KICDH activity reported in Chapter II decreased by 91% after a 96 h fast. In addition, it is generally accepted that adipose tissue KIC degradative activity of rats is decreased by fasting (Goldberg and Tischler, 1981). Thus, the slight increase (nonsignificant) in adipose tissue KICDH activity due to 84 h of fasting in the present study may be artifactual, and it is possible that more than 84 h of fasting is required to obtain a decrease in adipose tissue KICDH activity. That adipose tissue KICDH activity was unaffected by 48 h of fasting (Chapter II) adds some validity to the latter suggestion.

In the lambs fasted for 12 h, liver was the primary site of KICDH activity but after 84 h of fasting adipose tissue appeared to be the most important tissue. These results are certainly in conflict with the results reported in Chapter II since in that study the importance of adipose tissue as a site of KICDH activity diminished as length of fasting was increased to 96 h, but this discrepancy is not totally surprising in light of the discussion contained in the preceding paragraph.

KICDH and KIVDH activities were compared by analysis of variance and these means and approximate probability levels (F values) are shown in table III-7. Although the differences were not always significant, KICDH activity expressed per milligram of protein, per gram of tissue or per tissue was greater than KIVDH activity in crude homogenates of skeletal muscle, liver and kidney of fed or fasted lambs. Furthermore, the relative changes in KICDH and KIVDH activities in skeletal muscle, liver and kidney attributable to length of food deprivation were similar. On the other hand, adipose tissue KIVDH activity was higher (nonsignificantly) than KICDH activity. The parallel fluctuation in muscle, liver and kidney KICDH and KIVDH activities suggests that a single enzyme complex could be responsible for the decarboxylation of KIC and KIV. Most research workers agree that a single BCKA dehydrogenase

TABLE III-7. MEANS AND SIGNIFICANCE PROBABILITIES OF THE F STATISTIC FOR DEHYDROGENASE ACTIVITIES IN SEVERAL TISSUES EXCISED FROM FASTED RAM LAMBS

	Length of fast, h					
	12			84		
	Labeled substrate[a]			Labeled substrate[a]		
Tissue	Leucine	Valine	(p⁻)	Leucine	Valine	(p⁻)
	---pmol degraded x mg homogenate protein^{-1}x min^{-1}---					
Longissimus muscle	1.0	.4	.207	2.7	1.3	.460
Trapezius muscle	2.7	.9	.060	1.9	1.0	.671
Liver	31.9	14.7	.034	8.8	4.1	.031
Kidney	171.3	104.8	.178	108.7	65.6	.027
Subcutaneous adipose	29.7	42.8	.140	31.5	32.9	.867
	--------nmol degraded x g tissue^{-1}x mina^{-1}--------					
Longissimus muscle	.12	.05	.244	.27	.13	.449
Trapezius muscle	.19	.07	.088	.11	.04	.561
Liver	6.27	2.88	.040	1.61	.75	.024
Kidney	24.91	14.79	.122	14.91	9.03	.079
Subcutaneous adipose	.87	1.21	.356	1.05	1.12	.886
	--------nmol degraded x tissue^{-1}x min^{-1}[b]--------					
Skeletal muscle	1549	580	.012	1862	866	.305
Liver	4971	2305	.086	1171	538	.011
Kidney	3533	2162	.260	2101	1275	.150
Subcutaneous adipose	2202	3032	.370	3038	3280	.887
Total[c]	12256	8079	.066	8171	5959	.250
	-------------Contribution of tissue, %[b]-------------					
Skeletal muscle	12.9	7.0	.360	23.8	17.0	.612
Liver	40.2	27.8	.136	14.9	10.9	.505
Kidney	27.9	26.0	.730	25.6	21.7	.324
Subcutaneous adipose	19.0	39.2	.113	35.8	50.5	.334

[a] L-[1-^{14}C]-leucine or L-[1-^{14}C]-valine were supplied as substrate.

[b] Equations used to calculate activity per tissue and percentage contribution of each tissue are shown in Appendix II.

[c] Total represents the sum of activities in skeletal muscle, liver, kidney and subcutaneous adipose tissue.

complex is responsible for the degradation of all three BCKA, and tissue isozymes have not been observed (Danner et al., 1981; Randle et al., 1981). The results with adipose tissue homogenates are interesting and the data suggest that a ruminant adipose tissue isozyme of BCKA dehydrogenase may exist. That adipose tissue KIVDH activity was greater than KICDH activity can be made compatible with the theory of a single BCKA dehydrogenase complex; however, if we consider the results of Randle et al.(1981). They reported that the K_m and V_{max} for KIC were 8.7μM and 1.83 nmol/min, respectively, and the K_m and V_{max} for KIV were 15.6μM and 2.07 nmol/min, respectively. Furthermore, the aminotransferase activity in adipose tissue was high, BCKA concentrations may have approached saturation levels. Therefore, since the BCKA dehydrogenase complex apparently has a higher capacity for KIV than KIC, KIV would be degraded more rapidly. In other tissues with lower aminotransferase activities, the concentration of KIC and KIV could have been low enough that the ketoacid for which the dehydrogenase had the greater affinity (KIC) would have been degraded most rapidly. In any case these results suggest a single enzyme complex may be involved in the decarboxylation of KIC and KIV at least in skeletal muscle, liver and kidney. However, all of these results for dehydrogenase activities must be viewed with caution because

only the BCAA were supplied in the reaction medium and thus actual KIC and KIV concentrations are unknown.

In longissimus and trapezius muscle and adipose tissue, LAT activities expressed per milligram of protein ranged from 9- to 47-fold greater than the corresponding KICDH activities, and VAT activities ranged from 8- to 33-fold greater than the corresponding KIVDH activities (tables II-4 and 6) expressed per milligram of protein. In comparison, liver and kidney LAT activities ranged from 1.7- to 2.8-fold greater than the corresponding KICDH activities and VAT activities ranged from 1.4- to 2.0-fold greater than the corresponding KIVDH activities. These results are in close agreement with the results presented in Chapters I and II, and indicate that BCKA dehydrogenase is probably rate limiting in ovine skeletal muscle and adipose tissue while BCAA aminotransferase may be rate limiting in sheep liver and kidney. The study presented in Chapter IV in which labeled KIC was supplied as substrate verified the suggestion that the dehydrogenase enzyme is indeed rate limiting in ovine muscle and fat, and that the aminotransferase enzyme is rate limiting in sheep liver. However, in the study presented in Chapter IV the KICDH activity of kidney was slightly lower than LAT activity.

Summary and Conclusions

Three crossbred ram lambs were fasted for 12 h and three lambs were fasted for 84 h prior to slaughter to study the effects of fasting on the relative rates of leucine and valine degradation. L-[1-^{14}C]-leucine was utilized as substrate to measure LAT and KICDH activities, and L-[1-^{14}C]-valine was supplied as substrate to measure VAT and KIVDH activities. When fasting length was increased from 12 to 84 h, LAT, VAT, KICDH and KIVDH activities in skeletal muscle and adipose tissue were not significantly altered, but increasing the length of feed deprivation caused a decrease ($P<.05$) in ovine liver KICDH and KIVDH activities.

For lambs fasted 12 or 84 h, leucine was more rapidly transaminated than valine by homogenates of all of the tissues studied. The relative changes in LAT and VAT activities caused by increased fasting length were similar. The contributions of individual tissues to the sum of aminotransferase activities in all four tissues were similar whether leucine or valine was supplied as substrate and adipose tissue was the predominant site of LAT and VAT activities. Thus, it is possible that a single enzyme similar to isozyme I described by Ichihara (1975) is primarily responsible for transaminating both leucine and valine. Although dehydrogenase activities must be viewed

with caution because only the BCAA were supplied in the reaction medium and thus actual KIC and KIV concentrations are unknown, the results indicate a single enzyme complex may be involved in the decarboxylation of KIC and KIV in skeletal muscle, liver and kidney. LAT and VAT appeared to be rate limiting in liver and KICDH and KIVDH activities were apparently rate limiting in skeletal muscle and adipose tissue.

CHAPTER IV

COMPARISON OF THE TISSUE AND SUBCELLULAR DISTRIBUTION OF LEUCINE AMINOTRANSFERASE AND ALPHA-KETOISOCAPROATE DEHYDROGENASE ACTIVITY IN GROWING RATS, SWINE, CATTLE AND SHEEP

Introduction

The first two enzymes involved in the degradation of BCAA, BCAA aminotransferase and BCKA dehydrogenase, have been extensively studied in rats (Dancis and Levitz, 1978). Ichihara (1975) described three isozymes of BCAA aminotransferase (isozymes I, II and III). Isozymes I (found in most tissues) and III (found primarily in brain, ovary and placental tissues) actively transaminate all three BCAA, but isozyme II (found only in liver) is specific for transaminating leucine. While isozymes I, II and III are primarily localized in the cytosolic fraction, mitochondrial enzymes similar to isozymes I (Kadowaki and Knox, 1982) and II (Ikeda et al., 1976; Ichihara et al., 1981) have also been isolated. In fact Kadowaki and Knox (1982) reported that 66 and 74% of BCAA aminotransferase activity in liver and kidney, respectively, was located in the mitochondrial fraction. Most evidence indicates that a single BCKA dehydrogenase complex, which is bound to the inner surface of the inner mitochondrial membrane, is responsible for the

degradation of all three BCKA (Hinsbergh et al., 1978; Danner et al., 1979; Randle et al., 1981). In addition, there is apparently an oxidase in the soluble fraction of liver that decarboxylates KIC (Johnson and Connelly, 1972; Sabourin and Bieber, 1981). There has been little work relating to the subcellular distribution of BCAA aminotransferase and BCKA dehydrogenase activities in tissues from species other than the rat, although Johnson and Connelly (1972) reported that 87 to 89% of BCKA dehydrogenase activity in bovine liver was isolated from the mitochondrial fraction.

Distribution of BCAA aminotransferase and BCKA dehydrogenase is not uniform among tissues (Harper and Zapalowski, 1981). In rats, the BCAA are unique among essential amino acids, in that they are primarily catabolized by extrahepatic tissues (Miller, 1962; Odessey and Goldberg, 1972). On a total tissue basis skeletal muscle because of its bulk is probably the major site of BCAA aminotransferase activity (Harper and Zapalowski, 1981), but kidney (Ichihara et al., 1981) and adipose tissue (Goodman, 1963a,b; Leveille, 1966; Rosenthal et al., 1974; Tischler and Goldberg, 1980a) also actively degrade BCAA. On the other hand, while rat liver is low in BCAA aminotransferase, research workers generally agree liver is the primary site of BCKA dehydrogenase activity.

Rosenthal et al.(1974) suggested that adipose tissue is a major site of leucine degradation in the rat, second only to skeletal muscle. Goldberg and Tischler (1981) postulated that in older, more obese rats or in species that contain a high percentage of adipose tissue, such as humans or domesticated animals, adipose tissue may play an even more important role in BCAA degradation.

In ruminants, skeletal muscle appears to have a more limited capability to metabolize BCAA (Teleni et al., 1983) than in rats, while liver (Heitmann and Bergman, 1980) and adipose tissue (Chapters I and II) are relatively more important sites of BCAA metabolism in sheep than in rats.

Therefore, this study was conducted to compare the subcellular and tissue distribution of BCAA and BCKA degradative activities among selected species. Thus, LAT and KICDH activities were measured in crude homogenates, the mitochondrial and cytosolic fractions of skeletal muscle, liver, kidney and adipose tissue samples excised from growing rats, pigs, beef cattle and sheep.

Experimental Procedure

Materials

The sodium salts of alpha-ketoglutarate, CoA and thiamine pyrophosphate, dithiothreitol, NAD^+, EGTA, EDTA, catalase, L-amino acid oxidase (type IV), L-leucine and alpha-ketoisocaproic acid were obtained from Sigma Chemical Co. (St. Louis, MO). L-[1-^{14}C]-leucine used to synthesize [1-^{14}C]-alpha-ketoisocaproate was purchased from Research Products International Corp., (Mt. Prospect, IL). The L-[1-^{14}C]-leucine used directly in enzyme assays and the Aqueous Counting Scintillant were procured from Amersham Corp., (Arlington Heights, IL).

Experimental Design

Three 275 g male rats, three 106 kg male pigs, three 480 kg castrated male beef cattle and four 40 kg male sheep were fasted overnight before sacrifice (table IV-1). Rats were killed by decapitation, and boars, steers and rams were stunned and exsanguinated.

Tissue Preparation

Homogenates and cell fractions were prepared by a modification of the procedures of Ernster and Nordenbrandt

TABLE IV-1. EXPERIMENTAL DESIGN

Item	Species			
	Male rats	Boars	Steers	Rams
Number of animals	3	3	3	4
Live weight, average	275 g	106 kg	480 kg	40 kg

(1967), Johnson and Lardy (1967), Nelson and Butow (1967) and Odessey and Goldberg (1979). After sacrifice semimembranosus muscle, liver and kidney samples were excised and placed in ice cold buffer A containing 50 mM Tris/HCl (pH 7.5), .25 M sucrose, 50 mM KCl, 5 mM $MgCl_2$ and 5 mM EGTA (see flow diagram, figure IV-1). Tissues were then minced with scissors, passed through a tissue press and 2 g of the tissue preparation were suspended in 10 ml of buffer A. Suspensions were homogenized in Potter-Elvehjem ground glass homogenizing tubes with five passes of a motor driven Teflon pestle. Homogenates were transferred to a tight fitting Ten-Broeck all glass homogenizer and further homogenized with two strokes of the pestle. Homogenates were then transferred to 50 ml, plastic centrifuge tubes, and the homogenizing tubes and pestles were rinsed with enough of buffer A to bring the final homogenate volume up to 20 ml. Inguinal and epididymal fat pads from rats, and inguinal fat samples from boars, steers and rams were handled identically to the muscle, liver and kidney samples except they were placed and homogenized in buffer at room temperature. Sample weights and buffer volumes for adipose tissues were doubled to obtain sufficient mitochondria to assay. A 5 ml aliquot of each homogenate was then removed and assayed immediately to determine crude homogenate enzyme activities.

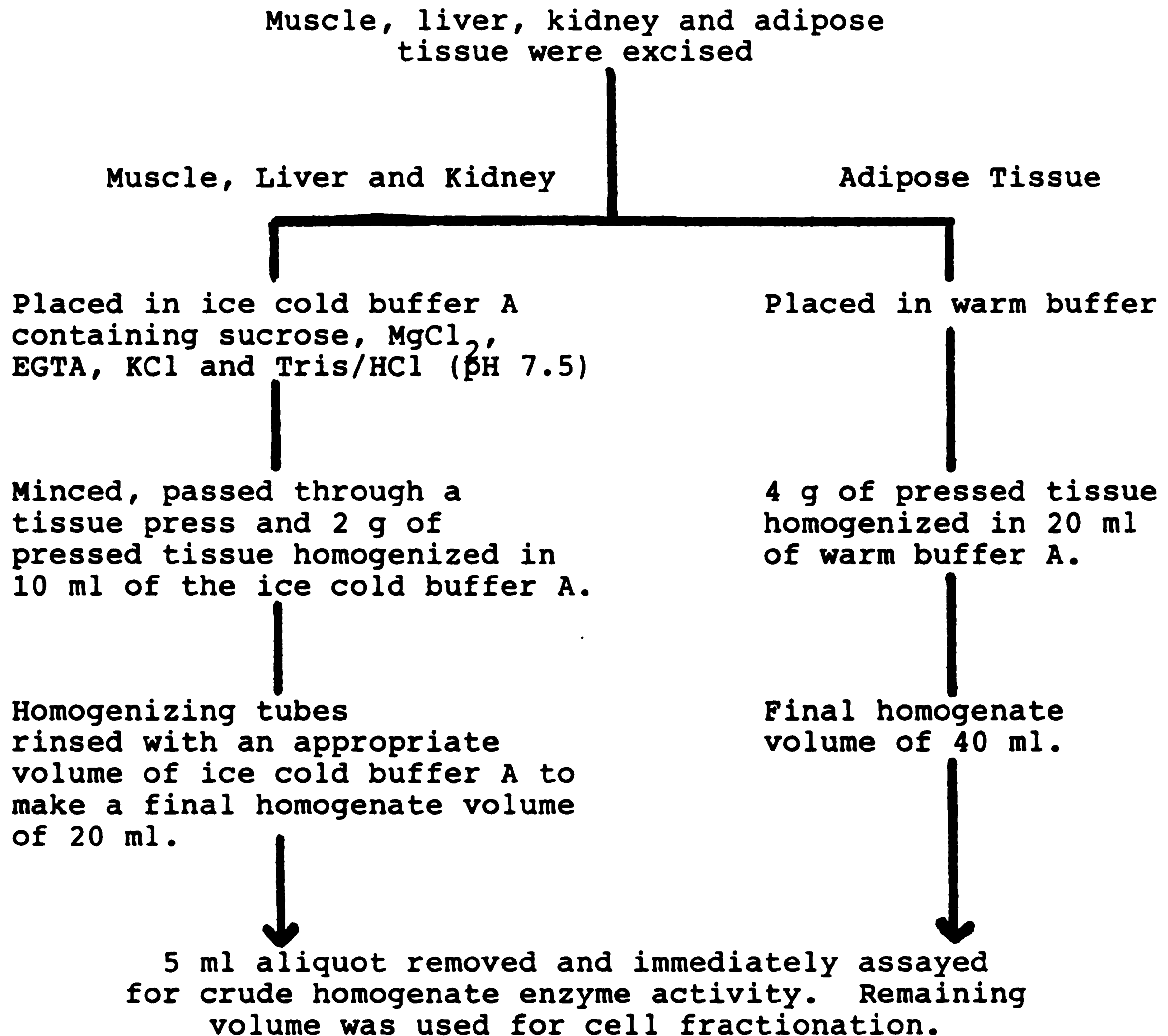


Figure IV-1. Flow diagram of tissue preparation.

Cell Fractionation

The remaining volume of crude homogenate (15 ml of muscle, liver and kidney and 35 ml of adipose tissue homogenate) was centrifuged at 600 x g for 5 min at 2 C to remove unbroken cells, separable lipid (from fat homogenates) and myofibrils (from muscle homogenates). Figure IV-2 is a flow diagram of the subcellular fractionation procedure. Muscle, liver and kidney 600 x g supernatants were rapidly decanted and the adipose tissue 600 x g infranatants were carefully, but quickly pipetted from beneath the lipid layer. The 600 x g pellets (lipid layer) were discarded (except where noted in results and discussion section) and the supernatants (infranatants) were centrifuged at 11,000 x g for 10 min at 2 C to obtain the mitochondrial fraction. The 11,000 x g supernatants from all tissue homogenates were assayed immediately (except where noted in results and discussion section). Muscle liver, kidney and adipose tissue 11,000 x g pellets were washed once in buffer B containing .25 M sucrose, 50 mM KCl, 5 mM $MgCl_2$, .1 mM EDTA and 50 mM Tris/HCl (pH 7.5) and recentrifuged at 11,000 x g for 10 min at 2 C. The washed 11,000 x g pellets of liver and kidney, and those of muscle and adipose tissue were resuspended in 10 and 5 ml, respectively, of buffer B, and assayed immediately. The

15 ml of muscle, liver and kidney homogenates
and 35 ml of adipose tissue homogenates were centrifuged
at 600 x g for 5 min at 2 C

600 x g supernatants (infranatants) were decanted
(removed by pipette) and centrifuged at 11,000 x g for
10 min to precipitate mitochondria

Liver and kidney 11,000 x g
pellets (mitochondrial
fractions) was washed once
in buffer B containing
sucrose, KCl, $MgCl_2$, EDTA
and Tris/HCl (pH 7.5). The
pellets were then resuspended
10 ml of buffer B and assayed
immediately.

Muscle and adipose tissue
11,000 x g pellets were washed
once in buffer B, and then
resuspended in 5 ml of buffer B
and assayed immediately

11,000 x g supernatants
(cytosolic fractions) from all
tissue homogenates were assayed
immediately

Figure IV-2. Flow diagram of cell fractionation procedure.

washed pellets and the supernatants of each tissue following centrifugation at the 11,000 x g will be referred to hereafter as the mitochondrial and cytosolic fractions, respectively.

Preparation of [1-^{14}C]-Alpha-Ketoisocaproate

[1-^{14}C]-alpha-ketoisocaproate was enzymatically prepared from L-[1-^{14}C]-leucine by the method of Rudiger et al.(1972) except that the catalase and L-amino acid oxidase enzymes were obtained from Sigma Chemical Co. L-amino acid oxidase was used to deaminate the labeled leucine and the resulting KIC was separated amino acid by ion exchange chromatography.

Enzyme Assays

In previous chapters, the rates of transamination and decarboxylation of L-[1-^{14}C]-leucine were referred to as LAT and KICDH activities, but in this chapter these activities will be referred to as LAT and leucine decarboxylation (LDC) activities, respectively. The rate of [1-^{14}C]-alpha-ketoisocaproate decarboxylation will be referred to as KICDH. LAT and LDC activities were determined as described previously (Chapter I) with minor modifications. The modifications were: 1) fresh crude homogenates, mitochondrial pellets and cytosolic fractions

were assayed instead of frozen crude homogenates; and 2) the contents of the reaction media were altered (see figure IV-3). The final reaction volume of 2.5 ml used in the assay of the crude homogenates and cytosolic fractions contained .2 mM thiamine pyrophosphate, .5 mM NAD^+, 5 mM alpha-ketoglutarate, 1 mM dithiothreitol, 50 mM sucrose, .1 mM CoA, 3 mM $MgCl_2$, 10 mM KCl, 1 mM EGTA, 2 mM L-leucine and 50 mM Tris/HCl buffer (pH 7.5). The reaction medium in which the mitochondrial fractions were assayed was identical to that used for the crude homogenates and cytosolic fractions except that .02 mM EDTA was included instead of 1 mM EGTA. KICDH activity was assayed identically to LDC activity except .5 mM [1-^{14}C]-alpha-ketoisocaproate (specific activity; 40 mCi/mol) was added at the start of the 20 min reaction period instead of 2 mM L-[1-^{14}C]-leucine (specific activity; 40 mCi/mol).

Protein content of the tissue preparations was determined by the method of Lowry et al.(1951). The data are expressed on a milligram of protein, per gram of tissue and estimated total tissue basis. The data were analyzed by one-way analysis of variance and significant differences ($P<.05$) among means were separated by Duncan's New Multiple Range Test as outlined by Steel and Torrie (1960).

Leucine aminotransferase and Leucine decarboxylation activity assay medium: 2.5 ml containing .5 ml of tissue preparation, .2 mM thiamin pyrophosphate, .5 mM NAD^+, 2 mM alpha-ketoglutarate, 1 mM dithiothreitol, 50 mM sucrose, .1 mM CoA, 3 mM $MgCl_2$, 10 mM KCl, 1 mM EGTA or .02 mM EDTA, 2 mM L-leucine and 50 mM Tris/HCl buffer (pH 7.5).

Alpha-ketoisocaproate dehydrogenase assay medium: Same as above except .5 mM alpha-ketoisocaproate was included instead of 2 mM-L-leucine

L-[1-^{14}C]-leucine specific activity: 40 mCi/mol

[1-^{14}C]-alpha-ketoisocaproate specific activity: 40 mCi/mol

Reaction temperature: 37 C

Preincubation period: 15 min

Reaction period: 20 min

Figure IV-3. Enzyme assay conditions.

Results and Discussion

Preliminary Experiment

LAT and LDC activities were measured in crude homogenates and in several subcellular fractions of skeletal muscle, liver, kidney and adipose tissue of two sheep and one rat. Six hundred x g pellets, 11,000 x g pellets (mitochondrial fractions), 150,000 x g pellets (microsomal fractions) and 150,000 x g supernatants (cell sap) were prepared from crude homogenates (see flow diagram, figure IV-4). A substantial portion of sheep skeletal muscle LAT activity was isolated with the 600 x g pellet (table IV-2). This activity in the 600 x g pellet may result from the entrapment of mitochondria within the network of myofibrils as they were precipitated, or within cells which were not completely disrupted during homogenization. The observation that a large portion of LAT activity was associated with the sheep muscle 600 x g pellets and not with rat muscle was not surprising because sheep skeletal muscle tissue was much more difficult to disrupt by homogenization than was rat muscle. When the fractions prepared from the 600 x g supernatants (mitochondrial, microsomal and cell sap fractions) from both the sheep and the rat are compared, 52 to 66% of the muscle and adipose tissue activity remained in the cell sap. Thirty to 40% of the LAT activity was

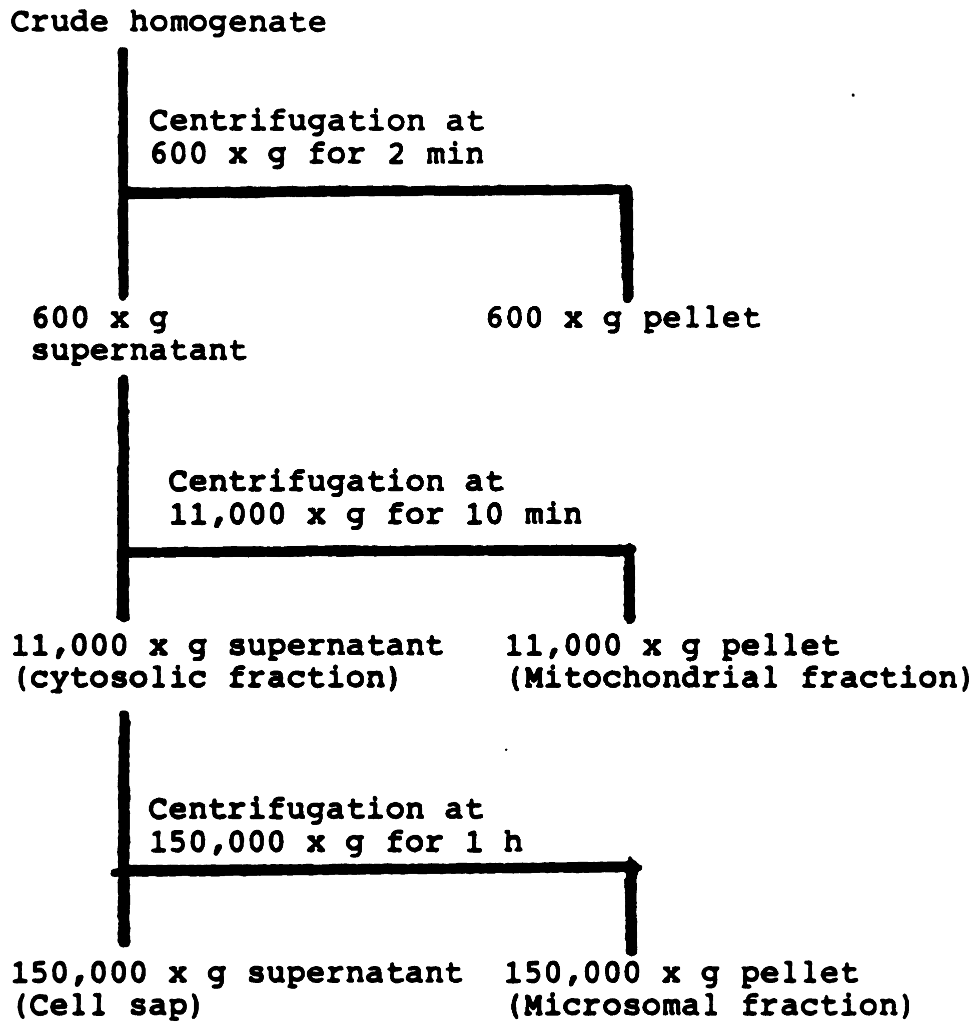


Figure IV-4. Flow diagram of cell fractionation procedure used for the preliminary experiment.

TABLE IV-2. SUBCELLULAR DISTRIBUTION OF LEUCINE AMINOTRANSFERASE AND LEUCINE DECARBOXYLATION ACTIVITIES

Species and fraction	Leucine aminotransferase				Leucine decarboxylation			
	Skeletal muscle	Liver	Kidney	Adipose tissue	Skeletal muscle	Liver	Kidney	Adipose tissue
	nmol leucine degraded x g tissue^{-1} x min^{-1}							
Ovine (n=2)								
Crude homogenate	1.9	12.0	67.0	21.9	.04	5.20	48.91	.97
600 x g pellet	2.7	11.9	14.6	7.4	0	2.90	5.17	.45
Mitochondrial	1.6	14.2	51.9	9.4	.90	4.19	14.02	.70
Microsomal	.3	.2	.4	2.0	.08	.16	.18	.38
Cell sap	2.1	2.3	15.5	16.4	.22	.50	.16	0
Rat (n=1)								
Crude homogenate	55.0	15.2	428.3	67.7	2.97	7.87	144.68	.37
600 x g pellet	1.1	.7	74.9	2.5	.29	.70	21.97	0
Mitochondrial	11.5	6.2	119.0	21.5	.59	4.33	26.72	1.08
Microsomal	2.4	.2	1.3	2.7	.03	.07	.18	0
Cell sap	18.0	1.0	108.8	47.0	.40	0	.53	.89

recovered in the mitochondrial fraction and a negligible amount was precipitated with the microsomal fraction. In rat and sheep liver and kidney, however, 52 to 85% of the 600 x g supernatant LAT activity was precipitated in the mitochondrial pellet and most of the remaining activity remained in the cell sap. Unlike LAT activity, most (55 to 98%) of the LDC activity for all four tissues was found in the mitochondrial fraction.

The cytosolic fraction (11,000 x g supernatant) contains the microsomal proteins as well as the soluble proteins; and undoubtedly the mitochondrial pellet also contains other intracellular membranes. However, little LAT or LDC activities were observed in the microsomal pellet. Thus, most of the enzyme activities in the mitochondrial fraction were probably localized in the mitochondria, and activities, subsequently measured in the cytosolic fraction, were primarily soluble. These results relating to the subcellular distribution of LAT and LDC activities in sheep and rat tissues are in agreement with others who have studied rat skeletal muscle, liver and kidney preparations (Odessey and Goldberg, 1979; Randle et al., 1981; Kadowaki and Knox, 1982). All subsequent enzyme assays were performed on crude homogenates, mitochondrial fractions and cytosolic fractions only.

Subcellular Distribution of LAT and KICDH Activities

Table IV-3 contains means and standard errors for LAT activity in crude homogenates, mitochondrial fractions and cytosolic fractions. Also included in table IV-3 are means and standard errors for the relative contribution of the mitochondrial fraction to the sum of the enzyme activity in the mitochondrial and cytosolic fraction expressed as a percentage. Thus, the relative contribution of the cytosolic fraction would be equal to 100 minus the mitochondrial fraction contribution. Relative differences in LAT activities between species were similar for all four tissues, whether activity was measured in crude homogenates, mitochondrial fractions or cytosolic fractions. The only important exception was for pig liver activity. Pig liver mitochondrial LAT activity was not significantly different from rat liver mitochondrial LAT activity, while pig liver crude homogenate and cytosolic LAT activities were, 3- and 11-fold greater, respectively, than those activities in the rat. These results indicate that the high activity of a soluble aminotransferase was probably responsible for the surprisingly high LAT activity observed in porcine liver.

In all four species more of the skeletal muscle and adipose tissue LAT activity remained in the cytosolic fraction than was found in the mitochondrial fraction, but in rat, bovine and ovine liver and kidney most of the LAT

TABLE IV-3. MEANS AND STANDARD ERRORS OF LEUCINE AMINOTRANSFERASE ACTIVITY IN SEVERAL TISSUES FROM RATS, PIGS, CATTLE AND SHEEP

Fraction and tissue	Species				Standard error
	Male rats	Boars	Steers	Rams	
	---nmol degraded x g tissue^{-1}x min^{-1}---				
Crude homogenate					
Muscle	31.31[b]	5.51[a]	1.53[a]	3.71[a]	4.22
Liver	15.91[a]	54.24[b]	5.11[a]	6.39[a]	4.67
Kidney	254.80[c]	121.18[b]	27.20[a]	41.54[a]	11.37
Adipose	24.73a[b]	7.11[a]	3.39[a]	7.81[a]	4.05
Mitochondrial					
Muscle	12.07[b]	2.02[a]	.62[a]	1.08[a]	1.98
Liver	6.22[ab]	8.62[b]	3.38[a]	3.92[a]	1.35
Kidney	90.20[c]	32.75[b]	12.22[ab]	9.63[a]	6.83
Adipose	5.01[b]	.51[a]	.29[a]	1.08[a]	1.10
Cytosolic					
Muscle	18.29[b]	4.18[a]	2.36[a]	1.31[a]	1.31
Liver	3.67[a]	41.07[b]	1.53[a]	2.66[a]	4.05
Kidney	80.98[c]	60.64[b]	5.78[a]	6.74[a]	4.56
Adipose	12.21[b]	7.54[ab]	2.98[a]	6.89[ab]	1.97
Mitochondrial activity[d]	------------------%------------------				
Muscle	37.6	30.2	24.4	47.3	8.3
Liver	64.2[c]	17.6[b]	70.2[c]	66.9[c]	9.0
Kidney	52.5[bc]	35.1[b]	67.9[c]	50.7[bc]	7.1
Adipose	26.3[c]	6.4[b]	10.2[b]	16.6[bc]	4.7

[a,b,c]Means in the same row with no superscript in common differ (P<.05).

[d]Mitochondrial activity means calculated as :

$$\frac{\text{Mitochondrial activity}}{\text{Mitochondrial activity + cytosolic activity}} \times 100.$$

activity was in the mitochondrial fraction (table IV-3). Ichihara et al.(1981) and Kadowaki and Knox (1982) obtained similar results with rat tissues. The subcellular distribution of BCAA degradative enzymes has not been extensively studied in domesticated animals. Interestingly, in the pig most of the liver and kidney LAT activity was found in the cytosolic fraction.

Means and standard errors of KICDH activity expressed per gram of tissue in crude homogenates, mitochondrial fractions and cytosolic fractions are shown in table IV-4. Means and standard errors for the relative contribution of the mitochondrial fraction to the sum of the enzyme activity in the mitochondrial and cytosolic fraction expressed as a percentage also are presented in table IV-4. Relative differences in KICDH activities between species were similar for muscle, liver and kidney, whether activity was measured in crude homogenates, mitochondrial fractions or cytosolic fractions. However, for adipose tissue crude homogenates, rats had higher ($P<.05$) KICDH activity than the boars and steers, but for the mitochondrial fractions of adipose tissue, no species differences were observed. Also worthy of note is the fact that, with the exception of sheep liver, from 19 to 84% of the crude homogenate KICDH activity was not recovered in the mitochondrial and cytosolic fractions. Some activity was undoubtedly lost in the 600 x g pellet,

TABLE IV-4. MEANS AND STANDARD ERRORS OF ALPHA-KETOISOCAPROATE DEHYDROGENASE ACTIVITY IN SEVERAL TISSUES FROM RATS, PIGS, CATTLE AND SHEEP

Fraction and tissue	Species				Standard error
	Male rats	Boars	Steers	Rams	
	---nmol degraded x g tissue^{-1} x min^{-1}---				
Crude homogenate					
Muscle	2.94	3.00	.41	1.22	.90
Liver	53.10	19.54	10.48	26.12	13.01
Kidney	47.92[b]	6.64[a]	10.86[a]	21.91[a]	4.61
Adipose	4.19[b]	.34[a]	.09[a]	2.72[ab]	.85
Mitochondrial					
Muscle	.71	1.27	.10	.54	.29
Liver	18.70	2.32	4.52	23.61	10.21
Kidney	14.02[b]	2.22[a]	3.76[a]	4.88[a]	2.13
Adipose	.39	.27	.05	.29	.13
Cytosolic					
Muscle	0	0	0	0	0
Liver	6.11[ab]	10.72[b]	1.46[a]	3.05[a]	1.67
Kidney	5.78[b]	.35[a]	.29[a]	1.75[a]	.94
Adipose	2.28	0	0	.14	.85
Mitochondrial activity[c]	---------%---------				
Muscle	100.0	100.0	100.0	100.0	0
Liver	76.5[b]	17.9[a]	67.7[b]	87.3[b]	9.3
Kidney	69.6	90.4	93.0	70.4	10.4
Adipose	20.3[a]	100.0[b]	100.0[b]	60.5[ab]	15.7

[a,b]Means in the same row with no superscripts in common differ ($P<.05$).

[c]Mitochondrial activity means calculated as :

$$\frac{\text{Mitochondrial activity}}{\text{Mitochondrial activity} + \text{cytosolic activity}} \times 100.$$

but the possibility exists that some enzyme inactivation occurred during the centrifugation procedure.

With the exception of rat adipose tissue and pig liver most of the KICDH activity was found in the mitochondrial fraction (table IV-4). These results (except for rat adipose tissue and pig liver) are in agreement with data from rat skeletal muscle, liver and kidney (Hinsbergh et al., 1978; Danner et al., 1979; Odessey and Goldberg, 1979; Randle et al., 1981), and from bovine liver (Johnson and Connelly, 1972). The subcellular localization of KICDH in rat adipose tissue and in porcine liver has not been reported previously.

Tissue Distribution of Crude Homogenate Enzyme Activities

Enzyme Activity Per Milligram of Protein. Means and standard errors for LAT, LDC and KICDH activities in tissue crude homogenates expressed per milligram of protein are presented in table IV-5. In semimembranosus muscle homogenates, LAT activity was 8-, 37- and 15-fold greater ($P<.05$) in rats than in boars, steers and rams, respectively. These results are at least in partial agreement with Lindsay (1982) who suggested that ruminant skeletal muscle has a more limited ability to degrade BCAA than skeletal muscle of nonruminants. He reported that rat

TABLE IV-5. MEANS AND STANDARD ERRORS OF ENZYME ACTIVITIES IN CRUDE HOMOGENATES OF SEVERAL TISSUES EXCISED FROM RATS, PIGS, CATTLE AND SHEEP[a]

Enzyme and tissue	Species				Standard error
	Male rats	Boars	Steers	Rams	
	---pmol degraded x mg protein^{-1} x min^{-1}--				
Leucine aminotransferase					
Muscle	754.70[c]	97.70[b]	20.30[b]	51.50[b]	74.50
Liver	91.30[b]	295.30[c]	11.30[b]	32.80[b]	41.80
Kidney	2406.70[d]	1370.00[c]	261.00[b]	400.50[b]	207.00
Adipose	1786.30	1512.30	1375.30	704.00	369.70
Leucine decarboxylase					
Muscle	26.00[c]	5.50[b]	.60[b]	7.90[b]	4.10
Liver	75.70[d]	46.50[cd]	15.20[b]	19.90[bc]	9.20
Kidney	647.00[c]	81.30[b]	110.50[b]	169.10[b]	65.50
Adipose	47.50[c]	65.20[c]	4.90[b]	38.60[bc]	11.90
Alpha-ketoisocaproate dehydrogenase					
Muscle	66.10[c]	55.50[bc]	5.60[b]	12.80[bc]	16.40
Liver	318.10	99.80	64.40	153.80	91.90
Kidney	444.00[c]	72.80[b]	103.80[b]	193.70[b]	39.70
Adipose	411.40[c]	43.50[b]	35.10[b]	248.30[bc]	100.30

[a]Leucine decarboxylation activity represents the enzymatic release of $^{14}CO_2$ when L-[1-^{14}C]-leucine was supplied as substrate, and alpha-ketoisocaproate dehydrogenase activity represents the enzymatic release of $^{14}CO_2$ when [1-^{14}C]-alpha-ketoisocaproate was supplied as substrate.

[b,c,d]Means in the same row with no superscripts in common differ ($P<.05$).

diaphragm muscles had a 10-fold greater ability to degrade BCAA than sheep diaphragm muscles. However, LAT activity in boar muscle homogenates was not significantly different than muscle homogenates of steers and rams. Therefore, the difference between LAT activity in rat and ruminant muscle could be due to metabolic body size rather than a difference in skeletal muscle BCAA metabolism between nonruminants and ruminants per se. LAT activity in liver homogenates was highest ($P<.05$) for boars, and liver LAT activities in male rats, steers and rams were not different from one another. LAT activity in kidney homogenates was highest ($P<.05$) for the rat, while kidney LAT activities in the two ruminant species were lower than in either of the nonruminant species. There were no significant species differences for LAT activity in adipose tissue homogenates. These results are in accord with previous observations in our laboratory (Busboom et al., 1983). The results indicated that when rat and sheep crude homogenates were compared, LAT activities in rat muscle and kidney were 10-fold higher than the activities in sheep, while LAT activities in rat and sheep adipose tissue were similar to one another. It is interesting that the ratios of adipose tissue to muscle LAT activities for rats, pigs, cattle and sheep were 2:1, 15:1, 64:1 and 14:1, respectively. Therefore, even though absolute adipose tissue LAT activity per milligram of

protein in the rat was the highest (nonsignificantly) of the four species studied, the adipose tissue LAT activity to skeletal muscle LAT activity ratio was actually lowest in the rat.

LDC activities in muscle, liver and kidney of rats were higher than in those tissues from either of the ruminant species (table IV-5). Muscle and kidney LDC activities were also higher in rats than in pigs, but liver LDC activities of male rats and pigs were similar. LDC activity in adipose tissue homogenates was lowest for steers, and the activities of rats, boars and rams were not significantly different from one another.

Muscle KICDH activity was significantly greater for rats than for steers, but muscle homogenate KICDH activity was not different among male rats, boars and rams. Skeletal muscle LDC activity was fivefold greater for the rat than for the boar, while when labeled KIC was supplied as substrate there was no difference in skeletal muscle dehydrogenase activity between rats and boars. This is undoubtedly due, at least in part, to the eightfold greater LAT activity that was present in rat skeletal muscle compared to that of boars.

There were no significant differences in liver KICDH activity between species, but kidney KICDH activity was higher ($P<.05$) in the rat than in the other three species

(table IV-5). Adipose tissue KICDH activity of rats was higher ($P<.05$) than that of boars or steers but was not different from that of the rams. Interestingly, adipose tissue LDC activity was slightly higher in boars than in rats, while adipose tissue KICDH activity was ninefold higher in rats than in boars. If adipose tissue LAT activity was higher in boars than in rats, this would explain why LDC activity was higher in boars than in rats, while KICDH activity was higher in rats than in boars. However, LAT activity was not higher in boars than in rats. A reasonable explanation for the LDC and KICDH activities in boars and rats is that the adipose tissue dehydrogenase is a lower affinity (higher K_m), higher capacity (higher V_{max}) enzyme in the rat than in the boar. When dehydrogenase activity was measured with labeled leucine as substrate, KIC concentration was low compared to the .5 mM KIC concentration utilized when KICDH activity was measured. Thus, if adipose tissue BCKA dehydrogenase has a lower K_m and lower V_{max} in the boar than in the rat it would be possible for boar adipose tissue homogenates to decarboxylate more KIC at low KIC concentrations (as in the LDC assay) and less KIC at high KIC concentrations (as in the KICDH assay) than rat adipose tissue homogenates.

KICDH activities expressed as a percentage of LAT activities are shown in table IV-6. These data indicate that

TABLE IV-6. MEANS AND STANDARDS ERRORS OF ALPHA-KETOISOCAPROATE DEHYDROGENASE ACTIVITY EXPRESSED AS A PERCENTAGE OF LEUCINE AMINOTRANSFERASE ACTIVITY IN CRUDE HOMOGENATES OF SEVERAL TISSUES FROM RATS, PIGS, CATTLE AND SHEEP[a]

Tissue	Species				
	Male rats	Boars	Steers	Rams	Standard error
	%				
Muscle	8.8	115.4	22.5	15.7	36.4
Liver	372.7	36.5	240.0	396.6	110.8
Kidney	19.2[bc]	5.5[b]	40.3[cd]	52.4[d]	7.7
Adipose	22.0	6.1	2.7	31.8	9.6

[a]Percentages =

$$\frac{\text{dehydrogenase activity (pmol x mg protein}^{-1}\text{ x min}^{-1}\text{)}}{\text{aminotransferase activity (pmol x mg protein}^{-1}\text{ x min}^{-1}\text{)}} \text{ x 100}$$

[b,c,d]Means in the same row with no superscripts in common differ ($P<.05$).

KICDH activity was rate limiting in rat, bovine and ovine skeletal muscle, kidney and adipose tissue, while LAT activity was rate limiting in liver homogenates from these species. Other data have shown that KICDH activity was rate limiting in rat muscle (Odessey and Goldberg, 1979), kidney (Dawson and Hird, 1968) and adipose tissue (Frick and Goodman, 1979), while LAT activity was rate limiting in rat liver (Livesey and Lund, 1980). In Chapters I and II, similar results were obtained in sheep tissues. KICDH activity also was found to be rate limiting in porcine kidney and adipose tissue homogenates and surprisingly KICDH activity was rate limiting in porcine liver homogenates as well. This finding is particularly significant since it had been generally assumed that LAT activity in liver was probably rate limiting in most species. The physiological importance of this observation in pigs requires further study.

The KICDH activity expressed as a percentage of LAT activity for boar skeletal muscle (115.4%) indicates that LAT activity was rate limiting (table IV-6). But it should be noted in table IV-5 that the mean for LAT activity was higher than the mean for KICDH activity in skeletal muscles of boars. This apparent discrepancy was due to one animal which had a twofold greater KICDH activity than LAT activity, thus skewing the percentage calculations. In any

case, the means for rat, beef cattle and sheep skeletal muscle LAT activities were from 4- to 11-fold greater than the respective KICDH activities, while in the pig, skeletal muscle LAT activity was only 1.8-fold greater than muscle KICDH activity (table IV-5).

Enzyme Activity Expressed on a Total Tissue Basis. Values for LAT, LDC and KICDH activity expressed on a total tissue basis are presented in table IV-7. These values were calculated by multiplying means of the activity expressed per gram of tissue by the weight of the respective tissue. Tissue weights were estimated from unpublished results in this laboratory and from published reports (Caster et al., 1956; Allen, 1966; Allen et al., 1976; Mostafavi, 1978; Mulvaney, 1984) in which tissue weights from similar animals were obtained. These weights and the references used to estimate the weights are shown in appendix table 4, and Appendix II-B contains the equations used to calculate activity on a per tissue basis. In the rat, values represent the contribution of total dissectable adipose tissue, but only the contribution of subcutaneous adipose tissue was included for boars, steers and rams. Since tissue weights were estimated, these data (table IV-7) were not statistically analyzed. The sums of LAT activities in the four tissues studied (skeletal muscle, liver, kidney and adipose tissue) were from 1.7- (in the sheep) to 4.5-fold

TABLE IV-7. ENZYME ACTIVITIES IN SEVERAL TISSUES FROM RATS, PIGS, CATTLE AND SHEEP EXPRESSED ON A TOTAL TISSUE BASIS[a]

Enzyme and tissue	Species			
	Male rats	Boars	Steers	Rams
	---nmol degraded x tissue^{-1} x min^{-1}---			
Leucine aminotransferase				
Muscle	3895	258600	275400	40810
Liver	178	122043	27090	5432
Kidney	586	23630	21760	6231
Adipose[b]	475	62923	86784	22649
Total[c]	5134	467197	411034	75122
Leucine decarboxylase				
Muscle	137	12238	8160	5720
Liver	148	20119	12974	3170
Kidney	156	1472	9148	2644
Adipose[b]	14	3009	256	1247
Total[c]	456	36838	30539	12782
Alpha-ketoisocaproate dehydrogenase				
Muscle	365	14107	73440	13420
Liver	595	43962	55562	22202
Kidney	110	1295	8686	3286
Adipose[b]	80	3009	2304	7888
Total[c]	1150	189273	139992	46796

[a]Values represent estimated total contribution of a particular tissue to enzyme activities. Appendix II-B contains equations involved in calculations.

[b]In the rat values represent the contribution of total dissectable adipose tissue, but only the contributions of subcutaneous adipose tissue were included for boars, steers and rams.

[c]Total represents the sum of activities in skeletal muscle, liver, kidney and adipose tissue[b].

(in the rat) higher than the corresponding KICDH activities. These four tissues are the most important sites of BCAA degradative enzymes in rats (Harper and Zapalowski, 1981), thus; in rats, boars, steers and rams, KICDH is probably the rate limiting enzyme of whole body leucine degradation. These observations are in agreement with previous results in rats (Goldberg and Tischler, 1981) and sheep (Chapters I and II).

The values from table IV-7 were used to calculate the contribution of individual tissues to LAT, LDC and KICDH activities, expressed as a percentage of the sum of the respective activities found in all four tissues. These percentages are shown in table IV-8. In the four species, the data indicate that skeletal muscle was the primary site of LAT activity, and in all species, except for the pig, liver was a relatively unimportant site of LAT activity. In all species, although the LAT activity per milligram of protein was high in kidney, this tissue appeared to be relatively insignificant when activity was expressed on a total tissue basis. In boars and especially in rats, adipose tissue did not appear to be an important site of LAT activity; but in the steer and ram, subcutaneous adipose tissue accounted for 21 and 30%, respectively, of the total LAT activity in the four tissues studied. It is important to note that in growing sheep, subcutaneous adipose tissue

TABLE IV-8. THE RELATIVE CONTRIBUTION OF SEVERAL TISSUES FROM RATS, PIGS, CATTLE AND SHEEP TO TOTAL ENZYME ACTIVITY IN THOSE TISSUES[a]

Enzyme and tissue	Species: Male rats	Boars	Steers	Rams
	-----Contribution of tissue, %------			
Leucine aminotransferase				
Muscle	75.9	55.4	67.0	54.3
Liver	3.5	26.1	6.6	7.2
Kidney	11.4	5.0	5.3	8.3
Adipose[b]	9.2	13.5	21.1	30.2
Leucine decarboxylase				
Muscle	30.1	33.2	26.7	44.7
Liver	32.6	54.6	42.5	24.8
Kidney	34.2	4.0	30.0	20.7
Adipose[b]	3.1	8.2	.8	9.8
Alpha-ketoisocaproate dehydrogenase				
Muscle	31.7	74.5	52.5	28.6
Liver	51.7	23.2	39.7	47.4
Kidney	9.6	.7	6.2	7.1
Adipose[b]	7.0	1.6	1.6	16.9

[a]Values represent the contribution of a particular tissue expressed as a percentage of the contribution from all four tissues. Appendix II-B contains equations involved in calculations.

[b]In the rat, values represent the contribution of total dissectable adipose tissue, but only the contributions of subcutaneous adipose tissues were included for boars, steers and rams.

constitutes approximately 44% of total body adipose tissue (Kauffman et al., 1963). Thus, if a similar percentage applies to steers, the contribution of all adipose tissue depots to LAT activity in ruminant animals would be greater than is indicated in table IV-8. In the rat, skeletal muscle because of its bulk is thought to be the primary site of BCAA transamination (Goldberg and Chang, 1978). The present results support this supposition, and skeletal muscle also appeared to be the primary site of LAT activity in growing boars, steers and rams. In boars the important contribution of liver to LAT activity was surprising, and the physiological implications of this observation requires further study.

In previous studies (Chapters I and II) it was reported that in growing lambs, adipose tissue accounted for most of the LAT activity, but in the present study skeletal muscle appeared to be more important. However, these results are not contradictory, because the lambs used in the present study were younger and leaner than those used in the study reported in Chapter II. Additionally, in Chapter I skeletal muscle was the predominant site of LAT activity in lambs up to at least 56 days of age. In the present study adipose tissue homogenates from male rats, boars, steers and rams actively transaminated leucine whether expressed on a per milligram of protein or per gram of tissue basis, lending

support to the suggestion of Goldberg and Tischler (1981) that in older more obese rats or in species that contain a higher percentage of adipose tissue, such as humans or domesticated animals, adipose tissue could play a very important role in BCAA transamination.

Skeletal muscle accounted for the highest percentage of KICDH activity in boars and steers, and was also a very important site of KICDH activity in rats and rams. Liver, on the other hand, was an important site of KICDH activity in boars and steers and was the most important site of KICDH activity in male rats and ram lambs. Kidney, because of its small mass was relatively unimportant in all species studied and only in the sheep did adipose tissue account for more than 10% of the total KICDH activity in all four tissues. Khatra et al.(1977b) and Harper and Zapalowski (1981) showed that liver is the primary site of BCKA dehydrogenase activity in rats, but skeletal muscle, because of its bulk also plays an important role in BCKA decarboxylation (Meikle and Klain, 1972). Heitmann and Bergman (1980) and Lindsay (1982) have indicated that the tissue distribution of BCKA dehydrogenase activity is probably similar in sheep. The present data are in general agreement with this supposition, although it appears that adipose tissue also could play a role in ovine BCKA oxidation. In boars and steers it seems

clear that, similar to the rat, skeletal muscle and liver are the two most important sites of KICDH activity.

It is also worthy of note that when L-[1-^{14}C]-leucine was supplied as substrate in this study the rate of liver dehydrogenase activity in the sheep (25%) was similar to that reported in Chapters I and II. Therefore, as was suggested in Chapters I and II, it is possible that liver plays a more active role in BCKA degradation than was indicated by the results reported in those chapters.

Summary and Conclusions

Comparative differences in the subcellular and tissue distribution of BCAA and BCKA degradative activities were studied in crude homogenates, mitochondrial fractions and cytosolic fractions of semimembranosus muscle, liver, kidney and adipose tissues from three 275 g male rats, three 106 kg male pigs, three 480 kg castrated beef cattle and four 40 kg male sheep. L-[1-^{14}C]-leucine was utilized as substrate to measure LAT and LDC activities, and [1-^{14}C]-KIC was supplied as substrate in separate reaction flasks to directly measure KICDH activity.

The subcellular distribution of BCAA aminotransferase and BCKA dehydrogenase activities in rat, steer and ram tissues appeared to be similar. In these three species,

skeletal muscle and adipose tissue LAT activities were primarily found in the cytosolic fraction, while most of the liver and kidney LAT activities and muscle, liver and kidney KICDH activities were precipitated in the mitochondrial fraction. The physiological significance of the cytosolic location of rat adipose tissue KICDH, porcine liver KICDH and porcine liver and kidney LAT activities requires further study.

Similar to the rat, LAT activity appeared to be rate limiting in bovine and ovine liver, and KICDH activity was rate limiting in ruminant skeletal muscle, kidney, adipose tissue and whole body BCAA decarboxylation. In the boar, however, not only was KICDH rate limiting for kidney, adipose tissue and whole body BCAA decarboxylation, but the dehydrogenase also was rate limiting in porcine liver homogenates. The present results were not clear as to whether LAT or KICDH was rate limiting in boar skeletal muscle crude homogenates. In muscle crude homogenates, LAT activity expressed per milligram of homogenate protein was 8-, 37- and 15-fold greater ($P<.05$) in rats than in boars, steers and rams, respectively. Rats also had the highest LAT activity in kidney homogenates while LAT activity in liver homogenates was highest for boars. Adipose tissue LAT activity was nonsignificantly higher for the rat than for the other three species. Skeletal muscle KICDH activity

expressed per milligram of protein was significantly greater for rats than for steers, but muscle crude homogenate KICDH activity was not different among male rats, boars and rams. There were no significant differences in liver KICDH activity in the other three species. Adipose tissue KICDH activity of the rat was higher than the boar or steer but was not different from that of the ram.

Skeletal muscle appears to be an important site of LAT activity in both the ruminant and nonruminant species studied. However, boar liver uniquely accounted for more than 25% of the LAT activity estimated in the four tissues studied. In addition, in sheep and cattle, and to a more limited extent in pigs, adipose tissue also appears to be an important site of LAT activity. The animals used in this study were relatively young, lean animals and results reported in Chapter I showed that in sheep, as the animals matured and fattened, adipose tissue increased in importance as a site of LAT activity. Thus, it is possible that in genetically obese rats or in more mature, fatter pigs, cattle and sheep, adipose tissue may exceed skeletal muscle as the primary site of LAT activity.

As expected, skeletal muscle and liver together accounted for 76 to 98% of the KICDH activity estimated in the four tissues studied. Only in sheep did adipose tissue make a significant contribution (16.9%) to total KICDH

activity in the four tissues. Adipose tissue dehydrogenase activity reported in Chapter I varied drastically as lambs matured from 1 to 365 d of age. The same variation with age may occur in the other species, thus longitudinal studies designed to determine the effect of age on KICDH activity are warranted.

APPENDICES

APPENDIX I

APPENDIX TABLE 1. MEANS AND STANDARD ERRORS OF LIVE BODY WEIGHT, CARCASS WEIGHT, DRESSING PERCENTAGE AND TISSUE WEIGHTS OF RAM LAMBS AT VARIOUS AGES

	Weight, Kg			Weight, g			
Day	Live body	Hot carcass	Dressing percentage	Longissimus muscle	Liver	Kidney	Adipose[a]
1	4.9^{c}	2.4^{c}	48.8^{c}	89^{c}	90^{c}	28^{c}	25^{c}
28	15.9^{d}	7.8^{d}	48.9^{c}	379^{d}	271^{d}	72^{d}	300^{cd}
56	27.2^{e}	13.3^{e}	48.8^{c}	715^{e}	549^{e}	108^{e}	1159^{d}
84	36.4^{f}	18.7^{f}	51.4^{d}	939^{f}	765^{f}	140^{f}	2405^{e}
112[b]	49.5^{g}	25.6^{g}	51.8^{d}	1274^{g}	1000^{g}	167^{g}	4213^{f}
140[b]	57.3^{h}	31.5^{h}	54.9^{ef}	1530^{h}	1165^{h}	189^{gh}	5288^{g}
168	68.1^{i}	36.1^{i}	53.1^{de}	1720^{hi}	1265^{hi}	198^{h}	6770^{h}
196	72.7^{i}	40.8^{j}	55.9^{f}	1767^{i}	1345^{ij}	204^{hi}	7400^{h}
224	82.2^{j}	46.6^{k}	56.7^{f}	2038^{j}	1444^{j}	210^{hi}	8714^{i}
365	121.4^{k}	68.8^{l}	56.7^{f}	3042^{k}	1477^{j}	224^{i}	14071^{j}
Stand. err	2.2	1.3	.7	67	49	8	330

[a]Perirenal adipose tissue weight was estimated for the 1-d old lambs from the report of Noble (1980). For the 28, 56, 84, 112, 140, 168, 196, 224 and 365 d old lambs subcutaneous adipose tissue weight was estimated from the results of Mostafavi (1978). See footnotes of Appendix II-B.

[b]Lambs were sheared between 112 and 140 days of age.

[c,d,e,f,g,h,i,j,k,l]Means in the same column with no superscripts in common differ ($P<.05$).

APPENDIX I, Cont.

APPENDIX TABLE 2. MEANS AND STANDARD ERRORS FOR LIVE AND CARCASS TRAITS OF WETHER LAMBS FED DIETS CONTAINING 8, 12 OR 18% CRUDE PROTEIN

Item	Dietary protein content,%			Standard error
	8	12	18	
Live body weight, kg				
Initial	46.4	46.0	46.2	1.6
Slaughter	54.0	57.6	56.5	2.0
Gain/d, kg	.21	.30	.26	.03
Hot carcass weight, kg	28.9	30.2	30.1	1.0
Dressing percentage	53.7	52.5	53.2	.7
Longissimus muscle weight,g	1244.0	1347.0	1355.0	64.6
Trapezius muscle weight, g	94.0	116.0	111.0	6.1
Liver weight, g	918.0	1129.0	1048.0	71.7
Kidney weight, g	125.0	138.0	151.0	8.4
Subcutaneous adipose tissue weight, g[a]	5142.0	5557.0	5421.0	262.1
Fat depth, cm	.67[c]	.39[b]	.59[b]	.05

[a]The equation used to calculate subcutaneous adipose tissue weight is shown in Appendix II-B.

[b,c]Means in the same row with no superscripts in common differ ($P<.05$).

APPENDIX I, Cont.

APPENDIX TABLE 3. MEANS AND STANDARD ERRORS FOR LIVE AND CARCASS TRAITS OF FASTED WETHER LAMBS

Item	Fast, h			Standard error
	0	48	96	
Live body weight, kg				
Initial	46.0	45.7	46.4	1.6
Prefasted	57.9	56.9	59.5	2.7
Slaughter	57.6	52.6	53.5	2.3
Gain/d, kg	.30	.29	.36	.05
Weight loss during fast, kg	.3[b]	4.3[c]	6.0[d]	.5
Hot carcass weight, kg	30.2	29.2	28.5	1.1
Dressing percentage	52.5[b]	55.4[c]	53.3[b]	.6
Longissimus muscle weight,g	1347.0	1301.0	1258.0	55.5
Trapezius muscle weight, g	116.0	102.0	110.0	7.0
Liver weight, g	1128.0[c]	850.0[b]	823.0[b]	61.9
Kidney weight, g	138.0	125.0	128.0	8.5
Subcutaneous adipose tissue weight, g[a]	5557.0	5181.0	5405.0	315.7
Fat depth, cm	.39[b]	.54[bc]	.58[c]	.05

[a]The equation used to calculate subcutaneous adipose tissue weight is shown in Appendix II-B.

[b,c,d]Means in the same row with no superscripts in common differ ($P<.05$).

APPENDIX I, Cont.

APPENDIX TABLE 4. TISSUE WEIGHTS USED IN COMPARATIVE STUDY TO CALCULATE ENZYME ACTIVITIES PER TISSUE (TABLE IV-7) AND REFERENCES CONSULTED TO ESTIMATE THESE WEIGHTS[a]

	Species			
Weight	Male rats	Boars	Steers	Rams
Live body	275.0	106.00	480.00	40.00
Hot Carcass		69.00	300.00	21.00
Skeletal muscle	124.4	46.95	180.00	11.00
Liver	11.2	2.25	5.30	.85
Kidneys	2.3	.20	.80	.15
Adipose tissue[b]	19.2	8.85	25.60	2.90
Reference	Caster et al. (1956)	Mulvaney (1984)	Allen (1966) Schroeder (Unpub.)	Mostafavi (1978) Appendix Table 1

[a]Rat tissue weights are expressed in grams and boar, steer and ram tissue weights are expressed in kilograms.

[b]In the rat, values represent the contribution of total dissectable adipose tissue, but only the contributions of subcutaneous adipose tissue were included for boars, steers and rams.

APPENDIX I, Cont.

APPENDIX TABLE 5. MEANS AND STANDARD ERRORS OF LEUCINE DECARBOXYLATION ACTIVITY IN SEVERAL TISSUES FROM GROWING RATS, PIGS, CATTLE AND SHEEP[a]

Fraction and tissue	Species				Standard error
	Male rats	Boars	Steers	Rams	
	---nmol degraded x g tissue^{-1} x min^{-1}---				
Crude homogenate					
Semimembranosis muscle	1.10	.26	.05	.52	.28
Liver	13.24^{e}	8.94^{de}	2.45^{c}	3.73^{cd}	1.84
Kidney	67.98^{d}	7.55^{c}	11.44^{c}	17.63^{c}	5.32
Adipose	.72	.34	.01	.43	.18
Mitochondrial					
Semimembranosis muscle	.41^{d}	.12^{c}	.06^{c}	.10^{c}	.07
Liver	5.32^{d}	1.21^{c}	1.07^{c}	1.56^{c}	.50
Kidney	13.69^{d}	2.13^{c}	5.77^{c}	2.87^{c}	1.65
Adipose	.20	.02	<.01	.13	.07
Cytosolic					
Semimembranosis muscle	.18	.05	.16	.02	.08
Liver	.44^{cd}	3.34^{d}	.12^{c}	1.46^{cd}	.88
Kidney	4.22^{e}	1.31^{d}	.18^{c}	.40^{c}	.28
Adipose	.29	.12	0	.10	.08
Mitochondrial activity[b]	---------------%---------------				
Muscle	71.5	77.0	69.8	68.3	19.4
Liver	92.1^{d}	24.3^{c}	93.3^{d}	70.1^{d}	12.0
Kidney	75.5^{cd}	59.0^{c}	97.8^{d}	85.8^{d}	7.0
Adipose	38.9^{cd}	17.5^{c}	100^{e}	65.9^{de}	13.4

[a]Leucine decarboxylation activity represents $^{14}CO_2$ production from L-[1-^{14}C]-leucine.

[b]Mitochondrial activity means calculated as :

$$\frac{\text{Mitochondrial activity}}{\text{Mitochondrial activity + cytosolic activity}} \times 100.$$

[c,d,e]Means in the same row with no superscript in common differ (P<.05).

APPENDIX I, Cont.

APPENDIX TABLE 6. MEANS AND STANDARD ERRORS OF LEUCINE DECARBOXYLATION ACTIVITY EXPRESSED AS A PERCENTAGE OF LEUCINE AMINOTRANSFERASE ACTIVITY IN CRUDE HOMOGENATES OF SEVERAL TISSUES FROM RATS, PIGS, CATTLE AND SHEEP[a]

Tissue	Species				Standard error
	Male rats	Boars	Steers	Rams	
	%				
Muscle	3.4	11.0	2.4	13.5	4.7
Liver	82.7[d]	15.8[b]	53.8[c]	59.5[c]	6.8
Kidney	26.4[c]	6.2[b]	42.4[d]	42.8[d]	3.4
Adipose	2.7	6.3	.4	6.0	1.9

[a]Values calculated as:

$$\frac{\text{decarboxylase activity (pmol x mg prot}^{-1}\text{ x min}^{-1})}{\text{leucine aminotransferase activity (pmol x mg prot}^{-1}\text{ x min}^{-1})} \times 100$$

[b,c,d]Means in the same row with no superscripts in common differ ($P<.05$).

APPENDIX I, Cont.

APPENDIX TABLE 7. MEANS AND STANDARD ERRORS OF ENZYME ACTIVITIES IN MITOCHONDRIAL FRACTIONS OF SEVERAL TISSUES EXCISED FROM RATS, PIGS, CATTLE AND SHEEP[a]

Enzyme and tissue	Species				Standard error
	Male rats	Boars	Steers	Rams	
	---pmol degraded x mg protein^{-1}x min^{-1}--				
Leucine aminotransferase					
Muscle	5836.3[c]	780.0[b]	135.0[b]	262.5[b]	717.3
Liver	146.0[bc]	264.3[c]	132.3[b]	111.0[b]	38.6[b]
Kidney	2977.0[c]	1045.3[b]	671.0[b]	407.5[b]	205.9
Adipose	11613.7	1911.7	10204.0	1010.2	3727.9
Leucine decarboxylase					
Muscle	217.4[c]	70.0[b]	11.2[b]	21.0[b]	36.6
Liver	125.3[c]	37.2[b]	40.5[b]	44.1[b]	13.7
Kidney	450.9[d]	66.0[b]	320.0[cd]	117.6[bc]	64.2
Adipose	304.3	73.0	62.6	130.3	89.3
Alpha-ketoisocaproate dehydrogenase					
Muscle	454.2	279.9	23.3	54.8	141.4
Liver	438.4[c]	72.2[b]	162.2[b]	200.0[b]	40.4
Kidney	459.4[c]	68.1[b]	203.1[b]	212.5[b]	70.9
Adipose	332.2	1097.7	1785.5	149.5	617.5

[a]Leucine decarboxylation activity represents the enzymatic release of $^{14}CO_2$ when L-[1-^{14}C]-leucine was supplied as substrate, and alpha-ketoisocaproate dehydrogenase activity represents the enzymatic release of $^{14}CO_2$ when [1-^{14}C]-alpha-ketoisocaproate was supplied as substrate.

[b,c,d]Means in the same row with no superscripts in common differ (P<.05).

APPENDIX I, Cont.

APPENDIX TABLE 8. MEANS AND STANDARD ERRORS OF ENZYME ACTIVITIES IN CYTOSOLIC FRACTIONS OF SEVERAL TISSUES EXCISED FROM RATS, PIGS, CATTLE AND SHEEP[a]

Enzyme and tissue	Species				Standard error
	Male rats	Boars	Steers	Rams	
	---pmol degraded x mg protein^{-1} x min^{-1}--				
Leucine aminotransferase					
Muscle	737.3[c]	84.3[b]	34.7[b]	25.5[b]	84.0
Liver	39.3[b]	359.3[c]	15.0[b]	21.2[b]	31.9
Kidney	1396.7[d]	1019.3[c]	124.7[b]	131.5[b]	61.9
Adipose	1577.7[b]	1261.7[bc]	726.7[b]	1034.0[bc]	175.3
Leucine decarboxylase					
Muscle	7.3[c]	.9[b]	2.3[bc]	.4[b]	1.7
Liver	4.9[b]	28.4[c]	1.1[b]	11.9[bc]	6.9
Kidney	73.4[c]	22.3[b]	4.6[b]	7.6[b]	5.6
Adipose	35.9	18.1	0	14.0	8.9
Alpha-ketoisocaproate dehydrogenase					
Muscle	0	0	0	0	0
Liver	68.9[cd]	94.8[d]	14.5[b]	22.7[bc]	14.9
Kidney	103.8[c]	5.7[b]	6.2[b]	37.8[bc]	19.3
Adipose	227.7	0	0	21.6	75.0

[a]Leucine decarboxylation activity represents the enzymatic release of $^{14}CO_2$ when L-[1-^{14}C]-leucine was supplied as substrate, and alpha-ketoisocaproate dehydrogenase activity represents the enzymatic release of $^{14}CO_2$ when [1-^{14}C]-alpha-ketoisocaproate was supplied as substrate.

[b,c,d]Means in the same row with no superscripts in common differ (P<.05).

APPENDIX II

Calculations

A. Estimate of percentage contribution of intramuscular adipose tissue to skeletal muscle enzyme activities.

1. Percentage contribution = $\frac{AT \times EE}{SM}$

2. In which: AT = Enzyme activity in adipose tissue, nmol x g tissue^{-1} x min^{-1}.

   EE = Percentage ether extractable lipid in muscle.

   SM = Enzyme activity in skeletal muscle, nmol x g tissue^{-1} x min^{-1}.

B. Activity per tissue and percentage contribution of each tissue.[a]

1. Skeletal muscle: $M = m_1 + m_2$ x skeletal muscle weight[b]
2. Liver: L = l x liver weight
3. Kidney: K = k x kidney weight (both)
4. Adipose tissue: AT = at x tissue weight[c]
5. Tissue percentage contribution:
   a. Skeletal muscle contribution = $\frac{M}{M + L + K + AT} \times 100$
   b. Liver percentage contribution = $\frac{L}{M + L + K + AT} \times 100$
   c. Kidney percentage contribution = $\frac{K}{M + L + K + AT} \times 100$

d. Adipose tissue percentage contribution =

$$\frac{AT}{M + L + K + AT} \times 100$$

6. In which: m_1 = Longissimus muscle enzyme activity, nmol x g tissue^{-1} x min^{-1}

m_2 = Trapezius muscle enzyme activity, nmol x g tissue^{-1} x min^{-1}

l = Liver enzyme activity, nmol x g tissue^{-1} x min^{-1}

k = Kidney enzyme activity, nmol x g tissue^{-1} x min^{-1}

at = Adipose tissue enzyme activity, nmol x g tissue^{-1} x min^{-1}

M = Total enzyme activity in skeletal muscle

L = Total enzyme activity in liver

K = Total enzyme activity in the kidneys

AT = Total enzyme activity in subcutaneous or perirenal adipose tissue

[a]In Chapter I, means for enzyme activity on a total tissue basis were used to calculate the percentage contribution of the individual tissues to the sum of activities in all four tissues, but in Chapters II and III the percentage contribution of the individual tissues was calculated for each lamb. In Chapter IV means for enzyme activity per gram of tissue were multiplied by estimated tissue weights to calculate enzyme activity on a total tissue basis. These enzyme activities on a total tissue basis were subsequently used to calculate the percentage contribution of the individual tissues.

[b]Ovine skeletal muscle weights were calculated according to the results of Kauffman et al. (1963) who found that longissimus muscle comprised 9.5% of total ovine skeletal muscle. Therefore:
Total skeletal muscle weight =

$$\frac{\text{longissimus muscle weight (both sides)}}{.095}$$

[c]Adipose tissue weights were estimated as follows:

Chapter I - Perirenal and subcutaneous adipose tissue weights for the 1 and 28 d old lambs, respectively, were estimated from the results of Mostafavi (1978) and Noble (1980). For the 56, 84, 112, 140, 168, 196, 224 and 365 d old lambs, adipose tissue weights were estimated by the following equation which was derived from the results of the ram lambs studied by Mostafavi (1978).

$$SAT = 136.48 \times LW_s + 2.86 \times EE - 2753.42$$

Chapter II - Adipose tissue weights were estimated by the following equation which was derived from the results of the ewe lambs studied by Mostafavi (1978).

$$SAT = (LW_r + LW_s) - 2 \times 125.4 - 1683.32$$

Chapter III - Adipose tissue weights were estimated by the regression equation which was used in Chapter I except that the quotient of the hot carcass weight divided by the average dressing percentage (50.62%) was substituted for live weight at slaughter.

$$SAT = HCW - .5062 \times 136.48 + 2.86 \times EE - 2753.42$$

In the above equations:

SAT = Subcutaneous adipose tissue weight, g.

LW_s = Live body weight at slaughter, kg.

LW_r = Live body weight when removed from feed, kg.

HCW = Hot carcass weight, kg.

EE = Ether extractable lipid content of the subcutaneous adipose tissue.

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