BRANCHED-CHAIN KETO ACID METABOLISM IN THE ISOLATED PERFUSED RAT HEART

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BY

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Abstract

The Langendorff once-through perfusion system was used to investigate the metabolism of branched-chain amino acids in rat heart. The effects of branched-chain amino acids on fatty acid metabolism was studied. The ketoacid of leucine (KIC) inhibited the oxidation of oleate in the perfused heart. Although the ketoacids of valine (KIV) and isoleucine (KIL) were decarboxylated in cardiac muscle, they did not affect fatty acid oxidation. This appears to be due to the fact that KIC is almost completely oxidized in the heart, whereas only 19% of KIV is completely oxidized.

3-Hydroxyisobutyrate, an intermediate in ketovaline metabolism, was shown to be released from heart when KIV was the substrate. 3-Hydroxyisobutyrate was shown to be a substrate for gluconeogenesis in isolated kidney cortical tubules and hepatocytes.

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ADP	adenosine diphosphate
ATP	adenosine triphosphate
BCAA	branched-chain amino acid
BCKA	branched-chain ketoacid
BCKDH	branched-chain ketoacid dehydrogenase complex
BSA	bovine serum albumin
CoA	coenzyme A
FAD	flavin adenine dinucleotide (oxidized form)
3-HIB	3-hydroxyisobutyrate
HPLC	high performance liquid chromatography
KIC	α-ketoisocaproate
KIL	α-keto-β-methylvalerate
KIV	α-ketoisovalerate
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)

INTRODUCTION

(1) GENERAL

The heart has a wide range of substrate adaptability. Among the substrates it can use are glucose, fatty acids, pyruvate, lactate, acetate, ketone bodies, triglycerides and certain amino acids. The availability of substrates and the metabolic state of the heart determine which substrates will be utilized by cardiac muscle for energy. Fatty acids are, however, said to be the preferential fuel of cardiac metabolism (Neely and Oram, 1972). This belief stems from the early experiments on cardiac metabolism which were carried out on fasted subjects. It is clear when fatty acid levels are high, (ie. during fasting) that they are the preferential fuel of the heart. With different metabolic conditions, the preferential fuel of the heart varies.

The effect of fatty acids on branched-chain amino acid metabolism in the heart has been investigated (Buxton et al., 1984; Buse et al., 1972; Harris & Paxton, 1985; Waymack, 1980; Buffington et al., 1979). The effects of branched-chain amino acids on fatty acid metabolism, however, have not been examined. The use of branched-chain amino acid- and ketoacid- supplemented enteral and parenteral nutrition formulas has been advocated in the treatment of hepatic encephalopathy, renal failure and sepsis. Due to the use of branched-chain amino acids and ketoacids in the treatment of clinical syndromes, we were interested in examining the effects of the branched-chain amino acids and their corresponding

ketoacids on cardiac fatty acid metabolism.

(2) REGULATION OF BRANCHED-CHAIN AMINO ACID CATABOLISM

(a) Common Enzymes in Branched-Chain Amino Acid Metabolism

The branched-chain amino acids leucine, isoleucine, and valine, are essential amino acids. Leucine is a ketogenic amino acid; valine is glucogenic; and isoleucine is both ketogenic and glucogenic. The first two steps in branched-chain amino acid catabolism are, however, catalyzed by two common enzymes.

The first enzyme is a reversible aminotransferase. This enzyme catalyzes the transfer of an amino group to an amino acceptor, alpha-ketoglutarate, to form glutamate and the corresponding branched-chain ketoacid. Leucine is transaminated to alpha-ketoisocaproate(KIC); valine to alpha-keto-isovalerate(KIV); and isoleucine to alpha-keto-beta-methylvalerate(KIL). It was previously thought that the bulk of the transaminase was cytosolic. However, recent studies have shown that the location of the branched-chain aminotransaminase varies with the tissue. In heart muscle, the transaminase is located exclusively in the mitochondrial fraction. In skeletal muscle, the location of the transaminase depends largely on the type of fibres present, in skeletal muscle with a high proportion of red fibres the bulk of the transaminase is mitochondrial, whereas in skeletal muscle with a high proportion of white fibres it is largely cytosolic (Hutson, 1988a, Hutson et al., 1988b). High transaminase activities are also found in the heart, brain and kidney (Ichihara & Koyama, 1966). Low transaminase activity is

present in the liver (Williamson, 1984).

The second step in branched-chain amino acid catabolism is catalyzed by an irreversible branched-chain ketoacid dehydrogenase. The branched chain ketoacid dehydrogenase catalyses the oxidative decarboxylation of the branched-chain ketoacids. The branched-chain ketoacid dehydrogenase is a large multienzyme complex. The reaction catalyzed by it is similar to that catalyzed by the pyruvate dehydrogenase complex. The initial step in the oxidative decarboxylation of branched-chain ketoacids requires thiamine pyrophosphate and involves the elimination of carbon dioxide. The thiamine pyrophosphate becomes covalently bound to the decarboxylated fragment; this step is irreversible. The second step is the oxidation of a thiamine pyrophosphate complex by lipoic acid; this step is catalyzed by enzyme B of the multienzyme complex. Reduced lipoic acid is covalently bound to the decarboxylated acid and thiamine pyrophosphate is liberated. The third step involves the displacement of lipoic acid with CoASH, thus forming the CoA derivative of the decarboxylated alpha-ketoacids. FAD and NAD^+ are used to regenerate lipoic acid. The complex also requires Ca^{2+} and Mg^{2+} for optimal activity (Patel & Olson, 1982).

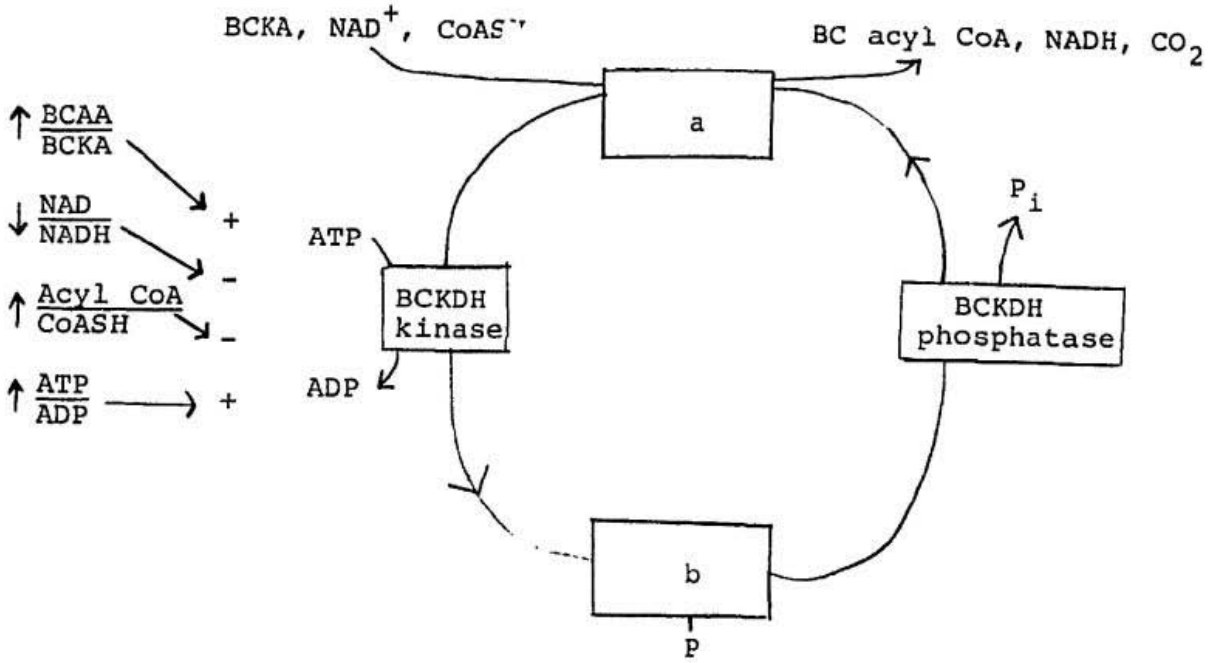


FIGURE 1: Regulation of the branched-chain ketoacid dehydrogenase complex. Phosphorylation inactivates the BCKDH (a) and dephosphorylation activates the complex (b).

The branched-chain ketoacid dehydrogenase is located in the mitochondria. Activities of the branched-chain ketoacid dehydrogenase are high in the liver and kidney (Wohlheuter and Harper, 1970; Shinnick and Harper, 1976; Lau et al., 1981). Activities of branched-chain ketoacid dehydrogenase have also been reported in skeletal muscle (Spydevold and Hockland, 1983; Spydevold, 1979; White and Brooks, 1981; Hagg et al., 1982), heart (Randle et al., 1983; Parker & Randle, 1978; Buffington et al., 1979), brain (Brosnan et al., 1985), and other tissues (Jones and Yeaman, 1986; Lau et al., 1981). The branched-chain ketoacid dehydrogenase, like pyruvate dehydrogenase, is subject to control by phosphorylation/dephosphorylation. Since the levels of ketoacids affect the activity of the branched-chain ketoacid dehydrogenase complex, the rate of interconversion of branched-chain amino acids and branched-chain ketoacids is also important. The branched-chain ketoacid dehydrogenase activity is also controlled by end-product inhibition. Products of the branched-chain keto acid dehydrogenase reaction, NADH and branched-chain acyl CoA derivatives, are competitive inhibitors of the complex. The ratios of NADH to NAD^+ and acyl-CoA to CoA exert control on the flux of branched-chain keto acids through the complex (Buffington & Olson, 1979). Using purified branched-chain ketoacid dehydrogenase from rat liver, Williamson et al. (1984) showed that the enzyme is inhibited by NADH, competitive with NAD^+, and by acyl CoA products competitive with free CoASH. The branched-chain ketoacid dehydrogenase complex is inactivated by covalent phosphorylation and reactivated by dephosphorylation as shown in Figure 1 (Paxton and Harris, 1984). Hughes & Halestrap (1981) showed that the branched-chain ketoacid dehydrogenase from heart can be

phosphorylated. Jones & Yeaman (1986) using isolated adipocytes showed that the regulation of branched-chain ketoacid dehydrogenase by phosphorylation is important in these intact cells. In heart, the branched-chain ketoacid dehydrogenase is also controlled by phosphorylation/dephosphorylation (Randle et al., 1983). Infusion of KIC or KIV, the natural substrates of the branched-chain ketoacid dehydrogenase, rapidly activates the branched-chain ketoacid dehydrogenase complex. It should be noted however, that under physiological conditions in the intact animal, flux through the branched-chain ketoacid dehydrogenase in different organs is limited primarily by the rate of delivery of the branched-chain ketoacids through the blood (Williamson, 1984).

Exercise has been shown to increase the activity of the branched-chain ketoacid dehydrogenase in skeletal muscle and heart. Kasperek et al. (1985) showed that exercise in rats does not affect the total activity of the dehydrogenase in heart, liver or skeletal muscle. However, the active fraction of the dehydrogenase was increased about ten-fold in muscle and five-fold in heart by exercise.

(b) Pathways of leucine, valine and isoleucine metabolism

The pathway of leucine catabolism is shown in Figure 2. Leucine is a ketogenic amino acid since the product of leucine catabolism is acetyl-CoA. Williamson et al. (1984) examined the regulation of flux through the pathway of branched-chain ketoacid metabolism in liver. In leucine catabolism, NADH is produced only at the branched-chain ketoacid dehydrogenase step. This NADH will have relatively little effect on the overall mitochondrial NAD^+ redox state in the intact mitochondria (Williamson, 1984). The steady state mitochondia NADH/NAD+ ratio reflects the overall substrate supply and energy state of the liver; therefore inhibition of the BCKDH by NADH is the main regulatory mechanism which prevents over production of ATP relative to the needs of the cell at high substrate concentrations of BCKA (Corkey et al., 1982). In liver, when KIC is metabolized in conjunction with substrates for other dehydrogenases that increase the mitochondrial NADH/NAD^+ ratio (e.g. fatty acids), flux through the branched-chain ketoacid dehydrogenase becomes inhibited (Williamson et al., 1979). This is probably a result of inhibition by NADH, however, it could possibly be due to a decrease in free CoA caused by fatty acid oxidation (Patel et al., 1981). The rate of KIC decarboxylation decreases with an increase of the mitochondrial NADH/NAD^+ ratio and the steady state content of acyl-CoA intermediates of the pathway fall (Patel et al., 1981).

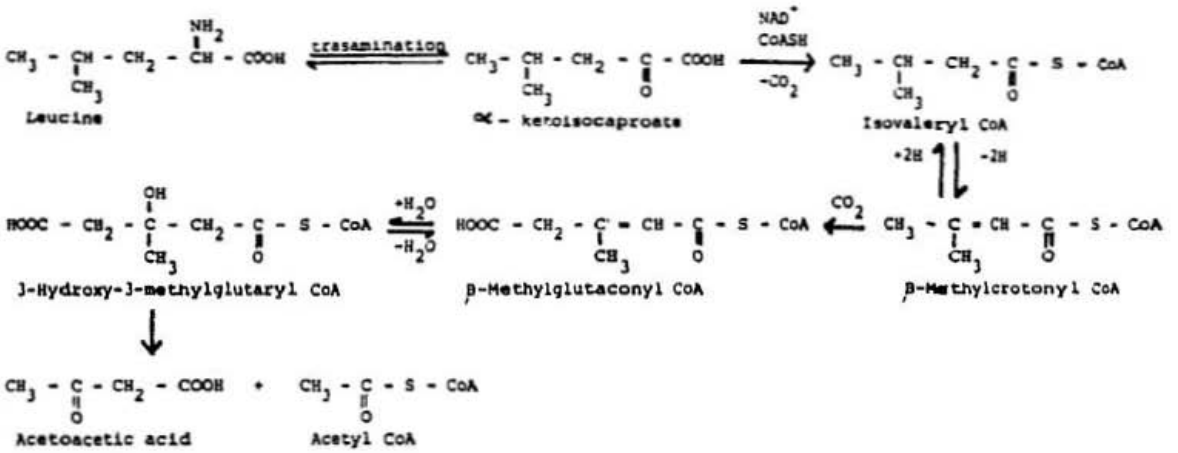


FIGURE 2: Pathway of leucine catabolism.

Williamson et al. (1984) have reported that in liver the branched-chain ketoacid dehydrogenase complex is also sensitive to inhibition by isovaleryl-CoA and beta-methylcrotonyl-CoA, both of which are intermediates in the catabolism of KIC. When acyl-CoA intermediates are decreased in liver, flux through the branched-chain ketoacid dehydrogenase complex is increased (Williamson et al., 1984).

The pathway for valine catabolism is shown in Figure 3. Valine is catabolized to succinyl CoA which in turn can be converted to malate through reactions of the citric acid cycle. In the liver, malate can be converted to glucose by the sequence of glucogenic enzymes in the cytosol. There is no problem in conserving the gluconeogenic potential of valine if branched-chain amino acid catabolism proceeds only as far as the ketoacid in muscle, and further metabolism occurs in liver. This has been suggested by several investigators (Wohlheuter and Harper, 1970; Odessey and Goldberg, 1979; Shinnick and Harper, 1976). This idea was originally based on the fact that in muscle, the activity of the branched-chain aminotransferase is high relative to the activity of the branchedchain ketoacid dehydrogenase. In liver, the reverse is true. Branched-chain ketoacid dehydrogenase activity is high in comparison to the activity of the aminotransferase (Buse et al., 1972; Lau et al., 1981; Shinnick and Harper, 1976). Such an arrangement implies that the gluconeogenic potential of valine and isoleucine, whose catabolism originates in muscle, would be conserved because the irreversible branched-chain ketoacid dehydrogenase would only be active in the liver. It is now apparent however, that there can be considerable flux through the muscle branched-

chain ketoacid dehydrogenase (Spydevold and Hockland, 1983; Spydevold, 1979; White and Brooks, 1981; Hagg et al., 1982). It has been shown in the perfused rat hindquarter, that valine is not completely catabolized in muscle, but that an intermediate, 3-hydroxyisobutyrate, is released (Spydevold, 1979; Lee and Davis, 1986). A similar observation has been made in mammary gland (Wohlt et al., 1977). 3-hydroxyisobutyrate could serve as an interorgan metabolite that would preserve the gluconeogenic potential of valine, provided that it can be taken up and converted to glucose by gluconeogenic tissues.

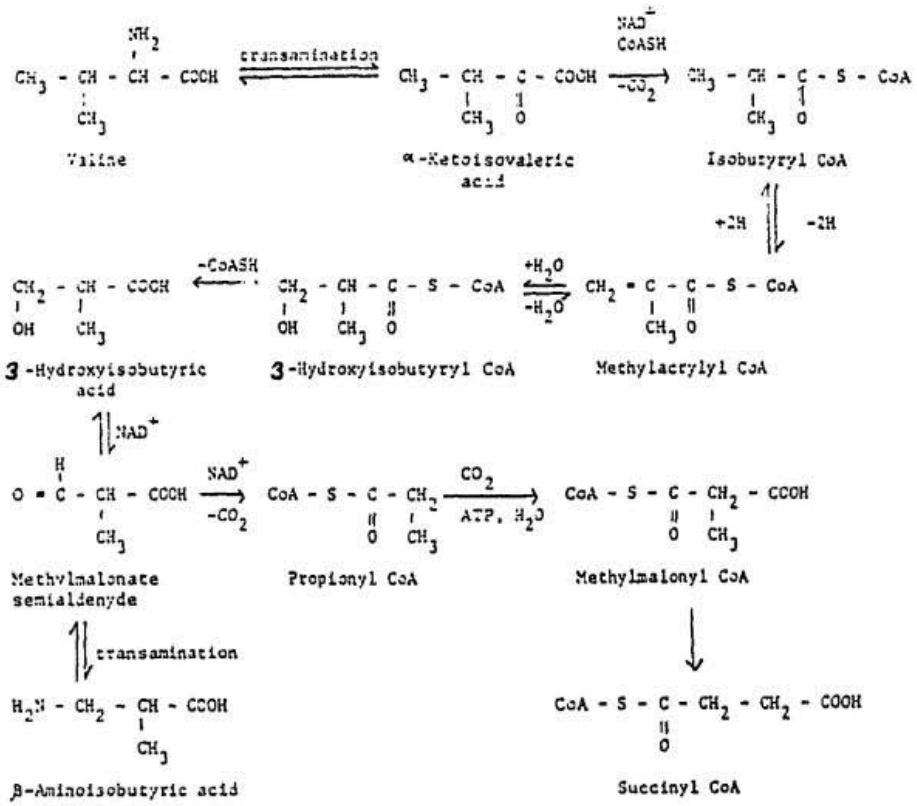


FIGURE 3: Pathway of valine catabolism.

The regulation of flux through the valine catabolism pathway in liver was summarized by Williamson et al. (1984). In valine catabolism NADH is produced at two dehydrogenase steps in addition to the branched-chain ketoacid dehydrogenase. As a result, the mitochondrial NAD^+ redox state may become more reduced with valine catabolism than with leucine catabolism (Williamson et al., 1984). Consequently, if inhibition of the branched-chain ketoacid dehydrogenase depended solely on the $NADH/NAD^+$ ratio, inhibition should be greater with KIV than with KIC. However in liver, flux through the branched-chain ketoacid dehydrogenase is somewhat higher with KIV than with KIC, as substrate with both isolated hepatocytes and isolated mitochondria. This possibly suggests a different effectiveness of feedback inhibition by accumulated acyl-CoA intermediates (Corkey et al., 1982). During valine catabolism, there is a loss of carbon from the pathway at substrate concentrations above approximately 0.1mM (Williamson, 1984), this is thought to be at the 3-hydroxyisobutyrate step. This may be due to the high permeability of 3-hydroxyisobutyrate across the mitochondria and plasma membranes relative to the acyl-CoA intermediates and the limited activity of 3-hydroxyisobutyrate dehydrogenase at the prevailing $NADH/NAD^+$ ratio (Williamson, 1984).

Metabolism of KIV causes an accumulation of propionyl-CoA and methylmalonyl-CoA and decreased levels of free Coenzyme A in hepatocytes. The changes in these metabolites, together with an increase of the $NADH/NAD^+$ ratio, causes an inhibition of the rate of acetyl-CoA production (Williamson, 1984).

The pathway for isoleucine catabolism is shown in Figure 4. Isoleucine is both ketogenic and glucogenic. The products of isoleucine catabolism are Acetyl CoA and Propionyl CoA. Isoleucine is incompletely metabolized in muscle (Scislowski & Davis, 1986). It appears that the catabolism of isoleucine in muscle is more rapid than that of valine (Scislowski & Davis, 1986; Wagenmakers, 1985 and Lee & Davis, 1986).

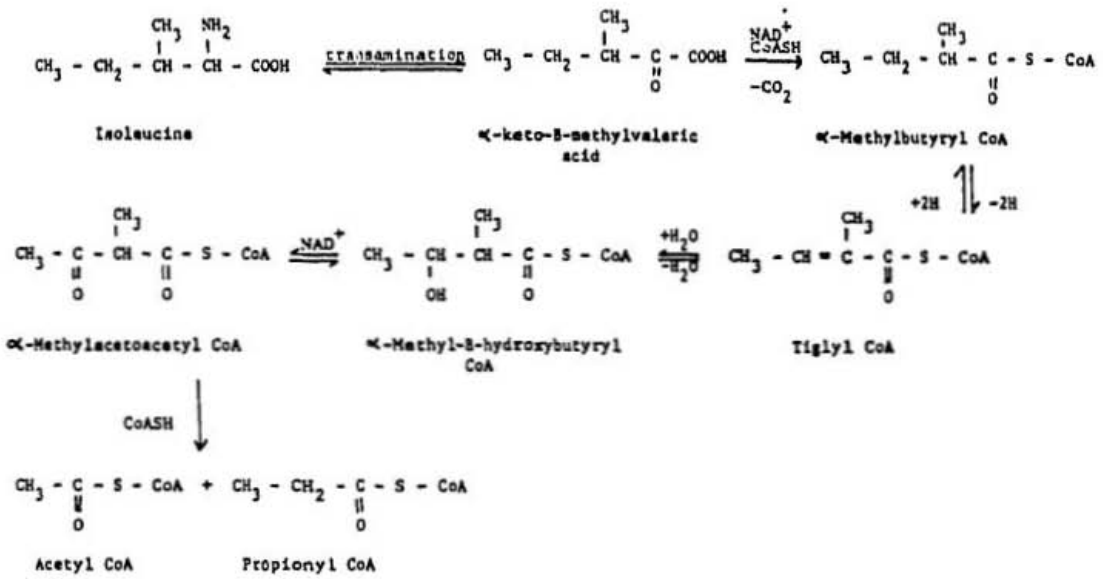


FIGURE 4: Pathway of isoleucine catabolism.

(3). EFFECTS OF BRANCHED-CHAIN AMINO ACIDS ON METABOLISM

Leucine, isoleucine and valine and their corresponding ketoacids affect the metabolism of protein, carbohydrate and lipids. The most potent effector of metabolism is leucine and its ketoacid alpha-ketoisocaproate.

(a) Protein Metabolism

As substrates for protein synthesis, branched-chain amino acids play a role in protein metabolism. However, in addition, Buse and Reid (1975) reported the stimulation of protein synthesis and the inhibition of proteolysis in muscle preparations when the branched-chain amino acids were added to the incubation or perfusion medium. Leucine appears to be the most important branched-chain amino acid with respect to influencing protein metabolism (Buse and Reid, 1975). It appears that leucine catabolism is not required for its effect on protein synthesis, but it is necessary for its effect on protein degradation. Tischler et al. (1982) showed that prevention of leucine transamination to KIC by L-cycloserine (a reversible inhibitor of leucine transamination) prevented the inhibition of proteolysis by leucine. However, cycloserine did not interfere with the ability of leucine to stimulate protein synthesis. KIC decreased proteolysis without stimulating protein synthesis. Therefore, while some intermediates or products of leucine metabolism inhibit proteolysis, muscle protein synthesis must be regulated by the intracellular availability of leucine (Tischler et al., 1982).

(b) Carbohydrate Metabolism

Carbohydrate metabolism is affected by the presence of leucine. In vitro studies show that in fasted rats, leucine inhibits glucose oxidation in skeletal muscle and heart (Chang and Goldberg, 1978b). The mechanism of inhibition does not involve alteration of glucose uptake or glycolysis, but seems to include inhibition of pyruvate oxidation (Chang and Goldberg, 1978b). Catabolism of leucine by muscle appears to be required for inhibition of pyruvate oxidation. When transamination is inhibited, the effect of leucine on pyruvate oxidation is abolished; the effect of KIC is however augmented (Chang and Goldberg, 1978b). In isolated rat hepatocytes KIC and KIV inhibit flux through the pyruvate dehydrogenase complex (Walajtys-Rode and Williamson, 1980). This inhibition is largely independent of pyruvate dehydrogenase interconversion between dephosphorylated (active) and phosphorylated (inactive) forms. A possible cause of decreased pyruvate dehydrogenase activity is inhibition, not only by increased ratios of acetyl-CoA/CoASH or NADH/NAD^+, but also, by acyl-CoA intermediates of the degradative pathways of the branched-chain amino acids (Walajtys-Rode and Williamson, 1980).

Leucine also inhibits glucose oxidation by brain cortex and liver slices of fed rats (Chang and Goldberg, 1978b). Leucine however, does not appear to have a consistent effect on glucose oxidation by liver slices and epididymal fat pads from starved rats (Hughes and Halestrap, 1981). This could be due to the fact that liver slices are not a good preparation for metabolic studies. The inconsistent effect of leucine on glucose

oxidation by different tissues of starved rats may be related to the varied effects of starvation on leucine oxidation i.e. starvation increases leucine oxidation in muscle but not in the liver (Adibi, 1974; Paul and Adibi, 1978). In isolated rat hepatocytes, KIC causes a mild stimulation of gluconeogenesis from lactate and alanine and a small increase in acetyl-CoA levels. KIV however, causes an inhibition of gluconeogenesis (Walajtys-Rode et al., 1979).

The secretion of insulin is stimulated by leucine and KIC (Hutton et al., 1980). Leucine and KIC stimulate insulin release from pancreatic islets from rats incubated in the absence of glucose (Hutton et al., 1980). Blood insulin levels are also important regulators of branched-chain amino acid levels. Insulin lack in diabetes mellitus is associated with increased plasma branched-chain amino acid concentrations (Crofford et al., 1964); this is possibly due to an increased proteolysis together with an inhibition of branched-chain amino acid oxidation. Insulin stimulates the oxidation of branched chain amino acids in the hearts of starved rats, whereas in fed rats, branched-chain amino acid oxidation is not affected by exogenous insulin (Buse et al., 1973).

(c) Lipid Metabolism

There has been no description of the regulation of lipid metabolism by branched-chain amino acids. Branched-chain amino acids do, however, appear to be important sources of substrates for lipid synthesis in adipose tissue (Rosenthal et al., 1974). In extrahepatic tissues, leucine

and isoleucine are metabolised to acetyl-CoA, which can be utilized for fatty acid synthesis (Rosenthal et al., 1974). Adipose tissue is a major extrahepatic site of fatty acid and sterol synthesis from leucine.

(d) Amino Acid Metabolism

Branched-chain amino acids also affect the metabolism of other amino acids. Branched-chain amino acids serve as a source of alpha-amino nitrogen in the synthesis of alanine and glutamine. Therefore, increased muscle catabolism of branched-chain amino acids, such as occurs in starvation, is associated with an increased release of alanine and glutamine (Garber, 1976; Chang and Goldberg, 1978a; Spydevold, 1979). This coupling serves as a control mechanism for the movement of alpha-aminonitrogen between peripheral and visceral tissue. Peripheral tissues, mainly skeletal muscle, are the principal sites for the uptake of branched-chain amino acids (Adibi, 1984).

The branched-chain amino acids not only affect the metabolism of other amino acids, they also affect the metabolism of each other. In this respect, leucine is the most potent of the branched-chain amino acids. When leucine is administered, the concentration of the other branched-chain amino acids in the plasma and tissue is lowered (Clark et al., 1968; Haggenfeldt et al., 1980). This is due to an activation of the branched-chain ketoacid dehydrogenase by virtue of KIC inhibition of the kinase (Hughes & Halestrap, 1980). This results in an increased degradation of isoleucine and valine. The K_i of the kinase for KIC is much lower than

that for KIL and KIV, thus accounting for the fact that leucine affects the catabolism of valine and isoleucine more than they affect the catabolism of leucine. In addition, leucine increases muscle protein synthesis and decreases muscle protein degradation, thereby decreasing the endogenous production of isoleucine and valine.

(e) Alanine and Glutamine Release From Skeletal Muscle

Although alanine and glutamine comprise only about 12% of the amino acid residues in skeletal muscle, they account for more than 50% of the total amino acids released from skeletal muscle (Snell, 1980). Alanine may be converted to glucose in the liver and as such, plays an important role in hepatic gluconeogenesis. Alanine may also serve as a transport system for ammonia from the periphery to the liver. Under normal conditions, glutamine is utilized by the intestine as its major fuel (Windmueller and Spaeth, 1980), however, it can also be converted to glucose by liver and kidney (Brosnan, 1982).

Alanine is formed from pyruvate and glutamate in muscle via the alanine amino transferase reaction. The origin of the carbon skeleton of the alanine released from skeletal muscle has been suggested as coming from two possible sources. Felig (1970) suggested glucose as the source of the carbon skeleton of alanine in starvation. The glucose-alanine cycle proposed by Felig (1970) involves muscle taking up glucose from the bloodstream to produce pyruvate via glycolysis. The amino nitrogen for alanine formation is derived via transamination from the amino acid

products of proteolysis, particularly, the branched-chain amino acids (Odessey et al., 1974). This scheme is concerned with the recycling of glucose carbon between muscle and liver and makes no net contribution to the total body glucose pool.

The second suggested source of carbon for alanine synthesis is from amino acids that are metabolized to TCA cycle intermediates. Conversion of amino acids to oxaloacetate and then to pyruvate via phosphoenolpyruvate carboxykinase and pyruvate kinase or malic enzyme would provide carbon skeletons for alanine synthesis. If this were the case, amino acids that can be metabolized to tricarboxylic acid cycle intermediates (i.e. valine, isoleucine, glutamate, aspartate and methionine) would be a source of alanine and thereby make a net contribution to the body glucose pool during starvation.

It would appear however, that alanine carbons are not derived from amino acids. Interpretation of the original data in support of this theory is changing as more data becomes available. A review of some of the early work in support of alanine synthesis de novo from amino acids and new evidence which alters the interpretations is given below. It has been shown that methionine is not metabolized in skeletal muscle in the presence of physiological concentrations of amino acids (Wu & Thompson, 1989). Therefore, it cannot provide the carbon skeleton of alanine.

Goldstein & Newsholme (1976) reported that alanine production and pyruvate content of isolated rat hemidiaphragm is markedly increased by

isoleucine and glutamate under conditions in which there is neither glycogen mobilization nor glucose utilization. This was interpreted as implying that the carbon skeletons of alanine or pyruvate must, in the absence of glycolytic flux, be derived from isoleucine and glutamate. Since branched-chain amino acids can contribute to alanine formation by acting as amino group donors, a more reliable index to determine the contribution of carbon skeletons from amino acid precursors is the production of lactate, pyruvate and alanine, the sum of which are not increased in quarter-diaphragms from 48hr starved rats by addition of leucine, valine or isoleucine (Caldecourt et al., 1985). Alanine release is increased by addition of branched-chain amino acids including leucine, which cannot be converted to oxaloacetate and pyruvate. Similar findings were reported by Chang & Goldberg (1978a). The stimulation of alanine production by leucine was therefore due to leucine being an amino group donor. These observations suggest that the production of alanine may be limited by the supply of amino groups for transamination.

The existence of a pathway of alanine synthesis de novo from branched-chain amino acids involving phosphoenolpyruvate carboxykinase, pyruvate kinase and alanine amino transferase has been advocated (Snell, 1980). Snell has shown that the maximal activity of malic enzyme surpasses that of phosphoenolpyruvate carboxykinase in muscle, yet malic enzyme shows no adaptive increase during starvation. Also, the inhibition of malic enzyme by hydroxymalonate has no effect on alanine released in vitro (Palmer et al., 1985b). The studies using enzyme inhibitors in support of a pathway for alanine synthesis from amino acids involving

phosphoenolpyruvate carboxykinase, pyruvate kinase, and alanine aminotransferase have been largely refuted. The observation that 3-mercaptopicolinate, an inhibitor of phosphoenolpyruvate carboxykinase, decreases the valine-stimulated increase in alanine production in vitro in diaphragms from 48hr. starved rats, (Snell & Duff, 1977) has been central to the argument that alanine can be synthesized de novo from branched-chain amino acids. Palmer et al. (1984a) showed that 3-mercaptopicolinate is not specific to phosphoenolpyruvate carboxykinase, but has various effects on intermediary metabolism. Mercaptopicolinate decreases the release of alanine and lactate in hemidiaphragms incubated with valine, leucine, isoleucine, or no added substrate. Irrespective of the substrate provided mercaptopicolinate also decreases the lactate/pyruvate ratio.

Dichloroacetate activates the pyruvate dehydrogenase complex through inhibition of pyruvate dehydrogenase kinase. Snell & Duff (1984) reported that dichloroacetate decreased valine-stimulated alanine release, and concluded that valine was involved in the formation of alanine by skeletal muscle. Studies by Palmer et al., (1984b) indicate that pyruvate dehydrogenase is not involved in the oxidation of tricarboxylic acid cycle intermediates and related amino acids to CO_2. This conclusion was based on the fact that in hemidiaphragms from fed and 40hr. starved rats dichloroacetate stimulates $^{14}CO_2$ production from [U-^{14}C] glucose but not from [U-^{14}C] valine, [1-^{14}C] valine, [U-^{14}C] glutamate or [U-^{14}C] aspartate (Palmer et al., 1985b).

2-amino-4-methoxy-trans-but-3-enoate (AMB) is an inhibitor of aspartate aminotransaminase. Snell & Duff (1985) have shown AMB to inhibit valine-stimulated and glutamate-stimulated alanine release by hemidiaphragms from 48hr. starved rats. AMB does not affect leucine-stimulated alanine release. Although these findings appear to support the hypothesis that alanine is derived from amino acid precursors, Snell & Duff (1985) also showed that the [lactate]/[pyruvate] ratio increases markedly with inhibition of diaphragm alanine release. Palmer et al. (1985b) suggest that this may be due to the decreased functioning of the aspartate aminotransferase isoenzymes in the transfer of reducing equivalents produced in glycolysis and may indicate a more general disturbance of metabolism by AMB. Therefore studies with AMB are indecisive.

Perhaps the best evidence that branched-chain amino acids do not make a major contribution of carbon for alanine synthesis was the fact that valine is not completely metabolised. Comparison of $^{14}CO_2$ production from [1-^{14}C] and [U-^{14}C] valine has shown that valine is not completely oxidized in muscle (Davis & Lee, 1985; Spydevold, 1979; Wohlt et al., 1977). There must therefore, be accumulation of intermediates, or products of valine metabolism.

These findings make it clear that valine is of little importance in providing the carbon skeleton of the alanine released from skeletal muscle. Carbons for glutamine synthesis may come from branched chain amino acids that enter the citric acid cycle. Chang and Goldberg (1978a) have shown that more than half of the valine and isoleucine carbon

entering the tricarboxylic acid cycle is released as glutamine, with less than 2% appearing as alanine carbon. The amount of valine carbon that is actually metabolised to tricarboxylic acid cycle intermediates is very small. Wagenmakers et al. (1985) estimate that one in six molecules of valine is completely metabolised to tricarboxylic acid cycle intermediates. Even though isoleucine is metabolised more fully, it is unlikely that more than 60% of the transaminated molecules deliver their carbon skeletons to the tricarboxylic acid cycle (Wagenmakers et al., 1985). Glutamine may provide a net contribution to the body glucose pool. Glutamine may serve directly as a renal glucogenic precursor or indirectly, through intestinal conversion to alanine, as a hepatic glucogenic precursor. In vivo, the major sites of glutamine metabolism are the kidney and small intestine (Chang and Goldberg, 1978b). In this way glutamine can serve as a gluconeogenic precursor. This role of glutamine may not be very important during starvation as the intestinal mucosa atrophies and intestinal glutamine metabolism is decreased.

Renal glutamine metabolism may contribute to the net body glucose pool. The carbon skeleton of the glutamine that is taken up by the kidney may be oxidized to CO_2 or used as a precursor in de novo glucose synthesis (Chang and Goldberg, 1978b). Glutamine is the major precursor for renal ammoniagenesis and thus plays a key role in counteracting metabolic acidosis. Therefore in starvation, renal glutamine metabolism may serve a dual purpose i.e. to convert glutamine carbon into glucose and to facilitate urinary excretion of ammonia (Chang and Goldberg, 1978b). Studies on valine catabolism show that only a part of the carbon from KIV

actually enters the tricarboxylic acid cycle, and that an intermediate, 3-hydroxyisobutyrate, is released (Spydevold, 1979; Lee & Davis, 1986). Therefore, only a small fraction of the valine carbon metabolised is actually used in glutamine synthesis.

(4) PATHOLOGICAL DISORDERS ASSOCIATED WITH ALTERED LEVELS OF BRANCHED-CHAIN AMINO- AND KETO- ACIDS

Altered circulating concentrations of branched-chain amino acids and branched-chain ketoacids are characteristic of several pathological conditions. The plasma concentrations of branched-chain amino- and keto-acids are very low in hepatic encephalopathy, chronic renal failure and sepsis. The theraputic use of branched-chain amino- and keto-acid supplemented enteral and parenteral nutrition formulas have been advocated in the treatment of these disorders. Plasma concentrations of branched-chain amino- and keto-acids are increased during short-term fasting, injury and uncontrolled diabetes.

(a) Hepatic Encephalopathy

Hepatic encephalopathy is a neuropsychiatric disorder associated with characteristic but non-specific histological lesions in the central nervous system. The clinical manifestations range from a slightly altered mental status to coma. The neuromuscular complications range from incoordination and tremor to orthaloplegia and incontinence (Fraser and Arieff, 1985).

In hepatic encephalopathy there is a decrease in serum branched-chain amino acid levels (Munro et al., 1975a). It has been suggested that the increased levels of insulin, seen in patients with decreased liver catabolism, stimulates the uptake and metabolism, of branched-chain amino

acids by muscle thereby lowering serum levels of the branched-chain amino acids.

While the levels of branched-chain amino acids may be decreased, blood concentrations of amino acids that are dependent on the liver for their catabolism are increased. Marked elevations in the plasma concentrations of aromatic amino acids, aspartate and glutamate have been observed (Munro et al., 1985b). The brain dysfunction seen in patients with hepatic encephalopathy is related to the altered plasma amino acid levels. A single transporter operates at the level of the blood-brain barrier for the transport of branched-chain and aromatic amino acids (Fraser and Arieff, 1985). The branched-chain amino acid/aromatic amino acid acid ratio is decreased, therefore, there is an increased transport of aromatic amino acids.

With the onset of encephalopathy and as a result of this altered transport, changes in the concentrations of neurotransmitters in the brain occur. In hepatic encephalopathy, "false" neurotransmitters (gamma-amino-butyric acid, octopamine, serotonin, histamine, phenylethanolamine and catecholamines) are produced locally in the brain to replace norepinephrine and dopamine (Mizock, 1985). These alterations in neuro-transmitter concentrations contribute to the symptoms of hepatic encephalopathy.

(b) Renal Failure

In chronic renal failure plasma concentrations of branched-chain amino acids are decreased (Kopple et al., 1980). The disability of patients with renal failure is partially due to malnutrition. This can be relieved to a large extent by a low protein/high energy diet. A low protein diet has also been shown to decrease urea production and uremic symptoms in renal patients (Richards, 1984). Since, the synthesis of essential amino acids from their alpha-ketoacid precursors in man has been shown (Richards, 1984) the keto acids of the essential amino acids may be used in the treatment of patients with renal failure.

(c) Therapeutic Use of Branched-Chain Amino- and Keto- acid Supplemented Solutions

The use of branched-chain amino- and keto- acid supplemented enteral and parenteral nutrition regimes has been advocated in the treatment of several clinical syndromes. The rationale for this is based on the fact that branched-chain amino acids can provide an energy source during periods of increased energy demands, particularly, when supply or utilization of the usual forms of energy substrates is impaired (Siegal et al., 1979) and also for the normalization of the amino acid ratios for brain transport and also to inhibit muscle proteolysis.

The branched-chain amino acid, leucine, increases protein synthesis and decreases protein breakdown during periods of stress (Siegal et al.,

1979). The mechanism for this anabolic effect is not known but seems to be related to the stimulation of the aggregation of oligoribosomes into polyribosomes, thus facilitating initiation of translation and muscle protein synthesis (Atwell et al., 1977).

The use of branched-chain amino acid supplemented solutions in hepatic encephalopathy normalizes the branched-chain amino acid/aromatic amino acid ratio. The anti-catabolic effect of the branched-chain amino acids is related to the metabolic regulator function of either leucine (Buse and Reid, 1975), or alpha-ketoisocaproate (Tischler et al., 1982). The transamination of leucine is not essential for its inhibitory effects on protein synthesis; it is however, essential for its inhibitory effects on proteolysis (Tischler et al., 1982).

The results of treating hepatic encephalopathy with branched-chain amino- and keto- acid supplemented solutions have been varied. This may be due to the fact that the patients receiving branched-chain amino- and keto-acid supplemented nutrition formulas exhibited different degrees of hepatic failure. Most studies show, however, that branched-chain amino- and keto-acid supplemented solutions did cause improvements in the patients' condition (Mizock, 1985; Siegal et al., 1979; Walser, 1984).

The use of a branched-chain amino- and keto-acid supplemented nutrition formula in the treatment of septic patients has also been advocated. In deteriorating sepsis, there is a reduced requirement for carbohydrate and an increased requirement for fat and branched-chain amino

acids (Siegal et al., 1979). In late sepsis triglyceride intolerance appears with the inability to clear a standard long-chain fat load (Mizock, 1985). The inability to utilize fatty acids may be due to a deficiency in carnitine (Atwell et al., 1977).

Low protein diets are advocated in the treatment of patients with renal failure. Long-term treatment with a markedly protein-restricted diet carries the risk of protein malnutrition. A low protein diet consisting of essential amino acids and their ketoanalogues has also been used in the treatment of patients with renal failure thereby improving nitrogen utilization and minimizing net protein loss. Low protein diets supplemented with essential amino acids or with essential amino acids plus their ketoacid analogues have both been claimed to retard the progression of chronic renal failure. Low protein diets supplemented with essential amino acids plus their ketoacid analogues have been suggested as being superior to low protein diets supplemented only with essential amino acids in retarding the progression of chronic renal failure (Heidland et al., 1978; Walser et al., 1973). The toxic effects of uremia are minimized with low protein diets. Diets supplemented with both essential amino acids and their ketoacid analogues are more effective than those supplemented only with essential amino acids since, they reduce the nitrogen load (Richards, 1984).

Studies on the effects of low protein diets, supplemented with both essential amino acids and their ketoacid analogues, on nitrogen balance, have been conducted. Most studies have been short-term in nature

(ie.) less than two months. In these studies, nitrogen balance has been found to become positive or neutral with this diet (Walser et al., 1973). In long-term studies, nitrogen balance was negative (Alverstrand et al., 1983). However, after long-term treatment with diets supplemented with essential amino acids and their ketoacid analogues (given continuously for 24 hrs. by nasogastric feeding) nitrogen balance was positive (Abras and Walser, 1982). In addition, several studies show that treatment with these diets resulted in a drop in both blood urea and the urea/creatinine ratio (Heidland et al., 1978; Bauerdick et al., 1978).

(d) Genetic Disorders of Branched-Chain Amino Acid Metabolism

Genetic disorders such as maple syrup urine disease, beta-methylcrotonic aciduria, isovaleric acidemia and hypervalemia lead to large increases in branched-chain ketoacids in the blood and/or urine.

Maple syrup urine disease is characterized by mental and growth retardation and frequent bouts of metabolic and neurological crisis. It is rare for a patient to live beyond the second year of life. In maple syrup urine disease, there is a reduction in the capability for oxidative decarboxylation of the three branched-chain ketoacids, therefore leading to an increased concentration of branched-chain amino acids and branched-chain ketoacids in the plasma (Buse and Reid, 1975). Plasma concentrations of the branched-chain ketoacids are normally 10-40 μM; concentrations do not usually rise above 0.1 mM. Branched-chain amino

acid concentrations in plasma are usually 60-250 μM and can rise to concentrations of several millimolar (Williamson, 1984). Treatment of maple syrup urine disease consists of decreasing the concentrations of branched-chain amino- and keto-acids in the diet (Buse and Reid, 1975).

Only one case of hypervalemia has been reported. In this patient, there was an increased concentration of valine in the blood due to a defect in valine metabolism. Isovaleric acidemia is characterized by an excess of isovaleric acid in blood and urine and is the result of a defect in leucine metabolism. In patients with beta-methylcrotonic aciduria, there is decreased activity of the beta-methylcrotonyl-CoA carboxylase. In the three cases reported, there is an increased excretion of beta-hydroxyisovaleric acid in the urine. Biotin induces a rapid improvement in the clinical symptoms of beta-methylcrotonic aciduria and also decreases the excretion of abnormmal metabolites. This suggests the possibility that the primary effect may be in biotin utilization, the enzyme affected, beta-methylcrotonyl-CoA carboxylase, is biotin-dependent. It is possible that the patients were biotin deficient.

(5) CONTROL OF FATTY ACID OXIDATION BY HEART MUSCLE

(a) Fatty Acid Utilization in Heart

The heart has the capacity to utilize a wide variety of substrates for energy production (ie.) glucose, lactate, pyruvate, acetate, amino acids, triglycerides and fatty acids. The availability of the various substrates is an important determinant of the substrate to be oxidized for energy at any given time. Most investigators feel that the preferential fuels for energy metabolism are fatty acids. In well oxygenated hearts, fatty acids can be completely oxidized via β-oxidation and the citric acid cycle. The NADH produced in the cycle or from the beta-oxidation of fatty acids is oxidized in the respiratory chain for ATP production.

The most important factor controlling the utilization of substrate by the heart is its work load (Taegtmeyer et al., 1980). Cardiac workload is defined as the volume of fluid to be pumped against the impedance of the vascular bed. Oram et al. (1973) have shown that the rate of fatty acid uptake increases as the concentration of fatty acids in the perfusate increases. However, the rate of fatty acid uptake is not linear and is saturable. This suggests that at high concentrations of fatty acids, intracellular processes become rate limiting for fatty acid utilization. Oram also showed that fatty acid uptake and oxidation were accelerated as cardiac workload was increased.

(b) Fatty Acid Oxidation

The first step in the oxidation of long-chain fatty acids is activation. Activation in heart occurs on the outer mitochondrial membrane where the long-chain fatty acids are linked to coenzyme A to form a long-chain acyl CoA ester. Because long-chain acyl CoA molecules do not readily traverse the inner mitochondrial membrane, a special transport system is required. Activated long-chain fatty acid molecules are carried across the inner mitochondrial membrane by carnitine. The acyl group is transferred from Coenzyme A to carnitine; this reaction is catalyzed by carnitine acyl transferase I. The reaction is reversed by carnitine acyl transferase II. It was originally thought that CAT I was located on the outer aspect of the inner mitochondrial membrane and that CAT II was located on the inner aspect of the outer mitochondrial membrane. Recent work by Murthy and Pande (1987) show that the CAT I is a transmembrane protein located in the outer mitochondrial membrane with the catalytic site on the inner side of the outer mitochondrial membrane. CAT II is located on the inner side of the inner mitochondrial membrane. Once the fatty acyl CoA molecule is inside the mitochondria, it enters the beta-oxidation sequence and is oxidized to acetyl CoA. In heart, the major fate of acetyl CoA is oxidation through the citric acid cycle.

Medium-chain fatty acids do not require the carnitine acyltransferase steps to enter the mitochondria as they directly to traverse the mitochondrial membrane and do not require prior activation. Their activation occurs in the mitochondrial matrix.

(c) Regulation of Fatty Acid Oxidation in the Perfused Heart

Oram et. al. (1973) examined the regulation of fatty acid metabolism in the isolated perfused rat heart. They used the long-chain fatty acid palmitate as a substrate in an attempt to localize the regulatory steps in fatty acid oxidation. At low levels of fatty acid (i.e. 0-0.6 mM) fatty acid uptake was proportional to its concentration in the perfusate. Fatty acid utilization appeared to be limited by the rate of fatty acid uptake or activation (Oram et. al., 1973). At high concentrations of fatty acid (0.6-1.2 mM), however, the rate of uptake and oxidation did not increase further. This work lead to the conclusion that at high levels of exogenous fatty acid, fatty acid uptake is limited by the rate of acetyl-CoA oxidation through the citric acid cycle. The activation or uptake of fatty acids into mitochondria may have been impaired by the high ratios of acetyl CoA to CoA and acetyl carnitine to carnitine. Levels of CoA + carnitine were not sufficient for optimal rates of fatty acid activation and oxidation (Oram et. al., 1973).

At increased levels of cardiac work Oram et. al. (1973) noted an increased oxidative phosphorylation and citric acid cycle rate (i.e. increase oxygen consumption and increased CO_2 production). Also an increase in uptake and β-oxidation of palmitate was observed. When the citric acid cycle was accelerated levels of acyl CoA, acetyl-CoA, acetylcarnitine and tissue content of fatty acid was decreased, while an increase in acylcarnitine, free CoA + free carnitine was observed (Oram et. al., 1973). Oxidation of octanoate, which does not require activation

for entry into the mitochondria, was fast enough to maintain high levels of acetyl-CoA when fatty acid oxidation was accelerated by increased cardiac work. This suggests that the translocation of the acyl units limited the rate of long-chain acyl carnitine oxidation at high levels of cardiac work (Oram et al., 1973).

In heart, the major fate of acetyl-CoA produced by beta-oxidation is oxidation through the citric acid cycle. The beta-oxidation sequence is coupled with the citric acid cycle by the mitochondrial pool of free Coenzyme A. Since 85% of tissue CoA is mitochondrial and 90% of tissue carnitine is extramitochondrial it has been suggested that there is a coupling of the intra- and extra- mitochondrial pools through carnitine acetyltransferase. In this scheme the mitochondrial acetyl CoA/CoA ratio would be in equilibrium with extramitochondrial acetyl carnitine/carnitine ratio. Therefore, mitochondrial increases in acetyl CoA/CoA ratio would adjust the extramitochondrial pools of free CoA and free carnitine which are available for fatty acid oxidation and acyl carnitine formation (Neely et al., 1972)

Despite this, the view put forth by Neely and Oram in 1972 must now be modified to take into account new discoveries. Malonyl-CoA has been shown to play a major role in the regulation of fatty acid utilization in the liver. Liver carnitine acyl transferase 1 is subject to inhibition by malonyl-CoA. When malonyl-CoA levels are high (ie.) during fatty acid synthesis, there is an inhibition of carnitine acyl transferase 1 and fatty acid oxidation is decreased (McGarry et al., 1983). It is felt that

this is important in the glucose sparing effect of fatty acids that is seen during starvation.

McGarry et al. (1983) proposed that malonyl CoA could also play a role in controlling long-chain fatty acid oxidation in non-hepatic tissues by inhibiting the carnitine acyl transferase system. Readily measurable levels of malonyl-CoA have been found in rat heart and although the distribution of malonyl-CoA in heart and skeletal muscle are not known, malonyl-CoA levels in heart do nonetheless decrease in fasting rats.

Saggerson (1982) has shown carnitine acyl transferase I in heart muscle to be even more sensitive to inhibition by malonyl-CoA than liver carnitine acyl transferase I. Since carnitine acyl transferase I displays different characteristics in different tissues, the possibility exists that other CoA esters might exert a significant inhibition on carnitine acyltransferase I in tissues where there is a high sensitivity to malonyl-CoA (McGarry et al., 1983). Indeed heart carnitine acyl transferase I has been shown to be sensitive to other CoA esters (i.e.) succinyl-CoA and methylmalonyl-CoA. The levels of these metabolites could regulate fatty acid oxidation. When levels of these metabolites are high, fatty acid oxidation may be inhibited.

(d) The Effects of Fatty Acids on Branched-Chain Amino Acid Metabolism

The metabolism of branched-chain amino acids is affected by the

oxidation of fatty acids. The relationship has been examined by several investigators (Buxton et al., 1984; Paxton and Harris, 1984; Harris and Paxton, 1985; Buse et al., 1972). The effect of fatty acids on branched-chain amino acid metabolism is dependent on the chain length of the fatty acid.

The effect of octanoate, a medium-chain fatty acid, on branched-chain amino acid catabolism, depends on the activity of the branched-chain ketoacid dehydrogenase. Buxton et al. (1984) showed that in perfused rat heart under conditions where the branched chain ketoacid dehydrogenase activity was low, i.e. short perfusion time or low activator concentration, octanoate stimulated flux through the enzyme. When the activity of the branched-chain ketoacid dehydrogenase is high, i.e. in perfusions where activator concentrations are high or perfusion times are longer, octanoate causes an inhibition of decarboxylation. The mechanisms for these various effects are not readily apparent. Buxton et al. (1984) showed that infusion of palmitate, a long chain fatty acid, in hearts perfused with leucine caused an 80% inhibition of flux through the branched-chain ketoacid dehydrogenase.

The effects of fatty acids on branched-chain amino acid metabolism have, therefore, been investigated (Buxton et al., 1984; Buse et al., 1972; Harris and Paxton, 1983; Paul and Adibi, 1976; Spydevold and Hockland, 1981). The effects of branched-chain amino acid metabolism on fatty acid metabolism, however, have not been investigated.

Fatty acids are said to be the preferential fuel of heart muscle. In certain clinical syndromes, the levels of branched-chain amino- and keto-acids are very high. Therapeutically, enteral and parenteral nutrition formulas suppplemented with branched-chain amino acids and their ketoacid analogues are being used in the treatment of several pathological conditions. This raises a question: will high levels of branched-chain amino- and keto-acids affect the oxidation of fatty acids in the heart? The branched-chain amino acids have different metabolic fates, therefore we were interested in investigating the metabolism of the branched-chain ketoacids and their effects on fatty acid oxidation in heart muscle.

As a model for the investigation of this problem we chose to study rat hearts perfused in the Langendorff mode. Using this system we could measure the rates of fatty acid oxidation in the presence and absence of BCKA's. For the purpose of our study we chose a long-chain fatty acid (oleate) and a medium chain fatty acid (octanoate). The ketoacids used were KIC and KIV since these are available radiolabelled. Since the branched-chain amino acids have different metabolic fates we were therefore interested in investigating the different effects of KIC and KIV on fatty acid oxidation in heart muscle.

We also examined the metabolism of KIC and KIV by heart. The release of an intermediate in KIV metabolism was observed and identified as 3-hydroxyisobutyrate. 3-hydroxyisobutyrate was then determined to be a gluconeogenic substrate in hepatocytes and kidney cortical tubules.

MATERIALS AND METHODS

(1) MATERIALS

(a) Reagents

Chemicals were of analytical grade or equivalent and were obtained from either Sigma Chemical Company (St. Louis, Mo.); Fisher Scientific Company (Fairlawn, NJ.); or Aldrich Chemical Company (Montreal. Que.). All enzymes were obtained from Sigma Chemical Company (St. Louis, Mo.) or Boehringer Mannheim (Montreal, Que.)

3-hydroxyisobutyrate was prepared from (S)-(+)-methyl 3-hydroxy-2-methyl propionate obtained from Aldrich Chemical Company (Montreal, Que.). The ester was incubated at room temperature for one hour with 10% molar excess of sodium hydroxide and an equivalent volume of methanol as a cosolvent. Solvents were removed by lyophilization. The ^{1}H NMR spectrum of the resulting sodium salt was identical to that of an authentic sample kindly supplied by Dr. A.P. Kozikowski, Dept. of Chemistry, University of Pittsburgh. The NMR was carried out for me by Dr. C. Jablonski, Dept. of Chemistry. M.U.N.

(b) Radioisotopes

L-[1-^{14}C] labelled oleate and octanoate were obtained from New England Nuclear. L-[1-^{14}C] and L-[U-^{14}C] labelled leucine and valine were

also obtained from New England Nuclear. Labelled ketoacids were synthesized from corresponding branched-chain amino acids by the method of Rudiger et al. (1972).

(c) Animals

Male Sprague-Dawley rats (Charles River Inc., La Prairie, Que.) weighing 300-400g were used in all experiments. They were allowed free access to water and Purina rat chow unless otherwise stated. The animal room was lighted from 08:00 to 20:00 hours and animals were used between 10:00 and 14:00.Starved rats were deprived of food for 48hr. Diabetes was induced by an intravenous injection of streptozotocin (100mg/kg bodyweight) under light ether anesthesia. Animals were maintained for 7 days on a daily injection (subcutaneous) of protamine-zinc insulin (3-5 U/day) adjusted so that weight gain was the same as control (Brosnan et al., 1983). Insulin was withdrawn 4 days prior to experiments. To examine the effects of dietary protein on gluconeogenesis from 3-hydroxyisobutyrate in kidney cortical tubules, animals were fed either a purified moderate protein diet (13% casein); a high protein diet (55% casein); or a high protein diet (55% casein) and 0.1N sodium bicarbonate substituted for drinking water (Brosnan et al., 1978). To examine the effects of acidosis on gluconeogenesis from 3-hydroxyisobutyrate in kidney tubules, 1.5% NH_4Cl was substituted for drinking water for 7 days.

(2) PERFUSIONS

(a) Langendorff Non-recirculating Perfusion

Langendorff non-recirculating perfusions were performed as described by Ross, 1972. Animals were anesthesized by intraperitoneal injection of 0.1 ml/100g bodyweight of a 65mg/ml solution of "Somnotol" (sodium pentobarbital, MTC Pharmaceuticals). The saphenous vein of the anesthesized animal was exposed and 100 units of heparin (Allen and Hanburys) were injected intravenously. After 30 seconds, the hearts were exposed and excised rapidly. Hearts were immediately plunged into ice-cold physiological saline. The hearts were cannulated on a stainless steel cannula and medium immediately allowed to perfuse the heart. This whole procedure from the first incision to the start of perfusion took up to one minute.

Hearts were perfused at a constant aortic pressure of 85cm of water with Krebs-Henseleit bicarbonate buffer medium containing 120mM NaCl, 5mM KCl, 2mM CaCl, 1mM KH_2PO_4, 1mM $MgSO_4$ and 25mM $NaHCO_3$ (Krebs-Henseleit, 1932). The medium was filtered through a Millipore HA 0.45um filter before being equilibrated with humidified 95% O_2/5% CO_2 for a half-hour before perfusion. The medium was gassed continuously throughout the perfusion. Stock solutions of 100mM octanoate and sodium oleate were prepared. Octanoate was added directly to the buffer as were the branched-chain ketoacids. Oleate was complexed to bovine serum albumin (final concentration 0.5% BSA).

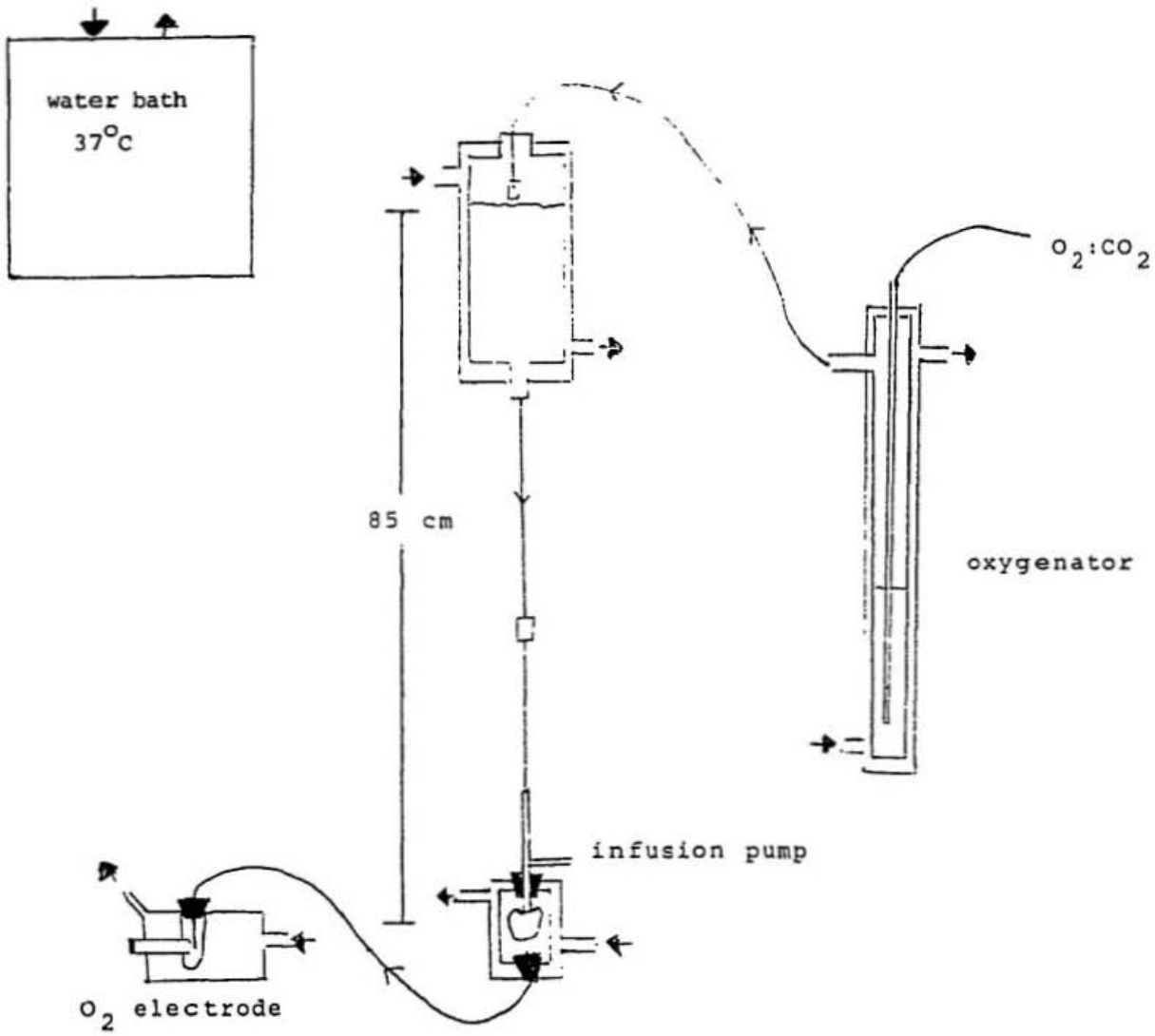


Figure 5a: Perfusion apparatus for the non-recirculating Langendorff Heart Perfusion. Solid arrows indicate water circulation.

Bovine serum albumin (Miles Scientific) was defatted as described by Chen (1967) and dialysed as described by Ross et al., (1974). The perfusion system is shown in Figure 5a. In experiments in which a second substrate was presented to the heart this was via an infusion pump as shown in Figure 5a, except when the second substrate was oleate. In this case the apparatus depicted in Figure 5b was used since it was not possible to make the fatty acid-oleate substrate sufficiently concentrated to use the infusion pump. For such experiments in which the effect of oleate on the metabolism of labelled keto-acids was examined Krebs-Henseleit bicarbonate buffer was prepared as described, labelled branched-chain ketoacids and cold branched-chain ketoacid were added to the medium. Medium was divided into two parts. Both were gassed for 30 min Each reservoir contained the same specific activity (i.e. approximately 1500 DPM/μmol). To reservoir B, fatty acids were added to give a final concentration of 0.1mM (Oleate complexed to 0.5% BSA). The medium in reservoir A was used for the first 25 min of the perfusion; for the second half of the perfusion, medium in reservoir B was used.

Oxygen consumption was measured by an inline oxygen electrode. The oxygen electrode was standardized with water at 37°C. Arterial readings were taken before and after perfusion using gassed medium equilibrated at 37°C. These two values were averaged for the arterial reading. When the apparatus described in figure 5b was used the arterial oxygen reading before perfusion was taken using medium A and that at the end of perfusion using reservoir B. Arteriovenous differences were calculated from oxygen electrode data. Oxygen consumption remained constant throughout all

experiments. Coronary flow rates were at least 10ml/min and heart rates 200 beats/min. Both remained constant throughout the perfusion period.

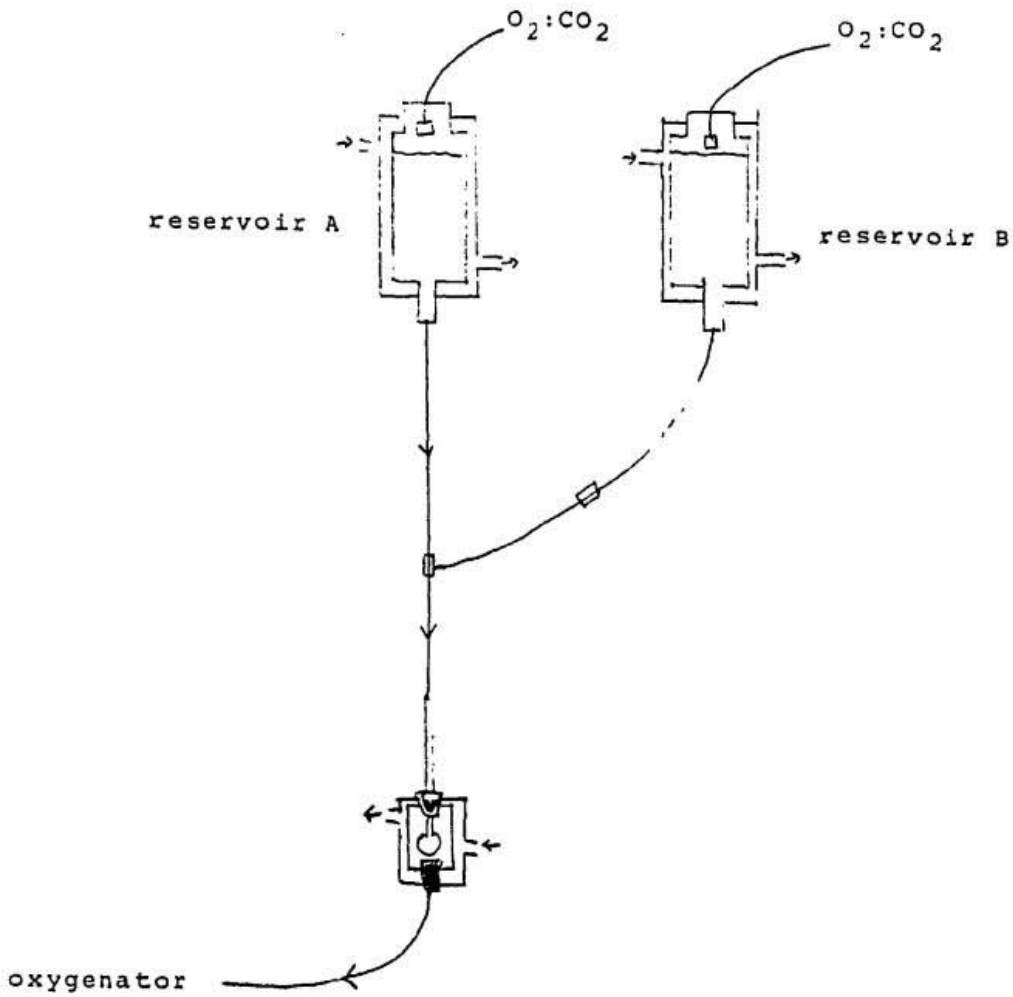


FIGURE 5b: Perfusion apparatus for the non-recirculating Langendorff mode adapted to allow for introduction of long chain fatty acids as the second substrate.

Hearts were allowed to stabilize for the first 15 minutes of the perfusion. Starting at 15 min, samples were collected every 5 min until the end of the perfusion. The samples were collected from perfusate effluent for 30 s, under mineral oil in preweighed test tubes. The flow rate was calculated from these samples. Oxidation rates were calculated per gram dry weight. Oxidation of substrates were calculated using the specific radioactivity of the substrate in the perfusion medium. Dry weights were obtained by drying hearts to constant weight (i.e.48hrs at 55°C).

(i) Quality Control

The parameters for quality control were set before perfusions were performed. Hearts were rejected on the basis of heart rate, oxygen consumption and flow rate. A separate set of experiments were performed to determine the levels of adenine nucleotides.

Heart rates were at least 200 beats per min; oxygen consumption was at least 15 μmol/min/g dry weight; and flow rates were at least 10 ml/min in each perfusion.

Adenine nucleotides were measured as described by Bergmeyer et al. (1974a). Hearts were perfused in the non-recirculating Langendorff mode for 30 min. Either 0.1mM oleate complexed to 0.5% BSA as the sole substrate or 1.0mM alpha-ketoisocaproate was infused for the last ten min of perfusion period and the hearts were clamped while on the cannula with aluminum tongs precooled in liquid nitrogen (with no prior interruption of perfusate flow). ATP values were 19.6±1.9(4) μmol/g dry wt.for oleate perfusion vs. 21.7±1.8(3) when KIC was infused; ADP values were 5.1±0.3(4) vs. 4.8±0.6(3); AMP values were 0.8±0.14(4) vs. 1.0±0.3 (3). There was no significant difference between the two substrates and these numbers agree well with published data for perfused hearts (Miller, 1979).

(ii) Sample Treatment

Samples of the effluent perfusate were collected at timed intervals in preweighed test tubes containing 1-2ml of mineral oil. Aliquots of these fractions (2.0ml) were injected into 25ml Erlenmeyer flasks. The flasks contained 2ml of 0.5M sodium citrate pH 6.0 and were sealed with rubber stoppers (Kontes Glass Company) equipped with plastic wells containing 0.3ml NCS tissue solubilizer (Amersham Searle). The flasks were agitated at 37°C for 60 min for collection of $^{14}CO_2$. The centerwells were then transferred to scintillation vials containing 10ml of toluene plus Omnifluor (New England Nuclear) for counting. Samples were counted in a Beckman LS-233 liquid scintillation counter. Quench correction was made by the channels ratio method. Citrate buffer (pH 6.0) was used to release $^{14}CO_2$ from the medium rather than the more conventionally used strong acids since at low pH a fraction of medium chain fatty acids (i.e. ^{14}C-octanoate) was volatilized and collected in the center-well thus giving an unacceptably high blank. Since the pK of the bicarbonate system is 6.1 it is possible to satisfactorily volatilize the CO_2 at higher pH provided there is an efficient CO_2 trapping system (Squires and Brosnan, 1978).

(b) Langendorff Recirculating Perfusions

Langendorff recirculating perfusions were performed as described by Ross (1972) using 750 ml of medium. Hearts were perfused for 60 min with 1.0mM [U-^{14}C] alpha-ketoisovalerate. Samples of perfusate were collected

every 15 min for HPLC analysis.

(3) HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

To identify the possible end-products of alpha-ketoisovalerate metabolism in the perfused rat heart, an HPLC system was developed to separate alpha-ketoisovalerate from these end-products. The system used was similar to that described by Womersley et al. (1985). Ion-mediated partition chromatography using low acidity and elevated temperature was found to give a good separation. The Waters HPLC system used consisted of a model 510 pump system, U6K injector, 481 spectrophotometer at 210nm and an Aminex HPX-87H, organic acid analysis column (Bio-rad). The isocratic mobile phase was 0.006N sulfuric acid, the flow rate of 0.4ml/min and the column temperature of 41°C.

In preliminary experiments I determined the elution times of the possible end products of valine catabolism, namely 3-hydroxyisobutyrate, alpha-ketoisovalerate,methylmalonate and propionate separately. Standards were dissolved in Krebs-Henseleit bicarbonate buffer, acidified to pH 5 with HCl to remove CO_2, standards were then injected onto the column and elution times were determined by changes in absorbance at 210nm. A standard solution containing all four compounds was used before and after each run to insure the column effectiveness. The elution conditions described above gave a good separation of the compounds (alpha-ketoisocaproate 14.5 min; 3-hydroxyisobutyrate 22.5 min; methylmalonate 17 min; and propionate 29 min) as shown in Figure 6. Samples of perfusate

were acidified to pH 5 with HCl to remove CO_2 and were injected onto the Aminex column. Fractions were collected from column eluent and counted in Aquasol to determine the elution times of the radioactive end-products of [U-^{14}C] KIV metabolism in perfused rat hearts. The times at which the peaks of radioactivity occurred were compared to the elution times of the compounds in the standard solution to determine which end-products were released from the perfused heart during KIV oxidation.

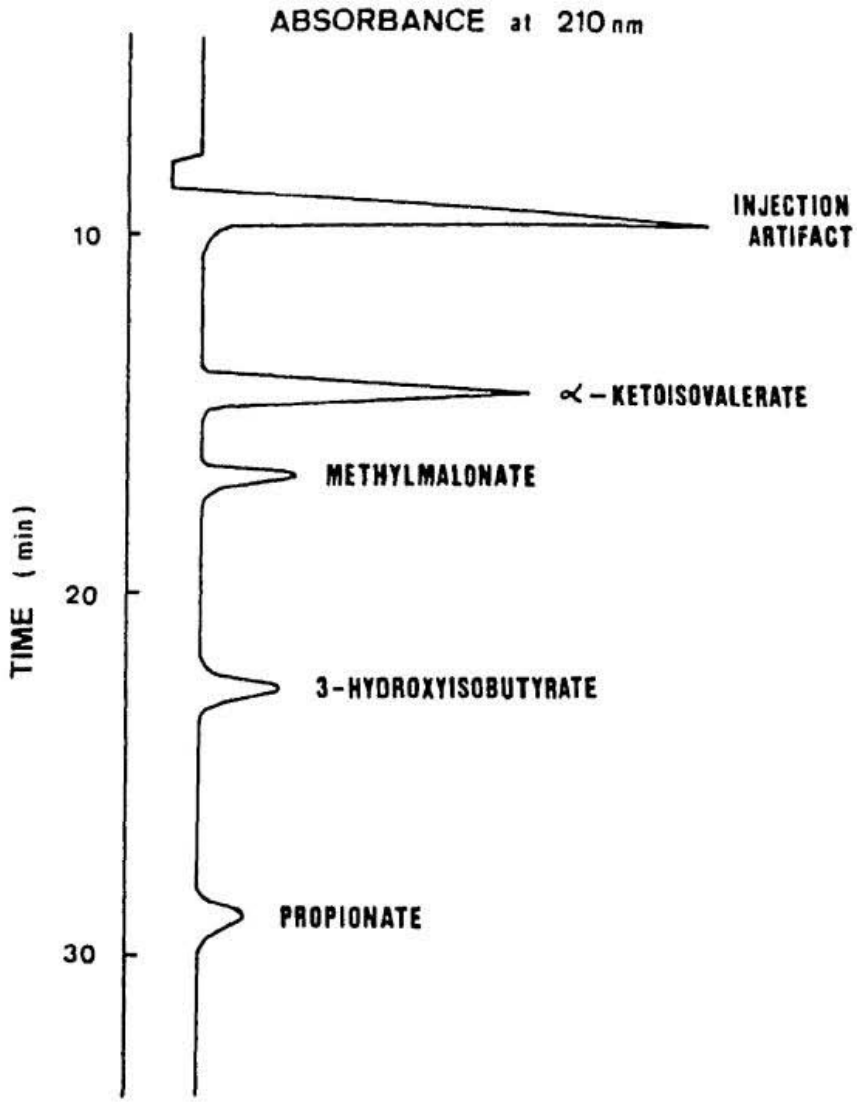


FIGURE 6: Separation of intermediates in valine catabolism by HPLC. Alpha-ketoisovalerate, methylmalonate, 3-hydroxyisobutyrate and propionate were separated on an Aminex-HPX organic acid analysis column. Mobile phase was 0.006N H_2SO_4. Absorbance was measured at 210nm.

(4) AMINO ACID ANALYSIS

Samples of the perfusate were also fractionated on a Beckman amino acid analyser so as to see whether KIV was converted to an amino acid. Effluent perfusate samples (10ml) from hearts perfused with 1.0mM [U-^{14}C] alpha-ketoisovalerate in the non-recirculating Langendorff mode were concentrated by lyophilization and then taken up in 2ml lithium citrate buffer containing 2% sulfosalicylic acid. They were run through the analyser without ninhydrin reaction and the effluent collected with a fraction collecter and counted in Aquasol in a Beckman LS-233 liquid scintillation counter.

(5) ASSAY OF INTERMEDIATES IN FATTY ACID METABOLISM

Hearts were perfused for 30 min with Krebs-Henseleit buffer containing either 1mM KIV, 1mM KIC or 0.1mM oleate complexed to 0.5% BSA. Hearts were freeze clamped at the end of perfusion period while still attached to the cannula using aluminum tongs that were precooled in liquid nitrogen. Hearts were powdered using a mortar and pestle precooled in liquid nitrogen. The powder was deproteinized with 6% perchloric acid and neutralized with KOH (Hems and Brosnan, 1970). The supernatant fraction was used to assay intermediates of fatty acid metabolism.

Free and acetyl-CoA were assayed spectrophotometrically as described by Alfred and Guy (1969). Free carnitine was measured using a radiochemical assay as described by McGarry and Foster (1976). Acetyl-l-

carnitine was measured as described by Bergmeyer (1974b). Long-chain acyl-CoA and long-chain acyl-carnitine were measured as described by Williamson and Corkey (1979).

(6) BRANCHED-CHAIN KETOACID DEHYDROGENASE

Hearts were perfused in the non-recirculating Langendorff mode with 0.1mM oleate complexed to 0.5% BSA for 30 min. During the last 10 min of perfusion either KIC or KIV were infused to a final concentration of 1.0mM. The active portion of the branched-chain ketoacid dehydrogenase activity was measured as described by Kasperek et al. (1985).

(7) ISOLATED KIDNEY TUBULES

Cortical tubules from kidneys were prepared by collagenase digestion as described by Lowry and Ross (1980). Tubules (about 10mg dry weight) were incubated with constant shaking in stoppered 25ml Erlenmeyer flasks for 30 min at 37°C in a total volume of 2mls of Krebs-Henseleit medium gassed with 95% O_2/5% CO_2. The incubations were terminated by addition of perchloric acid (final concentration 3%), extracts were neutralized with potassium phosphate and assayed for glucose.

(8) HEPATOCYTES

Hepatocytes were prepared by collagenase perfusion of the liver as described by Krebs et al. (1974). Hepatocytes (about 5mg dry weight) were

incubated in a total volume of 4mls Krebs-Henseleit medium gassed, with 95% O_2/5% CO_2, for 60 min at 37°C. Incubations were terminated by addition of perchloric acid (final concentrations 3%). Extracts were neutralized with potassium phosphate and assayed for glucose. Viability of hepatocyte preparations was assessed by determining the latency of lactate dehydrogenase (Morrison et al., 1966). In the cell preparations included in this study, lactate dehydrogenase was 96-98% latent.

(9) GLUCOSE

Glucose was measured by a standard enzymatic assay as described by Bergmeyer et al. (1974c). Results were corrected for glucose formed in the absence of added substrate.

(10) PRESENTATION AND ANALYSIS OF DATA

Data are reported as mean $\pm$S.D. with the sample size (n) in parentheses. Differences between labelled CO_2 production in samples collected from non-recirculating Langendorff perfusions at 15-20 min (averaged) and 45-50 min (averaged) for the same perfusion were compared by paired t-test. These $^{14}CO_2$ values show the initial (ie.) before infusion, and final (ie.) after infusion, oxidation rates of labelled substrates.

RESULTS AND DISCUSSION

(1) EFFECTS OF BRANCHED-CHAIN KETOACIDS ON FATTY ACID OXIDATION

A typical experiment is shown in Figure 7. In these experiments hearts were perfused in the non-recirculating Langendorff mode with 1-^{14}C oleate present as substrate throughout the perfusion. KIC was infused from 25 min to 50 min at a final concentration of 1.0mM. Infusion of KIC resulted in a 50% inhibition of oleate oxidation after 20-25 min of infusion without affecting the rate of oxygen consumption.

Oleate decarboxylation rates were calculated in the presence and absence of ketoacids. These rates were calculated using $^{14}CO_2$ production data. The rates of oleate decarboxylation in the absence of ketoacids is calculated from 15-20 min samples, at this time the decarboxylation rate is in a steady state. The decarboxylation rates in the presence of ketoacids are calculated from 45-50 min samples. At this time the decarboxylation rates have not yet reached a steady state. The perfused heart preparation used in these experiments were stable for 50 min, after this time there was a deterioration in some of the hearts being perfused. Since the competence of the hearts were only confirmed for 50 min it was decided that decarboxylation rates would be calculated from data obtained using 45-50 min samples. Our estimates of oleate decarboxylation were therefore slightly underestimated. KIC actually caused a greater inhibition of oleate decarboxylation than our data would suggest.

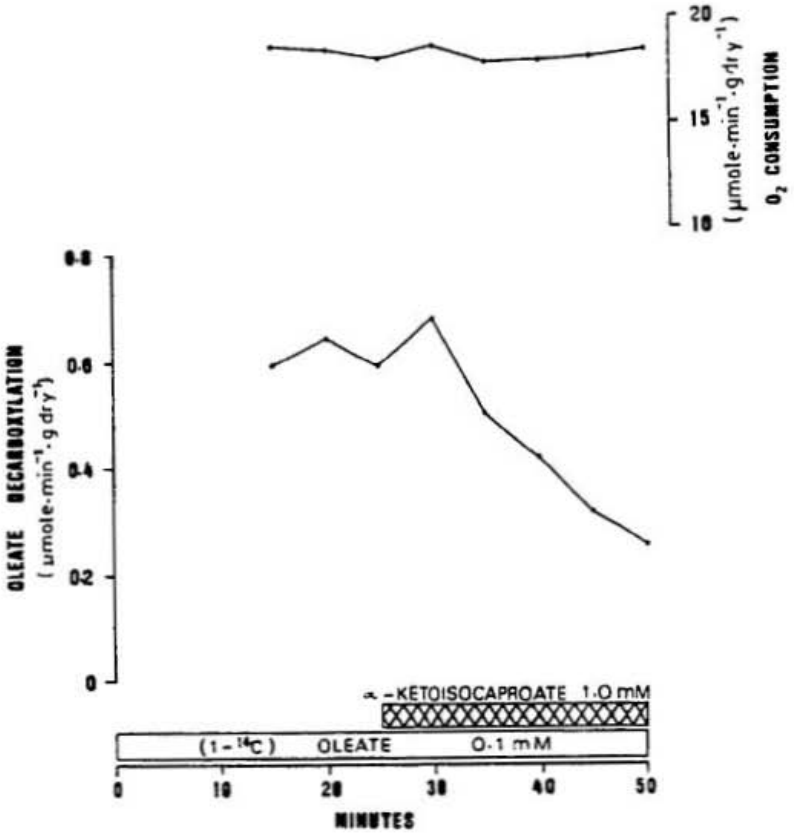


Figure 7: Inhibition of oleate oxidation by alpha-ketoisocaproate
Hearts were perfused for 50 min. with [1-^{14}C]-oleate present throughout. Alpha-ketoisocaproate (final concentration 1.0mM) was infused from 25-50 min. [1-^{14}C]-oleate$\rightarrow$$^{14}CO_2$ is expressed as umol $^{14}CO_2$/min/g dry wt.

The kinetics of the inhibition showed that a single flux component with a $t_{\frac{1}{2}}$ of about 15 min was involved. This was obtained from a semi-log plot of disintegrations per minute vs. time for the inhibition of oleate oxidation by alpha-ketoisocaproate. The $t_{\frac{1}{2}}$ is defined as the time taken for half the counts in CO_2 to disappear. The relatively slow decrease in $^{14}CO_2$ evolution does not mean that the inhibition of fatty acid oxidation occurs at the same rate. When oleate is oxidized the ^{14}C label is distributed throughout the Krebs cycle. In the Krebs cycle, CO_2 is released by the enzymes isocitrate dehydrogenase and alpha-ketoglutarate dehydrogenase. In a given turn of the cycle, the C atoms that are evolved as CO_2 come not from the acetyl groups that just entered the cycle but from the oxaloacetate with which it condenses. Therefore, inhibition of the entry of labelled ^{14}C-acetyl CoA into the Krebs cycle (i.e.) by inhibiting fatty acid oxidation, will not result in an equally rapid inhibition of $^{14}CO_2$ evolution. $^{14}CO_2$ will continue to be evolved from labelled Krebs cycle intermediates, and metabolites that can be converted to these intermediates. In the perfused heart, there is a pool of amino acids (glutamate, glutamine and aspartate) that are formed from and converted to Krebs cycle intermediates (alpha-ketoglutarate and oxaloacetate). This amino acid pool becomes labelled during the oxidation of ^{14}C-acetyl CoA in the Krebs cycle. When ^{14}C-acetyl CoA entry into the cycle is halted, the decrease in $^{14}CO_2$ evolution is determined by the size and degree of labelling of this pool and by the rate of cycle turnover. The inhibition of the β-oxidation of 1-^{14}C oleate by KIC shown in Figure 7 therefore, occurs at a faster rate than indicated by a $t_{\frac{1}{2}}$ of 15 min from $^{14}CO_2$ evolution. The inhibition of the β-oxidation of ^{14}C-oleate by KIC, which

has a $t_{\frac{1}{2}}$ of 15 min for $^{14}CO_2$ evolution is much slower however than the inhibition of ^{14}C-oleate by pyruvate, which has a $t_{\frac{1}{2}}$ of 3.5 min for $^{14}CO_2$ evolution (Forsey & Brosnan, 1987). The pathway for KIC oxidation to acetyl-CoA involves more steps than the oxidation of pyruvate to acetyl-CoA and it may be for this reason that the inhibition of oleate oxidation by pyruvate may occur at a faster rate than inhibition by KIC. The inhibition of oleate oxidation by KIC is dependent on the concentration of infused KIC (Figure 8). 1.0mM KIC causes a 50% inhibition of oleate oxidation whereas 0.1mM KIC causes a 10% inhibition after 20-25 min of infusion. The inhibition of oleate oxidation by KIC, is dependent on the concentration of KIC and is not linear with concentration. The inhibition of oleate oxidation by KIC is shown to be reversible (Figure 9). In these experiments, hearts were perfused in the non-recirculating Langendorff mode for 60 min with [1-^{14}C]-oleate present as substrate throughout the perfusion, KIC was infused from 20 to 40 min. At 35-40 min KIC had inhibited oleate decarboxylation by 45%. 15-20 min after KIC infusion ceased, $^{14}CO_2$ evolution had returned to pre-infusion levels. Oxygen consumption remained constant throughout the perfusion. Thus the inhibitory effect of KIC on oleate oxidation was readily reversed upon removal of KIC.

In the tables that follow, the rate of $^{14}CO_2$ evolution is given before (20-25 min) and during (45-50 min) infusion of an unlabelled potential competitor. As in Figure 7, the infusion of the ketoacid began at 25 min and was sustained to the end of the perfusion.

The physiological situation where fatty acid oxidation was most sensitive to inhibition by branched-chain ketoacids was assessed using 3 experimental models:

(1) 48 hr starved rats

(2) 48hr starved rats refed a high protein diet for 2 hr prior to use

(3) rats fed Purina rat chow

All rats were allowed free access to water. Hearts were perfused for 50 min with [1-^{14}C]-oleate as substrate, throughout.

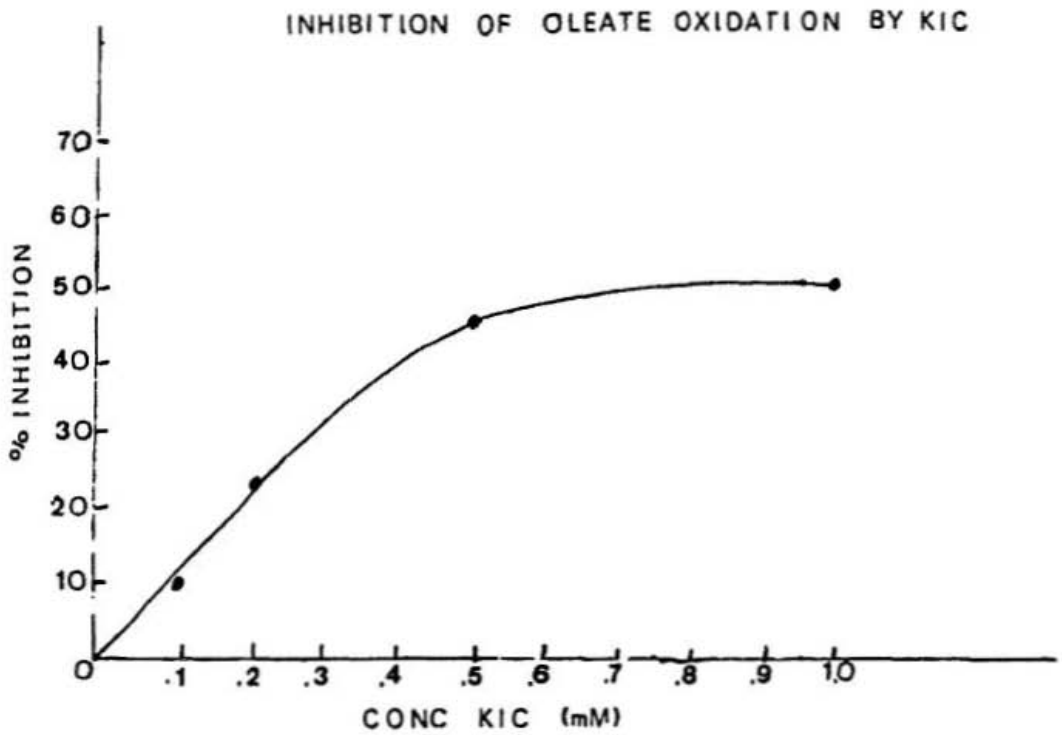


FIGURE 8: Percent inhibition of oleate decarboxylation by alpha-ketoisocaproate. Hearts were perfused for 50min. with 0.1mM [1-^{14}C]-oleate present throughout. Alpha-ketoisocaproate was infused from 25-50min. (final concentration 0.1mM - 1.0mM).

FIGURE 9: Inhibition of oleate oxidation by alpha-ketoisocaproate. Hearts were perfused for 60 min with 0.1mM [1-^{14}C]-oleate present throughout. KIC (final concentration 1.0mM) was infused from 20 - 40 min. [1-^{14}C]-oleate $\rightarrow$ $^{14}CO_2$ is expressed as umol $^{14}CO_2$/min/g dry wt.

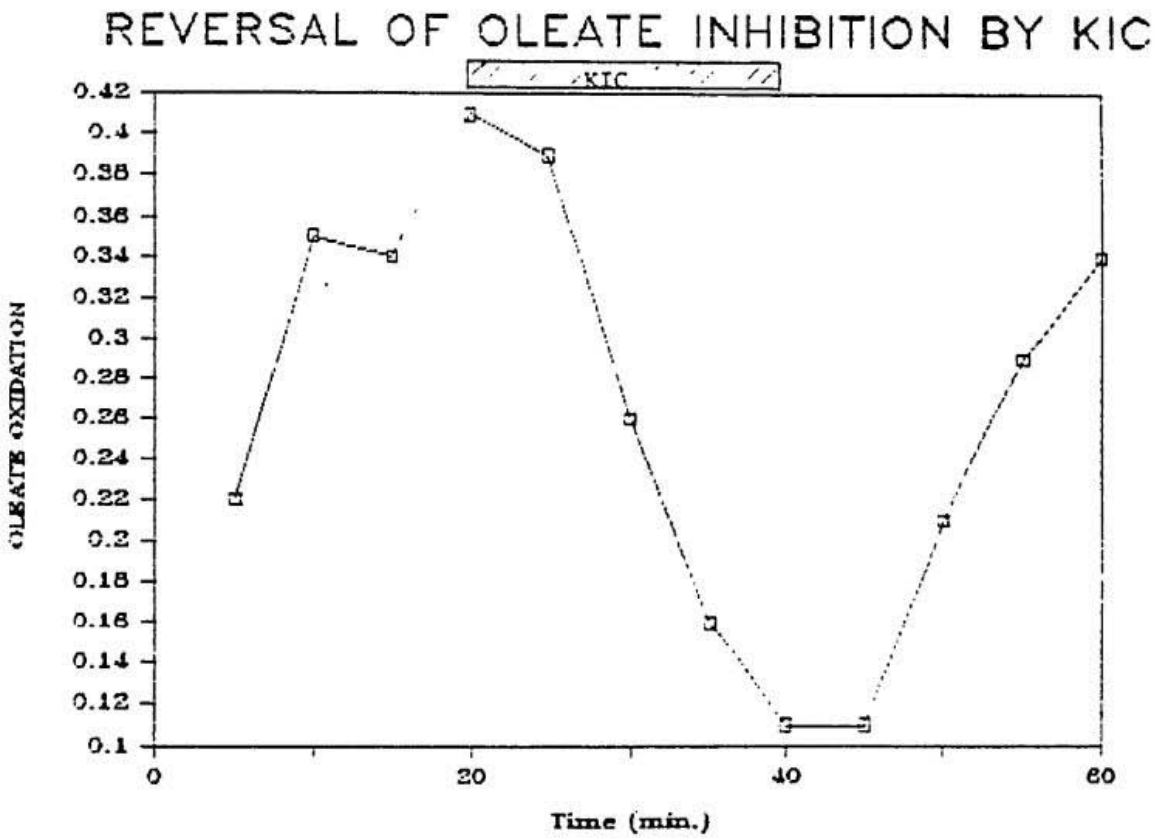


TABLE 1: Effect of nutritional state on the inhibition of oleate decarboxylation by KIC. Hearts were perfused for 50 min. with 0.1mM [1-^{14}C]-oleate present throughout. KIC (final concentration 0.5mM) was infused from 25 - 50 min. Decarboxylation rates shown are averaged for 15 - 20 min samples for pre-infusion values and 45 - 50 min for post-infusion values.

	Fatty acid oxidation (μmol $^{14}CO_2$/min/g dry wt.)	
Nutritional state	pre-infusion	post-infusion
48hr starved	0.58 ± 0.01 (3)	0.48 ± 0.04 (3)
48hr starved refed	0.58 ± 0.01 (3)	0.32 ± 0.05 (3)
fed	0.54 ± 0.01 (3)	0.31 ± 0.01 (3)

It has been shown in rat liver which contains approximately 70% of the total body branched-chain dehydrogenase complex, that the complex is mainly in the active (dephosphorylated) form in the fed state. In starvation, total activity of the complex and the proportion of the complex in the active form are decreased (Patston et al., 1984). Skeletal muscle contains a relatively small amount of the complex which is mainly in the inactive (phosphorylated) form. In skeletal muscle, fasting is associated with an increased rate of BCAA metabolism (Wagenmakers and VeerKamp, 1984). The 48 hr starved rats refed a high protein diet for 2 hrs showed the same sensitivity to KIC inhibition of oleate decarboxylation as the fed animals. In the 2 hr refeeding period enzymes involved in branched-chain amino acid catabolism may have been activated again, as the hearts from refed rats showed a response similar to normal rats. Since the chow fed rats gave as good a response as any other they were used in subsequent experiments.

Tables 2 and 3 contain data on oxygen consumption and oxidation of octanoate and oleate by perfused hearts in the presence and absence of KIC. The total oxidation of one mole of octanoate obliges the consumption of 11 moles of O_2, whereas oleate oxidation obliges the consumption of 25.5 moles of O_2 as described by the following equations:

Equations:

$$C_{18}O_2H_{34} + 25.5O_2 \text{ -----> } 17H_2O + 18CO_2$$

$$C_8O_2H_{16} + 11O_2 \quad \text{-----} > \quad 8H_2O + 8CO_2$$

The ratio of O_2 consumption to $^{14}CO_2$ evolution therefore provides information on the degree to which the fatty acid is able to satisfy the total fuel requirements of the heart. From the data in Table 2, the mean rates of octanoate oxidation and oxygen consumption for the period between 20-25 min, were 2.4 and 18.11 μmol/min/g dry wt., respectively. Thus the mean ratio of O_2 consumption to $^{14}CO_2$ production evolution was 7.5, implying that externally added octanoate can account for the entire oxygen consumption of the heart. This figure of 7.5 is low but the error is constant throughout all perfusions. One possible explanation, which we did not persue, is that a terminal fragment (C_2 or C_4) of the octanoate may escape oxidation and be released in the effluent. If a C_2 or C_4 fragment of octanoate does escape oxidation the number of mols of oxygen required for the complete oxidation of octanoate would be less than the 11 mols calculated above. Alternately we may have had a constant error. The oxidation of octanoate is slightly activated by the infusion of KIC (Table 2). This activation of octanoate oxidation is accompanied by a slightly increased oxygen consumption. Both oxidation rate and oxygen consumption data represent a significant increase.

The mean rates of apparent oleate oxidation to $^{14}CO_2$ and of oxygen consumption (Table 2) for the period between 20-25 min, were 0.65 and 18.33 μmol/min/g dry wt., respectively. The mean ratio of $^{14}CO_2$ production for the same period was 29.7, implying that externally added oleate can

account for,at most, 90% of the total oxygen consumption of the heart. The number of mols of oxygen required for the complete oxidation of oleate is 25.5. Since oxygen consumption was 29.7, some substrate other than oleate is being oxidized. As shown previously in Figure 7 and in Table 2. KIC caused a marked inhibition of $^{14}CO_2$ production from [1-^{14}C] oleate. There was approximately a 50% decrease in $^{14}CO_2$ production from [1-^{14}C] oleate after 25 min of KIC infusion.

TABLE 2: Effect of KIC on oxidation of fatty acids and oxygen consumption by perfused hearts. Hearts were perfused for 50 min with 0.1mM [1-^{14}C]-fatty acid present as substrate throughout the perfusion. KIC (final concentration 1.0mM) was infused from 25 - 50 min. Decarboxylation rates are expressed as umol $^{14}CO_2$/min/g dry wt. and oxygen consumption as μmol/min g dry wt.

*($p<0.05$ as compared to pre-infusion values).

	FATTY ACID	O_2 CONSUMPTION
	octanoate $\longrightarrow$ CO_2	O_2 uptake
pre-infusion	2.4 ± 0.5 (3)	18.11 ± 1.3
post-infusion	3.3 ± 0.6* (3)	20.40 ± 2.0*
	oleate $\longrightarrow$ CO_2	O_2 uptake
pre-infusion	0.65 ± 0.11 (4)	18.33 ± 1.84
post-infusion	0.31 ± 0.06* (4)	18.22 ±2.07

Inhibition of fatty acid oxidation was not accompanied by a decreased oxygen consumption. Therefore, the inhibition of fatty acid oxidation involved the partial replacement of fatty acid as fuels of respiration by the competing fuel rather than by an inhibition of respiration. The decreased evolution of $^{14}CO_2$ was therefore due to the dilution of the specific activity of the mitochondrial acetyl CoA pool by unlabelled acetyl CoA derived from the competing fuel. It was possible that the differences in the effect of KIC on oleate and octanoate could involve albumin since perfusions with long-chain fatty acids were carried out in the presence of 0.5% albumin, whereas those with medium-chain fatty acids were carried out without albumin. A series of control experiments were therefore performed in which octanoate perfusions were carried out in the presence of 0.5% albumin. KIC did not inhibit octanoate oxidation in the presence of albumin.

(2) EFFECTS OF FATTY ACIDS ON THE OXIDATION OF [1-^{14}C] AND [U-^{14}C] LABELLED KETOACIDS;

The inhibition of oleate oxidation was unique to the ketoacid of leucine (KIC). The other two branched-chain keto-acids, KIV and KIL, do not inhibit oleate oxidation (Table 3).

There are two possible explanations for the inhibition of oleate oxidation by KIC and not by KIV or KIL:

(1) The branched-chain ketoacid dehydrogenase could be activated to a greater extent by KIC than by KIV or KIL and therefore flux through this enzyme may be less with KIV than with KIC.

(2) KIC may be completely oxidized whereas KIV and KIL may be only partially oxidized.

To test the first of these possibilities, that the activity of the branched-chain ketoacid dehydrogenase complex varies depending on which ketoacid is present in the heart, I measured the active portion of branched-chain ketoacid dehydrogenase in extracts from hearts perfused in the non-recirculating Langendorff mode. Oleate was present throughout the perfusion (perfusion time 30 min). Alpha-ketoisocaproate or alpha-ketoisovalerate was infused during the last 10 min of perfusion (i.e. 20-30 min). A control where neither ketoacid was present (i.e. oleate alone) was also performed. The results are shown in Table 4. Both alpha-ketoisocaproate and alpha-ketoisovalerate activated the complex over oleate perfusion alone.

TABLE 3: Effect of branched-chain ketoacids on oleate oxidation. Hearts were perfused for 50 min with 0.1mM-[1-^{14}C]-oleate present throughout. Competing substrates were infused from 25 - 50 min. Decarboxylation rates for [1-^{14}C]-oleate $\rightarrow$ $^{14}CO_2$ and O_2consumption are expressed as μmol/min/g dry wt. Values shown are averaged for 15 - 20 min samples for pre-infusion and 45 - 50 min for post infusion.

*$P < 0.05$ as compared to pre-infusion values.

Infused substrate		Fatty Acid Oxidation	O_2Consumption
saline	pre-infusion	0.49 ± 0.06 (4)	18.30 ± 5.7
	post-infusion	0.54 ± 0.07 (4)	16.92 ± 1.62
KIC	pre-infusion	0.65 ± 0.11 (4)	18.33 ± 1.84
	post-infusion	0.31 ± 0.06* (4)	18.11 ± 1.3
KIV	pre-infusion	0.47 ± 0.10 (3)	15.70 ± 3.57
	post-infusion	0.47 ± 0.09 (3)	14.94 ± 3.57
KIL	pre-infusion	0.59 ± 0.05 (3)	16.08 ± 1.24
	post infusion	0.55 ± 0.005 (4)	16.64 ± 1.95

TABLE 4: Branched-chain ketoacid dehydrogenase activity. Hearts were perfused for 30 min with 0.1mM oleate present throughout. Competing substrates were infused from 20 - 30 min. Hearts were freeze clamped and extracts were assayed for branched-chain ketoacid dehydrogenase (BCKDH) activity. BCKDH activity is expressed as nmol/g dry wt./min.

Infused second substrate	BCKDH activity
none	11.62 ± 3.45 (3)
KIC	98.45 ± 43.71 (3)
KIV	58.82 ± 35.16 (3)

However, there is no statistically significant difference in branched-chain ketacid dehyodrgenase activity when hearts were perfused in the presence of either ketoacid (98.45 vs. 58.82). If more experiments were performed, alpha-ketoisocaproate might have been shown to be a better activator of the branched-chain ketoacid dehydrogenase complex than alpha-ketoisovalerate. This has been reported previously by Buffington et al. (1979) using perfused rat heart. As is, the variation are too large to permit us to identify any difference between the two ketoacids.

The activity of the branched-chain ketoacid dehydrogenase complex, (as measured by us using the method described by Kasperek et al., 1985) while being comparable to that obtained by other investigators was not sufficient to account for the decarboxylation rates of alpha-ketoisocaproate and alpha-ketoisovalerate observed in the perfused heart. The BCKA oxidation rates we obtained using the isolated perfused heart are similar to those reported by Hutson (1986). Similar discrepancies between the capacity of heart muscle to decarboxylate branched-chain ketoacids and the low branched-chain ketoacid dehydrogenase activity in heart as measured <u>in vitro</u> have been reported by several investigators (Waymack et al., 1980; Buffington et al., 1979; Shinnick and Harper, 1976). The prior metabolic condition of the tissue, the handling and the extraction procedure are all important considerations which can cause a marked variation in the measured enzymatic activity of the branched-chain ketoacid dehydrogenase. A loss of activity in the branched-chain ketoacid dehydrogenase complex during purification or during its separation from membranes has also been observed and is regarded as being characteristic of this enzyme complex (Waymack et al., 1980). Since the activity of the

branched-chain ketoacid dehydrogenase complex is consistently lower than the decarboxylation rates of alpha-ketoisocaproate and alpha-ketoisovalerate, the activity of the branched-chain ketoacid dehydrogenase complex must therefore have been under estimated.

The second possibility that alpha-ketoisocaproate is completely oxidized while alpha-ketoisovalerate is not, was examined by comparing $^{14}CO_2$ production from ketoacids that were uniformly labelled versus those that were labelled specifically in the first carbon atom. The basis for this comparison is shown in Figure 10a&b. If alpha-ketoisocaproate were completely metabolised in the heart, the $^{14}CO_2$ released when [U-^{14}C]-alpha-ketoisocaproate is the sole substrate should be 6 times that released when [1-^{14}C]-alpha-ketoisocaproate is the substrate. Similarly, the $^{14}CO_2$ released when [U-^{14}C] alpha-ketoisovalerate is the sole substrate should be 5 times that released when [1-^{14}C]-alpha-ketoisovalerate is the sole substrate. Since, radiolabelled alpha-keto-beta-methylvalerate is not available, it could not be included in this study.

$^{14}CO_2$ production from [1-^{14}C]-alpha-ketoisocaproate was 3.16 μmol $^{14}CO_2$/min/g dry wt. If alpha-ketoisocaproate were completely oxidized $^{14}CO_2$ production from [U-^{14}C] would be 19.08. The actual $^{14}CO_2$ production from [U-^{14}C]-alpha-ketoisocaproate was 16.85 μmol $^{14}CO_2$/min/g dry wt. (Table 5). In both sets of experiments oxygen consumption remained constant throughout the perfusion. These experiments indicate that 88% of the alpha-ketoisocaproate that is decarboxylated at the branched-chain dehydrogenase complex is completely oxidized in heart muscle.

TABLE 5: Effects of fatty acids on ketoacid decarboxylation. Hearts were perfused for 50 min with labelled ketoacid present throughout. Fatty acid was present in the perfusion medium from 25 - 50 min. Ketoacid decarboxylation is expressed as μmol $^{14}CO_2$/min/g dry wt. Oxygen consumption is expressed as μmol/min/g dry wt. Values shown are averaged from 15 - 20 min samples for initial rates and 45 - 50 min samples for post fatty acid rates.

	A		B		C	
	INITIAL	O_2 CONSUMPTION	POST OLEATE	O_2 CONSUMPTION	POST OCTANOATE	O_2 CONSUMPTION
[1-^{14}C]-KIC	3.16 ± 0.26 (6)	16.0 ± 1.6	2.54 ± 0.77 (3)	15.5 ± 1.4	0.27 ± 0.10 (3)	17.6 ± 2.6
Predicted for [U-^{14}C]-KIC	19.1		15.2		1.6	
Actual [U-^{14}C]-KIC	16.85 ± 0.39 (6)	16.7 ± 1.7	21.00 ± 0.85 (3)	15.7 ± 1.4	3.96 ± 0.08 (3)	17.8 ± 1.4
[1-^{14}C]-KIV	5.41 ± 0.94 (6)	18.3 ± 2.6	3.72 ± 0.77 (3)	19.0 ± 2.5	0.57 ± 0.28 (3)	16.9 ± 1.4
Predicted for [U-^{14}C]-KIV	27.0		18.6		2.8	
Actual [U-^{14}C]-KIV	9.57 ± 0.46 (6)	17.7 ± 1.6	7.00 ± 0.54 (3)	16.3 ± 3.2	1.90 ± 0.07 (3)	16.7 ± 1.3

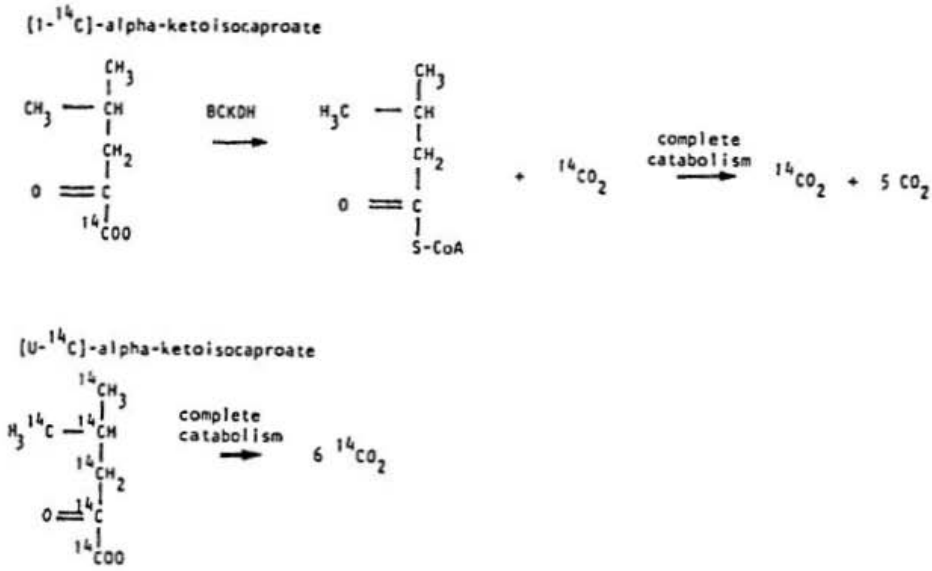
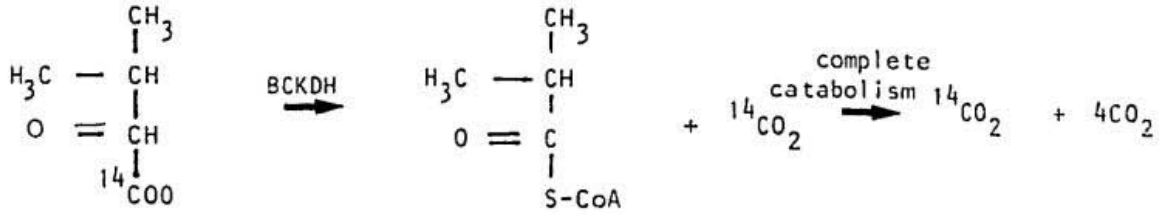


FIGURE 10a: Products of radiolabelled alpha-ketoisocaproate.

[1-^{14}C]-alpha-ketoisovalerate



[U-^{14}C]-alpha-ketoisovalerate

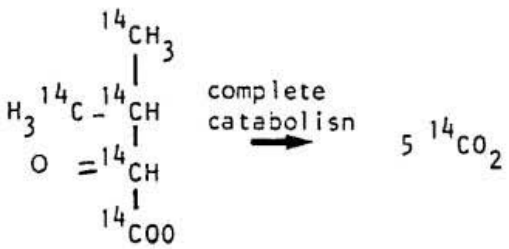


FIGURE 10b: Products of radiolabelled alpha-ketoisovalerate.

The $^{14}CO_2$ production from [1-^{14}C]-ketoisovalerate was 5.41 μmol $^{14}CO_2$ /min/g dry wt. Therefore, if alpha-ketoisovalerate were completely metabolized, the predicted value for $^{14}CO_2$ production from [U-^{14}C]-alpha-ketoisovalerate would be 27.1 However, $^{14}CO_2$ production from [U-^{14}C]-alpha-ketoisovalerate was only 9.57 indicating that only 19% of the alpha-ketoisovalerate decarboxylated by the branched-chain ketoacid dehydrogenase could have been completely metabolised. The flux through the branched-chain ketoacid dehydrogenase is greater with alpha-ketoisovalerate than with alpha-ketoisocaproate (5.41 vs. 3.16 μmol $^{14}CO_2$/ min/g dry wt.). This observation has been reported by several investigators (Waymack et al., 1980; Buffington et al., 1979).

Comparison of [U-^{14}C] data with [1-^{14}C] data, provides a possible explanation for the inhibition of oleate oxidation by KIC and not by KIV or KIL. If complete oxidation occurs, [U-^{14}C] values should be six times higher than [1-^{14}C] values for KIC, and five times higher for KIV (Figure 10a & b). Table 5 shows that initially, and after oleate infusion, KIC is probably completely oxidized, whereas KIV is not.

The oxidation of [1-^{14}C] octanoate as determined by $^{14}CO_2$ production is not decreased by the branched-chain ketoacids. The oxidation appears to be activated by infusion of KIC (Table 2). Conversely, when hearts were perfused with [1-14C] KIC, $^{14}CO_2$ production was all but completely eliminated (3.16 vs. 0.27 μmol/min/g dry) after 25 min of octanoate infusion. The inhibition of KIC oxidation by octanoate occurs much more rapidly than the inhibition of KIC oxidation by oleate. In fact 5 min

after the infusion of octanoate KIC, oxidation was decreased by 74%. Octanoate infusion also inhibited $^{14}CO_2$ production from hearts oxidizing [1-^{14}C] KIV (5.41 vs. 0.57 μmol/min/g dry wt.) as shown in Table 5. Octanoate can account for the entire oxygen consumption of the heart, and therefore completely blocks the oxidation of the branched-chain ketoacids.

(3) LEVELS OF KEY INTERMEDIATES IN FATTY ACID METABOLISM WITH INFUSION OF BRANCHED-CHAIN KETOACIDS

In an attempt to localize the step or steps at which alpha-ketoisocaproate inhibited oleate oxidation the levels of intermediates in fatty acid metabolism were measured. Changes in the levels of free CoA, carnitine and their acyl derivatives during oleate oxidation in the presence of alpha-ketoisocaproate and alpha-ketoisovalerate might provide a clue to the steps involved in the inhibition of oleate oxidation by alpha-ketoisocaproate.

Concentrations of intermediates were determined as described in "Materials and Methods". Hearts were perfused with oleate as substrate throughout the perfusion (perfusion time 30 min) Alpha-ketoisocaproate and alpha-ketoisovalerate were infused during the last 10 min of the perfusion. A control where neither ketoacid was present is shown in Table 6.

Levels of free CoA decreased significantly upon infusion of alpha-ketoisovalerate and alpha-ketoisocaproate. Acetyl-l-carnitine was decreased three-fold in hearts perfused with alpha-ketoisovalerate. The levels of the other intermediates measured were not altered significantly with infusion of either alpha-ketoisocaproate or alpha-ketoisovalerate. The basis for the decreased acetyl-l-carnitine with the infusion of KIV is not immediately apparent.

TABLE 6: Levels of key intermediates in fatty acid metabolism. Hearts were perfused for 30 min with 0.1mM oleate present throughout. Ketacids were infused from 20 - 30 min. Hearts were freeze-clamped and extracts were assayed for intermediates. *$P < 0.05$ as compared to oleate alone.

	INFUSION		
	none	KIV	KIC
Free CoA (nmols/g dry)	519.8 ± 1.20 (4)	198.3 ± 62* (3)	113.3 ± 65* (3)
Acetyl CoA (nmols/g dry)	47.5 ± 5.9 (4)	42.0 ± 16.0 (3)	53.4 ± 12.3 (3)
Long-chain acyl CoA (nmols/g dry)	96.3 ± 43.7 (4)	104.7 ± 27.3 (4)	64.6 ± 21.4 (4)
Free Carnitine (µmols/g dry)	5.7 ± 1.4 (4)	4.9 ± 1.3 (4)	5.0 ± 1.2 (4)
Acetyl-l-carnitine (µmols/g dry)	0.161 ± 0.04 (4)	0.055 ± 0.04* (4)	0.189 ± 0.03 (4
Long-chain acyl carnitine (nmols/g dry)	88.2 ± 19.5 (4)	118.3 ± 80 (3)	86.3 ± 18.3 (4)

Since alpha-ketoisocaproate inhibited oleate oxidation but not octanoate oxidation, we felt the inhibition might have been carnitine related. However, there is no suggestion that carnitine-dependent processes are limiting oleate oxidation upon infusion of KIC since acylcarnitine and long-chain CoA derivatives are not altered. The drop in free CoA levels with both infusion of KIV and KIC remain unaccounted for by changes in Acetyl-CoA and long-chain acyl-CoA. CoA intermediates in ketoacid metabolism probably accounted for the change in tissue CoA levels. This data does not, however, provide an explanation for the inhibition of oleate oxidation by alpha-ketoisocaproate.

(4) RELEASE OF AN INTERMEDIATE OF KIV METABOLISM

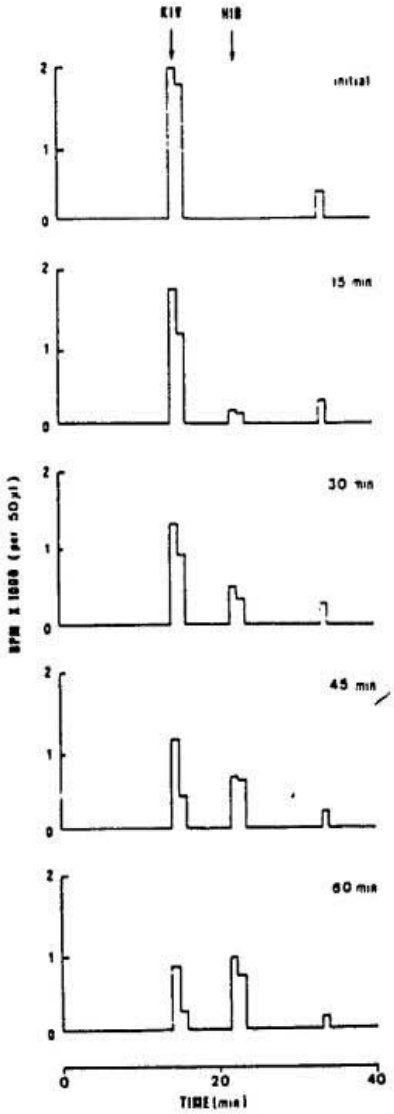
Comparison of $^{14}CO_2$ evolution from hearts perfused with [1-^{14}C] KIV and those perfused with [U-^{14}C] KIV show that only, at most, 19% of the KIV passing through the branched-chain ketoacid dehydrogenase complex is completely oxidized. Since KIV is not completely metabolised in the isolated perfused rat heart, either some some intermediate of KIV metabolism accumulates in the heart or is released. We first tested to see if the metabolite was an amino acid. It has been suggested that valine carbon can be utilized to form glutamine and this could account for incomplete oxidation of KIV. To determine whether this might be the case in our system, experiments with [U-^{14}C]-alpha-ketoisovalerate were carried out and we examined incorporation of label into amino acids. Hearts were perfused in the non-recirculating Langendorff mode with [U-^{14}C]-alpha-ketoisovalerate as substrate (perfusion time 60 min). Samples of

perfusion medium were collected at zero time and at 60 min. These samples were concentrated (10mls medium lyophilized and taken up in 2mls lithium citrate buffer pH 2.2 containing 2% sulfosalicylic acid). Samples of concentrated medium were then separated on an amino acid analyser the effluent was collected and counted. Due to the transamination of alpha-ketoisovalerate to valine, label was present in the valine and ketovaline fractions only. The glutamine fraction showed no change in radioactivity between the zero time and 60 min samples. Therefore, the released intermediate of valine catabolism is not glutamine.

Investigators using skeletal muscle or mammary gland have suggested that 3-hydroxyisobutyrate (an intermediate in KIV metabolism) was released during valine catabolism (Spydevold, 1979; Wohlt et al., 1977; Lee & Davis, 1986). Therefore, we felt that the intermediate released from our isolated perfused heart system during ketovaline catabolism could be 3-hydroxyisobutyrate. To test the possibility that 3-hydroxyisobutyrate was being released in our perfused heart system during valine catabolism, we perfused hearts in the recirculating Langendorff mode for 60 min with [U-^{14}C] KIV present as substrate throughout the perfusion. Samples of medium were collected (0, 15, 30, 45 & 60 min) and analysed by HPLC using a system that separated KIV from its metabolites, namely, propionate, methylmalonate and 3-hydroxyisobutyrate (Figure 6). Samples were separated on the column and fractions collected. Fractions were counted in Aquasol in a Beckman LS-233 liquid scintillation counter to ensure that the intermediate released was radioactive and therefore a metabolite of the labelled KIV. As shown in Figure 11a, there was a decrease in

radiolabelled KIV and an increase in radiolabelled 3-hydroxyisobutyrate as perfusion time increased. Figure 11b shows 3-hydroxyisobutyrate was the major end-product of KIV metabolism in the isolated perfused rat heart. At 30 min, 50% of the KIV removed could be accounted for as 3-hydroxyisobutyrate.

a.



b.

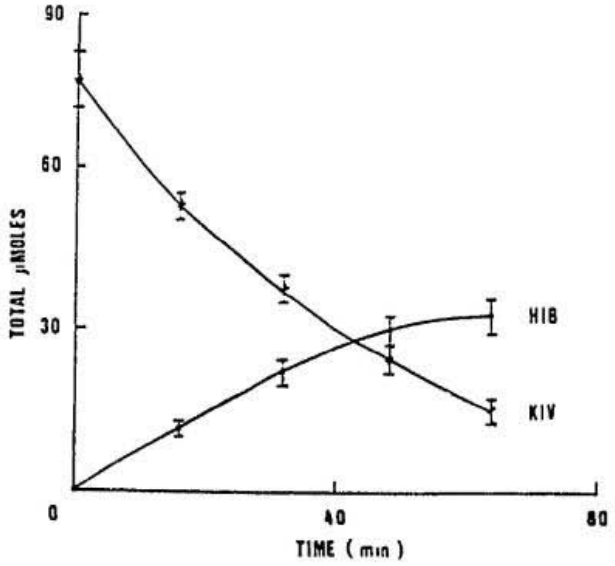
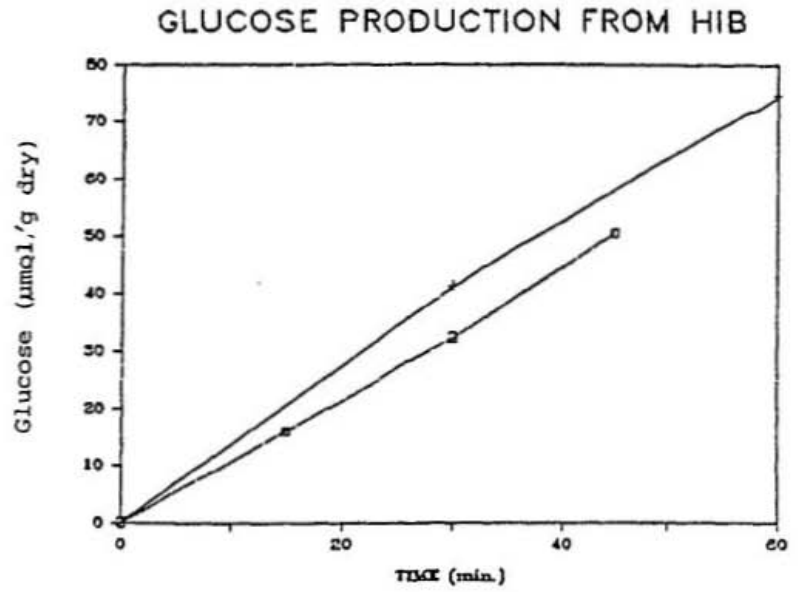


FIGURE 11a & b: Products of alpha ketoisovalerate catabolism. Hearts were perfused in the recirculating Langendorff mode for 60 min with [U-^{14}C]-KIV present throughout. Medium samples were collected at 15 min intervals. Medium was separated by HPLC using an Aminex HPX-87H organic acid analysis column. Radioactivity was measured in the collected fractions (a). Total μmol of HIB and KIV present in perfusion medium was calculated at 15 min intervals (b).

(5) 3-HYDROXYISOBUTYRATE AS A GLUCONEOGENIC SUBSTRATE IN ISOLATED KIDNEY CORTICAL TUBULES AND HEPATOCYTES

To test the hypothesis that the 3-hydroxyisobutyrate released from the heart during valine catabolism is gluconeogenic in the liver and kidney, we incubated isolated kidney cortical tubules or hepatocytes with 3-hydroxyisobutyrate. Glucose was readily formed from 3-hydroxyisobutyrate by both kidney cortical tubules and hepatocytes. Glucose production by isolated cortical tubules was linear for at least 45 min with either 3-hydroxyisobutyrate (Figure 12a) or KIV (Figure 12b) as substrates. Consequently, we used 30 min as the standard incubation time. Each incubation flask contained approximately 1.0mg dry wt. tubules. The effect of substrate concentration on glucose production is shown in Figure 13. Glucose production was maximal at 2mM 3-hydroxyisobutyrate in tubules (Figure 13a). There was no further increase in activity with substrate concentrations above 2mM. Maximal rates of glucose production were observed in kidney tubules with 0.5mM KIV (Figure 13b).

A.



B.

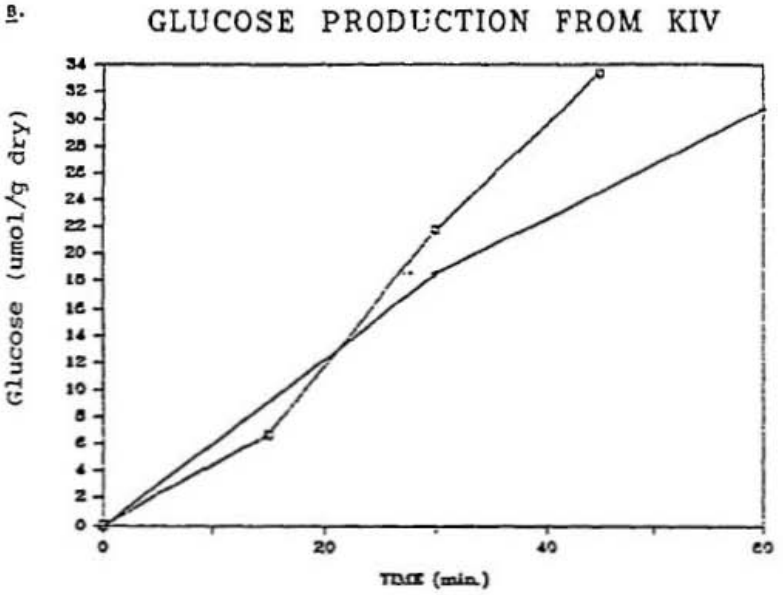


FIGURE 12: Glucose production as a function of time in hepatocytes and isolated kidney cortical tubules. Incubations contained either HIB (a) or KIV (b) as substrate. Hepatocytes from 48 h fasted rats were incubated with 5mM HIB or 2mM KIV for up to 60min (+ - +). Tubules from fed rats were incubated with 2mM HIB or 0.5mM KIV for up to 45 min (□-□).

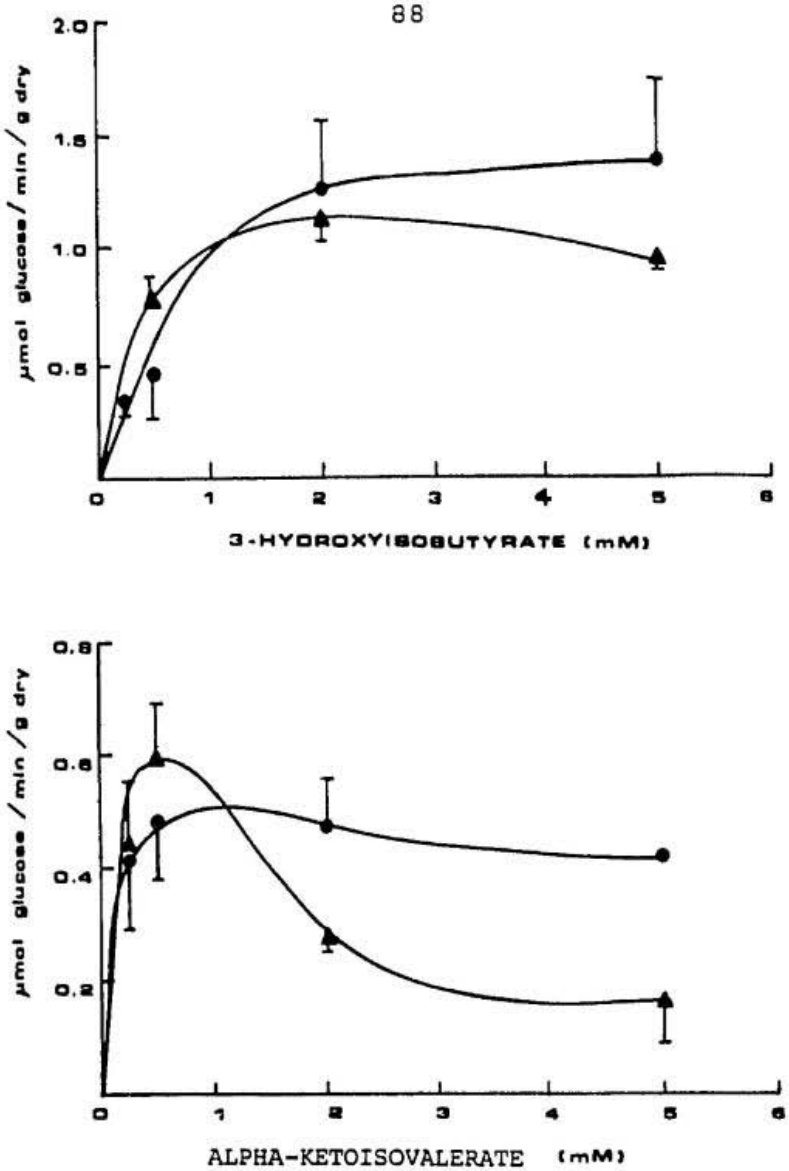


FIGURE 13 a & b: Glucose production as a function of substrate concentration in hepatocytes and isolated cortical tubules. Hepatocytes from 48 h fasted rats and kidney cortical tubules from fed rats were prepared as described in Materials and Methods. Incubations contained either 3-Hydroxyisobutyrate (a) or alpha-ketoisovalerate (b) as substrate. Hepatocytes were incubated for 60 min and kidney cortical tubules for 30 min. Data are means ± S.D. of five experiments for hepatocytes and three experiments for tubules with each substrate. In each panel, hepatocytes are shown as (● - ●) and kidney cortical tubules as (▲ - ▲).

A significant decrease in glucose production was observed in tubules incubated with concentrations of KIV above 0.5mM. Inhibition of gluconeogenesis in tubules at high concentrations of KIV have been reported by Strumpf and Kraus (1978).

In hepatocytes, glucose production from 3-hydroxyisobutyrate (Figure 12a) or KIV (Figure 12b) is linear for at least 60 min. Standard incubation times for hepatocytes were therefore 60 min. Each incubation flask contained approximately 5mg dry wt. of hepatocytes. The effect of substrate concentration on glucose production is shown in Figure 13a & b. Glucose production was maximal at 2mM 3-hydroxyisobutyrate in hepatocytes, as in tubules (Figure 13a). There was no statistically significant increase in glucose production with further increase in substrate concentration above 2 mM. Maximal rates of glucose production were seen at 0.5mM KIV in hepatocytes (Figure 12b). With hepatocytes no further increase in glucose production was observed up to 5 mM-α-ketoisovalerate. When valine was used as substrate, no glucose was produced.

Table 7 gives the rates of glucose production in isolated cortical tubules and hepatocytes physiological situations in which an elevated rate of gluconeogenesis has been reported were chosen. Tubules were isolated from 48hr. starved rats Tubules from streptozotocin- induced diabetic rats and from rats given 1.5% NH_4Cl in place of drinking water for 7 days. A series of experiments in which casein diets were fed to rats were also performed. Rats fed a 13% casein diet were the control group. Tubules from rats fed a 55% casein diet were examined to see the effect of a high

protein diet on glucose production from 3-hydroxyisobutyrate. A group fed a 55% casein diet and 0.1M $NaHCO_3$ ad libitum in place of drinking water was examined to determine differences in glucose production from 3-hydroxyisobutyrate in tubules from rats fed a high protein diet. This was to determine whether differences were due to protein intake or to the increased acid load associated with a high protein intake. Rats were maintained on these schedules for 7 days. The 0.1M $NaHCO_3$ was found to approximately neutralize the acid production from the high protein diet (Brosnan et al., 1978). Therefore, it is possible to differentiate between effects due to acidosis or those due to the high intake of amino acids.

In isolated cortical tubules from fed rats, the maximal rates of gluconeogenesis from 3-hydroxyisobutyrate and lactate were similar and the rates of gluconeogenesis were higher than from KIV. In all of the physiological states examined there was no difference in glucose production from 3-hydroxyisobutyrate. In kidney cortical tubules, glucose production from lactate doubled in rats given ammonium chloride in place of drinking water, as compared to fed rats (Table 7). Similar results were also observed by Brosnan et al. (1978). Starvation also increased glucose production from lactate. This is in agreement with the report of Iynedjian & Peters (1974). These increases in gluconeogenesis from lactate are paralleled by an increase in phosphoenolpyruvate carboxykinase activity in NH_4Cl fed rats (Brosnan et al., 1978) and in starved rats (Iyndjian & Peters, 1974).

TABLE 7 a&b: Glucose production in isolated cortical tubules (a) and hepatocytes (b) under various physiological conditions. Kidney cortical tubules and hepatocytes were prepared and incubated as described in Materials and Methods. Substrate concentrations were 2mM HIB, and 5mM lactate for tubules and 5mM HIB, 2mM KIV and 10mM lactate for hepatocytes.
*P<0.05 as compared to fed rats.

A.

Condition of rat	HIB	Lactate
Fed	1.2 ± 1.18 (4)	1.42 ± 0.27 (3)
48h starved	0.99 ± 0.31 (3)	2.45 ± 0.48*(3)
Diabetic	0.98 ± 0.24 (3)	1.82 ± 0.10 (3)
1.5% NH_4Cl	0.77 ± 0.34 (3)	2.68 ± 0.91*(3)
13% casein	0.87 ± 0.31 (6)	1.87 ± 0.33 (6)
55% casein	0.74 ± 0.20 (6)	2.12 ± 0.38 (6)
55% casein & 0.1M $NaHCO_3$	0.81 ± 0.22 (6)	1.79 ± 0.30 (6)

B.

Condition of rat	HIB	KIV	lactate
48h starved	1.20 ± 0.68(5)	0.52 ± 0.14 (5)	2.60 ± 0.79 (3)
Diabetic	1.14 ± 0.24 (4)	0.44 ± 0.20 (4)	3.80 ± 0.58*(4)

Data on glucose production by hepatocytes are given in Table 7b. The maximal rate of glucose production from 3-hydroxyisobutyrate by hepatocytes from starved rats was about half of that from 10mM lactate. Diabetes did not significantly influence the rate of gluconeogenesis from any of these substrates. The maximal rate of gluconeogenesis from 3-hydroxyisobutyrate was always greater than that from alpha-ketoisovalerate.

In an attempt to determine the pathway of gluconeogenesis from 3-hydroxyisobutyrate, we used mercaptopicolinate (0.6mM), an inhibitor of phosphoenolpyruvate carboxykinase (Di Tullio et al., 1974). Inhibition of glucose production from 3-hydroxyisobutyrate in tubules was observed. The rates were 11±0.10 (uninhibited) vs. 0.09±0.04 (inhibited) μmols/min/g dry wt.) and hepatocytes 1.14±0.24 (uninhibited) vs. 0.43±0.04 (inhibited) μmols/min/g dry wt.). Thus gluconeogenesis from 3-hydroxyisobutyrate requires phosphoenolpyruvate carboxykinase, as does that from other four carbon intermediates.

GENERAL DISCUSSION

Both heart (Davis and Bremer, 1973) and skeletal muscle (Spydevold et al., 1976) catabolize to a certain extent all three of the branched-chain amino acids. Indeed, our work shows alpha-ketoisocaproate to be an inhibitor of the oxidation of the long-chain fatty acid oleate in the isolated perfused rat heart. Since oxygen consumption was unchanged, ketoleucine must serve as a fuel for this tissue. Since ketoleucine inhibits long-chain fatty acid oxidation, the limiting step could be at one or more of several sites (such as the activation of long-chain fatty acids, the carnitine acyl transferase I or II, or the translocase). Our work also shows that KIV and KIL are good substrates but will not inhibit oleate oxidation.

Since valine is a glucogenic amino acid and leucine is ketogenic, the effects of the different branched-chain ketoacids might be expected to affect fatty acid metabolism differently. This was indeed the case; inhibition of oleate oxidation was unique to alpha-ketoisocaproate. Alpha-ketoisovalerate and KIL did not inhibit oleate oxidation. Two possible explanations for the inhibition of oleate metabolism by KIC and not by KIV or KIL were postulated and tested. The first possibility, that the irreversible enzyme involved in the catabolism of valine and leucine would be activated more by ketoleucine than ketovaline, was not the case. The flux through the branched-chain ketoacid dehydrogenase was greater with KIV as substrate was greater than with KIC as substrate both in the absence (5.41 vs. 3.16 μmol/min/g dry wt) and presence (3.72 vs. 2.54

μmol/min/g dry wt) of oleate (Table 5). This was supported by the fact that branched-chain ketoacid dehydrogenase activities were not greater with alpha-ketoisocaproate than with alpha-ketoisovalerate. Similar findings are reported by Hutson (1986) and Bufferington et al. (1979).

The second possibility, that alpha-ketoisocaproate is completely oxidised whereas alpha-ketoisovalerate is not, is supported by our data. Comparison of [1-^{14}C] and[U-^{14}C] data show that whereas, only 19% of alpha-ketoisovalerate is almost completely oxidised, the alpha-ketoisocaproate is completely oxidised.

The release of 3-hydroxyisobutyrate (an intermediate in valine metabolism) during valine catabolism, has been reported in perfused rat hindquarter (Spydevold, 1979; Lee and Davis, 1986) and in lactating bovine mammary gland (Wohlt, 1976). We have identified 3-hydroxyisobutyrate release in perfused rat hearts metabolising ketovaline. We have also shown that 3-hydroxyisobutyrate is gluconeogenic in kidney and liver. Indeed, maximal rates of gluconeogenesis from 3-hydroxyisobutyrate were greater than from alpha-ketoisovalerate. Studies on the interorgan movement of 3-hydroxyisobutyrate in vivo have not been made. Nevertheless, 3-hydroxyisobutyrate has been reported to be a normal constituent of plasma and indeed, is excreted in the urine of diabetics (Landass, 1975). In normal humans, the concentration of 3-hydroxyisobutyrate in serum is 1-4mg per liter (O.A. Mamer, personal communication) (10-45 μM). This compares with a KIV concentration in plasma from normal humans of 8 μM (Hutson & Harper, 1981). Therefore, we

postulated 3-hydroxyisobutyrate to be an interorgan metabolite in the catabolism of valine (Letto et al., 1986). The levels of branched-chain ketoacids used in our experiments (1.0 mM) are higher than the concentrations found in normal human plasma. The concentrations used approach those seen during the therapeutic administration of branched-chain ketoacids and branched-chain amino acids in the treatment of certain clinical syndromes i.e. sepsis, renal failure and hepatic encephalopathy. A possible pathway is illustrated in Figure 14. When valine catabolism is initiated in muscle, some of the carbon is released as alpha-ketoisovalerate and some is metabolised via the branched-chain ketoacid dehydrogenase and subsequent enzymes of valine catabolism. The intermediates of this pathway are all CoA derivatives until 3-hydroxyisobutyrate which is formed by hydrolysis of 3-hydroxyisobutyryl CoA. The removal of Coenzyme A facilitates transport through membranes so that 3-hydroxyisobutyrate may now leave the muscle and be converted to glucose in the liver and the kidney. Such a scenario requires that 3-hydroxyisobutyrate be a good substrate for transport out of muscle mitochondria and muscle cells on the one hand, and across the cell membranes and mitochondrial membranes of liver and kidney on the other hand. Whether this is the well-described monocarboxylate transporter remains to be determined. Coronel et al. (1986) showed that in mouse spermatozoa, the branched-chain ketoacids do not utilize the monocarboxylate carrier to enter the mitochondria. The situation for 3-hydroxyisobutyrate remains to be determined.

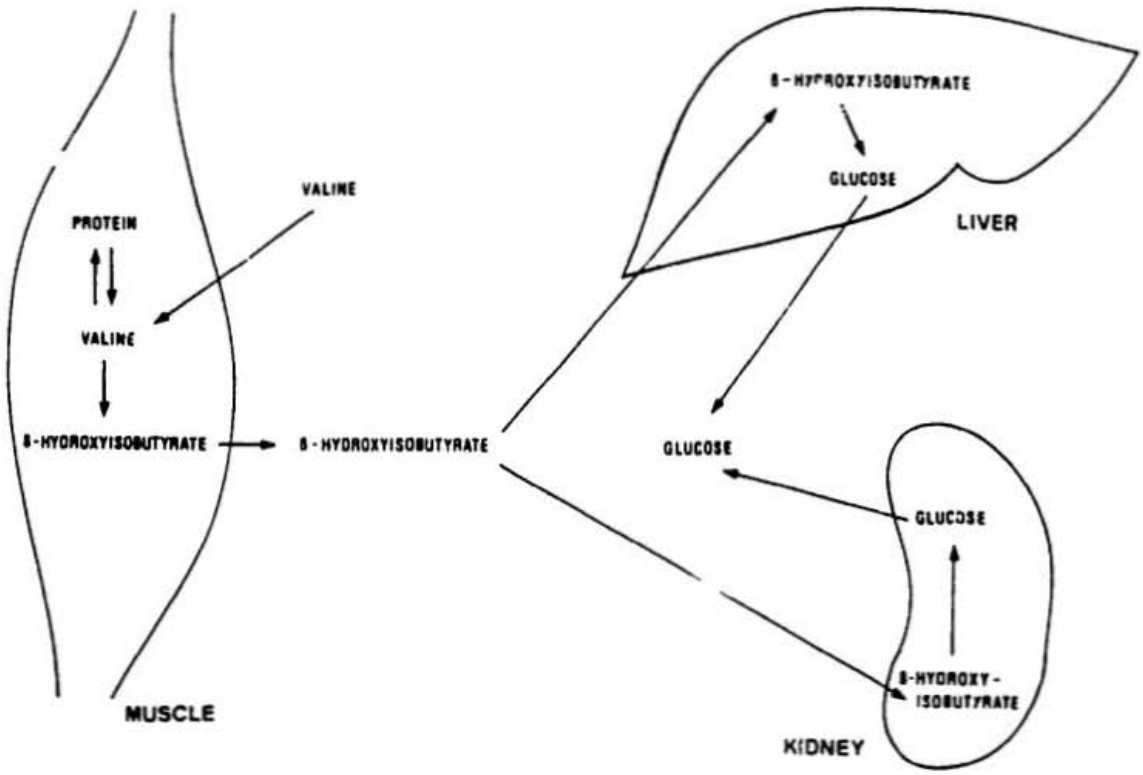


Figure 14: Proposed pathway for the release of 3-hydroxyisobutyrate during valine catabolism in muscle. Valine catabolism in muscle results in the release of alpha-ketoisovalerate and 3-hydroxyisobutyrate. 3-hydroxyisobutyrate leaves muscle and is taken up by gluconeogenic tissues, the liver and kidney, for production of glucose.

The experiments with mercaptopicolinate suggest that gluconeogenesis from 3-hydroxyisobutyrate requires phosphoenolpyruvate carboxykinase and presumably occurs via the conventional gluconeogenic pathway. The maximal rate of gluconeogenesis from 3-hydroxyisobutyrate, however, was always lower than that from lactate. In addition, the maximal rate of gluconeogenesis from 3-hydroxyisobutyrate did not increase in kidney cortical tubules during starvation, a situation in which the maximal rate of gluconeogenesis from lactate did increase, and where the activity of phosphoenolpyruvate carboxykinase has been reported to be increased (Henning et al., 1966). Thus phosphoenolpyruvate carboxykinase may not exert a significant rate limiration on the maximal rate of gluconeogenesis from 3-hydroxyisobutyrate. There are two irreversible reactions between 3-hydroxyisobutyrate and succinyl-CoA (conversion of methylmalonyl semialdehyde to propionyl CoA and propionyl CoA carboxylase) which probably determine the rate of gluconeogenesis from 3-hydroxyisobutyrate.

It has been postulated (Snell, 1980; Spydevold, 1976;Goldstein & Newsholme, 1976; Chang and Goldberg, 1978a & b; Davis & Bremer, 1973) that branched-chain amino acids play a role in providing the carbon skeleton of the alanine and glutamine which is released from skeletal muscle during fasting, in proportions more than present in muscle protein. The nitrogen of alanine and glutamine probably originates from the branched-chain amino acids. It has been suggested that the carbon skeleton of valine provides the carbon necessary for glutamine synthesis. Lee and Davis (1986) showed that in perfused rat hindquarter that some valine carbon reaches the

citric acid cycle and therefore can be oxidized to CO_2 or be converted into other products. Radioactivity was incorporated into citric acid cycle intermediates as well as glutamate, glutamine, lactate and alanine. 2-chloro-4-methyl-valerate stimulates the branched-chain ketoacid dehydrogenase and the removal of endogenous branched-chain amino acids by perfused hindquarter (2-chloro-4-methylvalerate inhibits the kinase which inactivates the dehydrogenase). They also showed that the bulk of the valine was released as 3-hydroxyisobutyrate and a lesser quantity as ketovaline. In hindquarters perfused in the absence of 2-chloro-4-methyl-valerate, incorporation was mainly into 3-hydroxyisobutyrate followed by KIV, lactate, glutamine and glutamate. In hindquarters perfused in the presence of 2-chloro-4-methylvalerate, much more 3-hydroxyisobutyrate accumulated and more radioactivity was found in glutamine, glutamate and alanine. Flux of carbon to all products past the first decarboxylation step was greatly enhanced by 2-chloro-4-methylvalerate. It is clear that the pathway for valine metabolism is regulated at more than one place and that both the branched-chain keto acid dehydrogenase and β-hydroxyisobutyrate dehydrogenase are important.

In heart muscle as in diaphragm, skeletal muscle and mammary gland, valine is not completely metabolised. Approximately only one out of six molecules of valine are catabolised as far as succinyl CoA in these tissues (Wagenmakers et al., 1985). Glutamine is not therefore the major end product of valine catabolism.

It may be, however, that the 3-hydroxyisobutyrate released into the

circulation is taken up by the liver and kidney for gluconeogenesis. In this way, valine, may provide net glucose for energy production. Leucine is completely metabolised in heart muscle, thereby providing energy by the oxidation of acetyl-CoA in the citric acid cycle.

SUMMARY AND CONCLUSIONS

1) The oxidation of the long-chain fatty acid oleate is inhibited by KIC, but not by KIV or KIL.

2) The oxidation of octanoate is modestly activated by KIC.

3) KIC is almost completely oxidized whereas only 19% of KIV is completely oxidized.

4) When KIV is oxidized in the heart there was an intermediate of KIV catabolism released. The intermediate was identified as 3-hydroxyisobutyrate.

5) 3-Hydroxyisobutyrate was shown to be a gluconeogenic substrate in hepatocytes and kidney cortical tubules.

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