ALANINE FORMATION AND BRANCHED-CHAIN AMINO ACID METABOLISM BY RAT SKELETAL MUSCLE

By

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ABSTRACT

Alanine is synthesised de novo by skeletal muscle and released for glucose formation in the liver. For alanine to make a net contribution to the body glucose pool in times of dietary insufficiency its carbon must be derived from non-carbohydrate sources.

A relationship between the metabolism of branched-chain amino acids (BCAAs) and alanine release observed in the present study using muscle preparations incubated with clofibrate acid or hypoglycin, suggested that BCAAs contributed carbon for alanine formation. A pathway for alanine formation from valine was proposed and developed. The phosphoenolpyruvate carboxykinase (PEPCK) inhibitor, 3-mercaptopicolinate, inhibited alanine formation and correlations were observed between dietary stimulation of BCAA oxidation, PEPCK activity and alanine formation. Amino-methoxybutenoate, an inhibitor which blocks mitochondrial aspartate transfer, inhibited BCAA-stimulated alanine release by muscle from 48 h-starved rats. Aspartate aminotransferase was therefore implicated in the pathway.

The subcellular locations of enzymes implicated in this pathway were studied. Using fractional extraction techniques muscle branched-chain aminotransferase activity was found to be associated with the mitochondria-containing fractions. Aspartate aminotransferase, alanine aminotransferase and PEPCK were present in both the soluble and mitochondria-containing fractions.
When verification of the pathway was sought, incubating muscle preparations in the presence of [U-\(^{14}\)C]-valine, negligible amounts of \(^{14}\)C were recovered in the alanine released from the muscle. It was concluded that the pathway for formation of the alanine released from starved muscle preparations therefore does not utilise exogenous BCAA carbon for pyruvate formation to any great extent but still involves PEPCK and aspartate aminotransferase. The source of pyruvate for alanine formation in these studies is therefore probably the amino acids derived from muscle protein breakdown.
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The work described in this thesis was carried out between 1978 and 1982 during which time the author was registered as a part-time Ph.D. student.

All amino acids mentioned in this thesis are the natural L-form unless otherwise stated.
Author's Publications


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CHAPTER ONE

INTRODUCTION
INTRODUCTION

1.1 METABOLIC FUELS AND FASTING

1.1.1 Requirement for Gluconeogenesis

Fasting in man can be considered to consist of three metabolically distinct phases, namely the postabsorptive state (6 - 12 h after food intake), short-term starvation (lasting up to 7 days) and prolonged starvation, which are characterised by changes in the utilisation of fuels by various tissues of the body. During the postabsorptive period adipose tissue releases free fatty acids to meet the fuel requirements of muscle, heart and parenchymal tissues (liver and kidney) (Felig, 1979), while carbohydrate utilisation occurs primarily in the brain and, to a lesser extent, in red blood cells, kidney medulla and testis. The daily glucose requirement for those tissues has been estimated as approximately 120 g for brain and 40 g for the kidney medulla, testis and red cells combined (Newsholme & Start, 1973). Muscle also uses carbohydrate as a fuel but the amount that is consumed is dependent upon the extent and duration of mechanical activity, the respiratory quotient of resting muscle being close to 0.7 (Andres et al., 1956). The minimum glucose requirement for an average man at rest is therefore approximately 160 g per day.

During the preliminary stages of fasting, glucose production is limited to the liver (Felig, 1979). Maintenance of circulating glucose levels depends on the liver releasing glucose at a rate equal to glucose utilisation in brain and other tissues. In the
absence of hepatic glucose production the blood glucose pool would be halved in 40 - 60 min. Approximately 75 per cent of the glucose released by liver after an overnight fast is derived from glycogen (Felig, 1973). Liver glycogen stores (approximately 70 g per 70 kg man) are rapidly depleted as starvation extends beyond 18 - 24 h (Hultman & Nilsson, 1971) and so can supply the glucose requirement of the brain for less than 24 h. Therefore as fasting progresses beyond the post-absorptive period hepatic glucose output is more dependent on hepatic gluconeogenesis than glycogenolysis.

In man an increase in gluconeogenesis can be detected after only a few hours fasting (Exton et al., 1970; Schimmel & Knobil, 1970).

1.1.2 Precursors for Hepatic Gluconeogenesis

Gluconeogenesis refers to the processes whereby glucose molecules are synthesised from precursor molecules, the main substrates being amino acids, glycerol, lactate and pyruvate. Muscle lacks the enzyme glucose 6-phosphatase and therefore its glycogen cannot be mobilised into the circulation as glucose. However lactate and pyruvate formed from muscle glycogen are released to the liver where they act as precursors for glucose synthesis thus enabling muscle glycogen to contribute to blood glucose homeostasis (Sugden et al, 1976). The average man (70 kg body weight) has only 120 g of muscle glycogen (Newsholme & Start, 1973) and even assuming total mobilisation to the liver, muscle glycogen is not sufficient to provide substantial amounts
of precursors for hepatic gluconeogenesis over a period of days. Lactate and pyruvate are also produced from glucose degradation in glycolytic tissues such as the kidney medulla and red blood cells. Gluconeogenesis from these substrates in this case is merely a recycling of carbon and does not represent net production of glucose for complete oxidation by the brain or other tissues.

During fasting adipose tissue triacylglycerol stores are mobilised yielding fatty acids and glycerol. While the fatty acids provide a fuel of respiration for several tissues, the glycerol is transported to the liver where it acts as a gluconeogenic precursor.

Glycerol, however, only plays a minor role in gluconeogenesis in man during both brief fasting, when it has been calculated that glycerol release during the breakdown of depot fat can provide only about 16 g out of a total of 180 g of glucose needed over 24 hours (Cahill et al., 1966), and during prolonged starvation (Cahill, 1970; Exton, 1972).

In man, as fasting proceeds beyond the postabsorptive period into the short-term fasting phase, the relative contribution of amino acids derived from body protein breakdown to total hepatic glucose production increases. It has been calculated that in fasting adult man, approximately 50% of net glucose production is derived from amino acid precursors, 30% from lactate and 10% from glycerol (Cahill, 1970). Similarly, when livers from fasted rats were perfused with mixtures of lactate, glycerol and amino acids in physiological concentrations, over
50% of the glucose formed was derived from the amino acids (Exton & Park, 1967; Exton et al., 1970). Amino acids have also been found to be major substrates for hepatic glucose formation in human insulin-dependent diabetes, a situation where gluconeogenesis is elevated above normal (Wahren et al., 1972).

Although all amino acids, with the exception of leucine, are potentially glucogenic (Krebs, 1964), only alanine, serine, threonine and glycine have been found to give rise to significant amounts of glucose in perfused rat liver in vitro (Ross et al., 1967) and much evidence has been presented to show that alanine is the major amino acid involved in hepatic gluconeogenesis.

1.1.3 Importance of Alanine as a Gluconeogenic Precursor

Transhepatic catheterisation studies in man suggest that over 50% of the glucose derived from gluconeogenic amino acids is formed from alanine, and the plasma alanine concentration exhibits the steepest decline of all amino acids during fasting in both adults and children (Adibi, 1968; Felig et al., 1969a; Pagliara et al., 1972; Pagliara et al., 1973).

Alanine is taken up by rat liver in vivo (Ishikawa, 1977) and in vitro (Fisher & Kerly, 1964), and by the human splanchnic bed in vivo (Felig et al., 1969a), to a greater extent than any other amino acid. It has been shown to be the best amino acid substrate for hepatic gluconeogenesis in the rat (Exton & Park, 1967; Ross et al., 1967) and specific stimulation of alanine transport into hepatocytes has been observed in starvation.
(Mallette et al., 1969a) and after glucagon stimulation of gluconeogenesis (Fehlmann et al., 1979). Indeed a major factor in the increase in gluconeogenesis in short-term fasting has been proposed to be the increased hepatic extraction of alanine, despite a fall in its plasma concentration (Felig et al., 1969a). This suggestion receives support from the findings that the rate of precursor supply to rat liver has been demonstrated to limit the rate of gluconeogenesis in the rat \textit{in vivo} (Aikawa et al., 1972) and in the perfused rat liver \textit{in vitro} (Exton & Park, 1967) as the physiological concentrations of these precursors in blood lie well below saturating levels for hepatic gluconeogenesis. In addition recent evidence from Tager's group (Sips et al., 1980; Groen et al., 1982) shows that the plasma membrane transport of alanine is probably rate-limiting for its utilisation by liver.

During prolonged starvation when the ketone bodies, acetoacetate and 3-hydroxybutyrate, progressively replace glucose as a fuel for the brain (Owen et al., 1967), the need for gluconeogenesis is decreased and the release of amino acids from body protein is reduced (Owen et al., 1969; Cahill, 1970). The infusion of small amounts of alanine to humans in this situation results in a rapid rise in the blood glucose concentration confirming that a rate-limiting step to gluconeogenesis in fasted man is the concentration of the substrate, alanine, available to the liver (Felig et al., 1969b).

The key role of alanine as a glucose precursor which is
limiting to gluconeogenesis is also demonstrated in several conditions in which a deficiency in circulating alanine is associated with fasting hypoglycemia. Normal pregnancy results in an exaggeration and acceleration of the hypoglycemic, hyperketonemic and hypoinsulinemic response to starvation in humans (Felig & Lynch, 1970) and in rats (Scow et al., 1964). In humans (Felig et al., 1972) and rats (Metzger et al., 1971) blood alanine levels are significantly reduced and fall more rapidly during starvation in pregnancy. The infusion of alanine into pregnant humans increased plasma alanine levels and a prompt rise in blood glucose was observed (Felig et al., 1972), implying that the fasting hypoglycemia of pregnancy was due to gluconeogenic substrate limitation. The importance of glucogenic amino acids as gluconeogenic substrates is also shown by the finding that in 48 h-starved pregnant rats circulating lactate, pyruvate and glycerol concentrations did not fall below the values observed in virgin rats, but a rapid and profound decrease was noted in the levels of circulating glucogenic amino acids, including alanine, coincident with the development of hypoglycemia (Metzger et al., 1971).

Ketotic hypoglycemia is a disorder of childhood characterised by recurrent episodes of hypoglycemia and ketosis, associated with hypoinsulinemia. A decreased circulating alanine concentration has been shown, but rapid restoration of blood glucose to normal was found following alanine administration (Pagliara et al., 1972) or corticosteroid-induced hyperalaninemia (Haymond et al., 1974).
Hypocalaninemia may also contribute to ethanol-induced hypoglycemia. When ethanol is administered to normal (Kreisberg et al., 1972) and diabetic subjects (Kalkhoff & Kim, 1973) circulating alanine levels rapidly decrease.

Alanine availability may be the key factor in the sex- and age-related variability in tolerance to starvation found among healthy subjects. Normal women exhibit a greater degree of hypoglycemia during fasting than men (Merimee & Fineberg, 1973) and healthy children show an exaggerated fall in blood glucose compared to adults (Chaussain, 1973). These findings correspond with the observations that alanine availability during fasting is highest in adult men, intermediate in women and lowest in children (Santiago, J., Haymond, M., Karl, I., Clarke, W., Pagliara, A. & Kipnis, D (1974) "unpublished results" referred to in Felig, 1975).

1.1.4 Sources of Alanine for Gluconeogenesis

Alanine is capable of being released into the circulation by a number of tissue. Using arteriovenous measurements in vivo, isolated organ perfusion systems or isolated tissue preparations in vitro, alanine release has been demonstrated from skeletal muscle (London et al., 1965; Felig et al., 1969a; Pozefsky et al., 1969; Marliss et al., 1971; Ruderman & Lund, 1972; Aikawa et al., 1973; Ruderman & Berger, 1974; Garber et al., 1976a), heart (Taegtmeyer et al., 1977), small intestine (Windmueller & Spaeth, 1974; 1975; Hanson & Parsons, 1977), kidney (Owen & Robinson, 1963; Aikawa et al., 1973) and adipose tissue (Snell & Duff, 1977a; Tischler & Goldberg, 1980).
By far the most important of these tissues in supplying alanine to the bloodstream is skeletal muscle. This tissue comprises about 45% of the adult human body weight (Miller, 1969) and contains almost 60% of the total body protein (Snell, 1980), thus representing a relatively large depot of gluconeogenic precursors which can be made available by proteolysis. Several investigators reporting the release of amino acids from postabsorptive human forearm by arterio-venous difference measurements have shown that alanine and glutamine together account for about 50% of the total amino acids released (Pozefsky et al., 1969; Marliss et al., 1971; Aoki et al., 1974). Similar findings have also been obtained from the patterns of amino acid release by the perfused hind-quarter of the 48 h-starved rat (Ruderman & Lund, 1972; Ruderman & Berger, 1974) and by intact epitrochlaris muscle preparations (Garber et al., 1976a). The release of large amounts of these two amino acids from skeletal muscle during fasting thus makes alanine and glutamine available for hepatic gluconeogenesis and, as noted above (1.1.3), hepatic extraction and gluconeogenesis from alanine is increased in fasting. The fate of the glutamine released by skeletal muscle, however differs from that of alanine. Although it has been observed that a large amount of circulating glutamine is extracted by the splanchnic bed (liver and gut) (Marliss et al., 1971), it has been found that most of the glutamine extracted is taken up by the intestinal tract rather than the liver, in man (Felig et al., 1973a), in fasted rat (Ishikawa et al., 1972) and in fasted dog
The small intestine (Windmueller & Spaeth, 1974), and more specifically the mucosal epithelial cells of the small intestine, have been identified as the major sites of glutamine utilisation in the non-hepatic splanchnic bed (Hanson & Parsons, 1977; Windmueller & Spaeth, 1978). Comparison of arterio-hepatic venous differences have shown that the intestinal tract in fasting man releases alanine into the circulation (Felig et al., 1973b). To this extent, measurements of alanine uptake across the splanchnic bed underestimate the hepatic uptake of alanine. The gut from the starved rat has also been found to release large amounts of alanine (Ishikawa et al., 1972) and several preparations of rat small intestine have shown that glutamine can contribute both carbon and nitrogen to the alanine released (Windmueller, 1980). Hence glutamine released by skeletal muscle can act indirectly as a precursor for hepatic gluconeogenesis via its contribution to alanine formation and release by the small intestine.

The kidney also takes up glutamine from the circulation and releases alanine (Owen & Robinson, 1963; Aikawa et al., 1973). Kidney utilises glutamine to generate ammonia for the neutralisation of acid urine (Pitts, 1964), kidney extraction of glutamine from the circulation being elevated under acidotic conditions and in starvation. Glutamine can also serve as a substrate for renal gluconeogenesis (Hems, 1972), a process which becomes increasingly significant with prolonged starvation (Cahill & Owen, 1968). Studies with fasted rats, however,
have shown that evisceration causes a far greater accumulation of plasma glutamine than does nephrectomy (Matsutaka et al., 1973) thus indicating that the gut is responsible for the greater proportion of glutamine catabolism compared to the kidney.

1.2 DE NOVO MUSCLE ALANINE FORMATION

1.2.1 Alanine and Glutamine Release by Muscle

The myofibrillar proteins constitute approximately 70% of the intracellular protein of muscle (Perry & Corsi, 1958), and the proteins, myosin and actin, make up approximately 60% and 30% respectively of the proteins of the myofibril (Waterlow et al., 1978a). Basing the amino acid composition of muscle protein on a theoretical protein containing actin and myosin in the proportions as they exist in skeletal muscle (Kominz et al., 1954), alanine and glutamine together have been estimated to comprise only about 13% of the amino acid composition of muscle protein (Ruderman & Lund, 1972; Ruderman & Berger, 1974). A similar estimate has been derived by Waterlow et al., (1978b).

During starvation muscle proteins, including those of myofibrils, undergo net breakdown (Haverberg et al., 1975) and, as noted above (1.1.4), alanine and glutamine together account for approximately 50% of the amino acids released by skeletal muscle from fasted subjects. Muscle release of these two amino acids is therefore far in excess of the relative amounts expected from their content in muscle protein. Chang & Goldberg (1978a)
directly confirmed these calculations with diaphragm preparations from rats starved for two days. They determined the amino acids released by the tissue incubated in vitro in Krebs-Ringer bicarbonate buffer containing 5 mM-glucose. Total muscle protein contained similar proportions of alanine and glutamine as the myofibrillar proteins (Ruderman & Berger, 1974; Chang & Goldberg, 1978a) and both glutamine and alanine were again released out of proportion to their percentage compositions of the tissue protein.

It is possible that preferential breakdown of labile proteins containing high proportions of glutamine and alanine residues could account for these observations (Ruderman & Lund, 1972). However, in the absence of evidence for the existence of alanine- and glutamine-"rich" proteins in muscle, an explanation for the disproportionate alanine and glutamine release by muscle is that, in addition to the alanine and glutamine derived from protein breakdown, alanine and glutamine are synthesised de novo within muscle.

The nitrogen of alanine and glutamine constitutes the major part of the nitrogen of the amino acids released from skeletal muscle. This suggests that amino acids released from muscle proteins in fasted rats are metabolised within the muscle and transfer their nitrogen for the formation of alanine and glutamine, which are then released from muscle into the circulation (Ishikawa, 1977). The output of valine, leucine and isoleucine from human forearm muscle (Pozefsky et al., 1969; Felig et al., 1970) and isolated rat diaphragm (Odessey et al., 1974; Chang & Goldberg, 1978a) accounts for only 10 - 12% of
the total amino nitrogen released by muscle and yet these amino acids represent 20% of the residues of muscle protein (Kominz et al., 1954; Odossey et al., 1974; Chang & Goldberg, 1978a), which suggests substantial catabolism of the amino acids in muscle. Similarly aspartate and asparagine together comprise 12% of the amino acid composition of muscle, yet only make up 0.8% of the total amino acids released by muscle (Chang & Goldberg, 1978a). It would appear that these amino acids are the donors of the nitrogen released from muscle in the form of alanine and glutamine. Muscle does not possess the enzymes required for the synthesis of urea and therefore, by releasing nitrogen as alanine and glutamine, muscle may be able to use amino acids as a fuel without accumulating, or releasing into the circulation, large quantities of potentially toxic ammonia (Ruderman, 1975).

The release of alanine from muscle has been found to be more closely related to nutritional variation than that of glutamine. Glutamine release from perfused rat hind-limb preparations was comparable in recently-fed and starved rats, whereas alanine release was negligible in fed rats but markedly increased after 24 - 48 h starvation (Ruderman & Berger, 1974; Ruderman, 1975). Also, glutamine release, measured by arterio-venous difference across rat hindquarters was unaffected by starvation whereas alanine was taken up by hindquarters of fed rats but released from 52 h-starved rats (McDonald et al., 1976). This suggests that alanine transports most of the extra nitrogen generated when the catabolism of amino acids in muscle is increased.
during starvation.

1.2.2 Transamination of Pyruvate

Alanine can be formed de novo in skeletal muscle by the transfer of amino groups to pyruvate. This direct transamination of pyruvate in muscle is restricted to glutamate (Rowsell, 1956; Rowsell & Corbett, 1958) and is catalysed by alanine aminotransferase which is present in muscle in high activity (Williamson et al., 1967; DeRosa & Swick, 1975).

Evidence that some of the alanine released from muscle does not arise from selective proteolysis of an alanine-rich protein but from de novo synthesis has been obtained using inhibitors of alanine aminotransferase. Garber et al., (1976a) showed that increasing amounts of the aminotransferase inhibitor, aminooxyacetate (Hopper & Segal, 1962), caused progressive decrease in muscle alanine release by intact rat epitrochlaris muscle in vitro. Similar findings were obtained in vivo by Blackshear et al., (1975) using the aminotransferase inhibitor, L-cycloserine (Barbieri et al., 1960). In starved rats, after removing the liver from the circulation, the accumulation of alanine in the blood was decreased by 80% after intravenous injection of L-cycloserine.

The reaction catalysed by alanine aminotransferase:

\[ \text{L-glutamate} + \text{Pyruvate} \rightleftharpoons 2\text{-oxoglutarate} + \text{L-alanine} \]

is freely reversible and has an equilibrium constant, \( K \), of 0.66 (Krebs, 1953; Williamson et al., 1967) where
\[
K = \frac{[\text{glutamate}] \cdot [\text{pyruvate}]}{[\text{2-oxoglutarate}] \cdot [\text{alanine}]}
\]

The concentrations of the reactants of the alanine aminotransferase system in the abdominal wall muscle of the rat have been reported (Krebs, 1975) and suggest that at the measured concentrations of glutamate, 2-oxoglutarate and pyruvate, alanine must be formed until its concentration is approximately 3 - 4 mM. The measured alanine concentration in the muscle was much lower and hence equilibrium was not attained. The reason for the non-attainment of equilibrium is possibly the inability of the muscle cell membrane to retain alanine at a high concentration against a tissue/plasma concentration gradient for alanine which has been measured between skeletal muscle (pectoralis major) and blood to be about 4 (Banos et al., 1973; Krebs, 1975). Therefore a reason for non-equilibrium would be the removal of alanine from the cell before equilibrium concentration is reached. The low concentration of alanine in the blood of fasted rats may therefore contribute to the high rate of alanine release observed from the hindquarters of fasted rats (MacDonald et al., 1976).

The abdominal wall muscle study showed a large deviation from equilibrium of alanine aminotransferase after muscular activity, due mainly to the accumulation of pyruvate which rose more than 8-fold after 10 s tetanus. Thus altering the intracellular availability of the reactants of the alanine aminotransferase system alters the rate of alanine production because of the tendency of the enzyme to establish equilibrium between
its reactants. Increased intracellular pyruvate concentrations after infusing pyruvate into postabsorptive man increased the release of alanine into the circulation (Pozefsky & Tancredi, 1972). Perfusing rat hindlimb preparations with lactate, and hence increasing intracellular pyruvate, also increased alanine release (Ruderman & Berger, 1974). Increasing pyruvate availability has also been shown to increase muscle alanine release when rat diaphragm preparations were incubated with glucose (Odessey et al., 1974) and rat epitrochlaris muscles were incubated with pyruvate (Garber et al., 1976a).

Decreasing the intracellular pyruvate available for transamination can be achieved by stimulating pyruvate oxidation with dichloroacetate which activates pyruvate dehydrogenase by increasing the proportion of active pyruvate dehydrogenase in muscle by inhibiting the pyruvate dehydrogenase kinase (Whitehouse & Randle, 1973; Whitehouse et al., 1974). Reduction of alanine release was observed from rat hindquarters perfused with dichloroacetate (Goodman et al., 1978). Similarly, after functional hepatectomy, rats injected with dichloroacetate showed highly significant decreases in the rate of accumulation of circulating alanine compared to controls (Blackshear et al., 1974). These findings also demonstrate that pyruvate availability affects alanine production by muscle.

The amino groups for alanine synthesis may be derived from amino acids other than glutamate if transamination first occurs with 2-oxoglutarate to form glutamate for transamination via alanine aminotransferase as below:
amino acid + 2-oxoglutarate $\rightleftharpoons$ glutamate + 2-oxo acid

\[
\text{glutamate} + \text{pyruvate} \rightleftharpoons \text{2-oxoglutarate} + \text{alanine}
\]

\[
\text{Sum: amino acid} + \text{pyruvate} \rightleftharpoons \text{2-oxo acid} + \text{alanine}
\]

As transamination of 2-oxoglutarate in muscle has only been found to occur to any great extent with aspartate and the branched-chain amino acids, valine, isoleucine and leucine (Krebs, 1975), these four amino acids and glutamate must be the principal amino group donors for muscle de novo alanine formation.

The equilibrium constant, \(K\), of the leucine aminotransferase catalysed reaction:

\[
\text{leucine} + 2\text{-oxoglutarate} \rightleftharpoons \text{glutamate} + 4\text{-methyl-2-oxopentanoate},
\]

defined by the expression:

\[
K = \frac{[2\text{-oxoglutarate}] [\text{leucine}]}{[\text{glutamate}] [4\text{-methyl-2-oxopentanoate}]} \approx 0.65
\]

(Taylor et al., 1970). Since the equilibrium constant for alanine aminotransferase is 0.66, the overall equilibrium constant for the sum of the two freely reversible transaminations is 0.43.

Thus at equilibrium the value of \([\text{alanine}] \times [4\text{-methyl-2-oxopentanoate}]\) is twice the value of \([\text{pyruvate}] \times [\text{leucine}]\).

The provision of branched-chain amino acids or aspartate to muscle should therefore make more glutamate available to the alanine aminotransferase catalysed reaction, displacing equilibrium to increase alanine formation and its subsequent release. Indeed,
*in vitro* experiments incubating preparations of diaphragm muscle in the presence of the three branched-chain amino acids (Odessey et al., 1974), incubating isolated epitrochlaris muscle in the presence of either leucine, isoleucine, valine or aspartate (Garber et al., 1976b), and perfusing hind-limb preparations with leucine or valine (Ruderman & Berger, 1974) have demonstrated increased alanine release by the muscle preparation.

1.2.3 **The Glucose-Alanine Cycle**

In view of the requirement for muscle alanine formation and release on the provision of adequate pyruvate, it has been proposed that the alanine released by muscle is part of a glucose-alanine cycle (Fig. 1.1) operating between the liver and muscle (Mallette et al., 1969a; Felig et al., 1970). It was suggested that blood glucose taken up by skeletal muscle serves by glycolysis, or via glycogen by glycogenolysis, as the source of pyruvate for alanine synthesis. The alanine formed is released by the muscle, extracted by the liver as a gluconeogenic substrate and the glucose so-formed is returned to the circulation. The amount of alanine formed in muscle is thus in part, dependent upon the rate of muscle glycolysis or glycogenolysis.

This proposed cycle is similar to that described between the liver and skeletal muscle for the interconversion of lactate and glucose (Cori, 1931). Lactate is formed in muscle during contraction by anaerobic glycogenolysis. The lactate is
FIG. 1.1 The Glucose-Alanine Cycle (As Proposed by Felig et al., 1970)
released by the muscle to the liver where it acts as a gluconeogenic precursor. The glucose formed is then returned to the blood by the liver, taken up by muscle and reincorporated into muscle glycogen (Cori, 1931).

Alanine formation in muscle may function as an alternative fate of glycolytically-derived carbon to lactate, thus lessening an accumulation of lactate which could disturb the cellular redox state. Another possible role of the proposed glucose-alanine cycle is a consequence of muscle not possessing the enzymic apparatus to synthesise urea. Therefore when amino acids are deaminated in muscle, ammonia must be removed. This is required when amino acids are utilised as a fuel by muscle and may also be of importance during exercise. Exercise has long been known to increase the production of ammonia by muscle (Parnas, 1929; Schwartz et al., 1958). Ammonia formation in muscle occurs by virtue of a cyclic interconversion of the purine nucleotides, adenosine monophosphate and inosine monophosphate, known as the purine nucleotide cycle (Lowenstein & Tornheim, 1971; Lowenstein & Goodman, 1978). The ammonia liberated may possibly be used for the synthesis of glutamate (by way of the glutamate dehydrogenase catalysed reductive amination of 2-oxoglutarate) which can then undergo transamination with pyruvate to form alanine, or further amination to form glutamine. During exercise net alanine release from muscle rises in proportion to the work intensity and hence the extent of anaerobic glycolysis (Wahren, 1979). An increase in alanine release of 50% has been observed with light work and rises of up to 500% have been found during
heavy exercise (Felig & Wahren, 1971). A function of alanine release by muscle may therefore be to provide a non-toxic alternative to ammonia for the transport of amino groups from muscle to the liver.

The glucose-alanine cycle is more useful to muscle than the Cori cycle with respect to ATP production. When lactate is produced from glucose there is a net production of 2 moles ATP per mole of glucose. However, when glucose metabolism results in alanine formation via transamination of pyruvate, two moles of NADH, produced per mole of glucose catabolised from the glyceraldehyde 3-phosphate dehydrogenase step, which would otherwise have been oxidised by lactate dehydrogenase, remain reduced. Since oxidation of NADH through the cytochrome electron-transport system leads to the formation of three high-energy phosphate bonds i.e. 3 moles of ATP are formed per mole of NADH oxidised, the benefit to muscle of glucose metabolism resulting in alanine and not lactate formation is 6 ATP per mole of glucose. Conversion of glucose to alanine therefore provides a net yield of 8 moles of ATP compared to 2 moles provided by conversion to lactate, and also makes available the 2-oxoacid remaining after transamination for possible oxidation by muscle (Odyssey et al., 1974).

It should be noted, however, that the operation of the glucose-alanine cycle as suggested by Mallette et al., (1969a) and Felig et al., (1970) does not allow for the provision of net carbon for new glucose synthesis during fasting. Carbon is merely recycled without any input into the total body glucose pool.
It is doubtful whether the glucose-alanine cycle always operates as originally proposed with the pyruvate for alanine formation being derived entirely from glycolysis. Situations in which the rate of alanine production is not directly dependent on the rate of muscle glycolysis and in which the pyruvate, subsequently transaminated for alanine formation, is derived from non-glycolytic sources, are discussed in Chapters 3 and 4.

1.3 **BRANCHED-CHAIN AMINO ACID METABOLISM**

1.3.1 **Muscle Branched-Chain Amino Acid Metabolism**

The findings that branched-chain amino acids readily transaminate with 2-oxoglutarate in muscle (Krebs, 1975) suggests their usefulness in providing the amino groups required for alanine synthesis de novo in muscle.

It has been found that $^{14}$C-labelled branched-chain amino acids injected intravenously in eviscerated rats are catabolised producing $^{14}$CO$_2$ while other amino acids are hardly oxidised (Miller, 1962). Also, after hepatectomy of dogs, plasma concentrations of branched-chain amino acids are decreased while those of others are increased (McMenamy et al., 1965). These results suggest that branched-chain amino acids are at least in part metabolised extrahepatically. Muscle preparations in vitro have been shown to oxidise branched-chain amino acids to CO$_2$ (Johnson et al., 1961; Manchester, 1965; Buse & Buse, 1967; Buse et al., 1972; Odessey & Goldberg, 1972; Goldberg & Odessey,
1972). Comparing the ability of different tissues to oxidise \( [1-^{14}C] \)-leucine, the order of the rate of leucine derived \(^{14}CO_2\) per gram of tissue has been found to be: kidney > brain > adipose tissue > muscle > liver (Odessey & Goldberg, 1972). However, if the oxidative capacity is expressed per total weight of the tissue, the total capacity of muscle to decarboxylate branched-chain amino acids far exceeds that of other tissues (Adibi, 1976).

The first two steps in the metabolism of the branched-chain amino acids are transamination, followed by oxidative decarboxylation of the resulting 2-oxo acids. The ability of tissues to metabolise branched-chain amino acids is therefore dependent on the amounts and activities of the enzymes catalysing these first two steps.

Branched-chain transaminase activities measured in homogenates of various tissues have been found to be highest in heart and kidney, followed by brain, diaphragm and leg muscle in decreasing order of activity, and lowest in liver (Ichihara & Koyama, 1966; Ichihara et al., 1967; Mimura et al., 1968; Wohlhueter & Harper, 1970; Sketcher et al., 1974; Sketcher & James, 1974; Adibi et al., 1975; Shinnick & Harper, 1976). Taking into account the mass of each tissue, Shinnick & Harper (1976) calculated the tissue activity distribution of branched-chain aminotransferase in the rat. Muscle made by far the largest contribution to total activity (84 %) whereas liver accounted for less than 2 % of total branched-chain aminotransferase activity. Similar findings were reported by other investigators (Ichihara et al., 1973;
Cappuccino et al., 1978).

The low capacity of the liver for the transamination of branched-chain amino acids appears to be a major hindrance to the oxidation of these amino acids.

In experiments using chicks, addition of kidney homogenate, which is rich in branched-chain aminotransferase activity, to homogenates of liver and muscle did not increase CO$_2$ production from leucine by the muscle but markedly increased CO$_2$ from leucine by the liver (Featherston & Horn, 1973). With rat tissue homogenates, oxidation of leucine by liver homogenates low in branched-chain aminotransferase activity, was vastly increased when heart homogenate was added, much more than would be expected from the oxidation of leucine by heart homogenate alone (Shinnick & Harper, 1976). These experiments verify that removal of the amino group is a necessary first step for the oxidation of branched-chain amino acids and that liver has a lower capacity to transaminate BCAAs than some other tissues.

The activity of the enzyme catalysing the second step of BCAA metabolism, the branched-chain 2-oxo acid dehydrogenase, has also been determined in various tissues. Several investigators failed to detect branched-chain 2-oxo acid dehydrogenase activity in muscle homogenates (Dancis et al., 1961; Connelly et al., 1968) or tissue slices (Wohlhueter & Harper, 1970) but found high activity in the liver. These findings led to the suggestion that branched-chain 2-oxo acids are produced by transamination of BCAAs in muscle and are then transferred to the liver for oxidation (Wohlhueter & Harper, 1970; Krebs & Lund,
1977). This scheme would account for the presence of branched-chain 2-oxo acids in the circulation (Kaser et al., 1960).

Indeed, it has recently been shown by arteriovenous difference measurements that considerable amounts of branched-chain 2-oxo acids are released by rat skeletal muscle into the circulation and similar amounts are removed by the liver (Livesey & Lund, 1980). Enzyme activity measurements, using tissue homogenates, subsequent to those of Wohlhueter & Harper (1970), have found a small but significant activity of branched-chain 2-oxo acid dehydrogenase activity in muscle (Noda & Ichihara, 1974; Shinnick & Harper, 1976), but the percentage contribution by muscle to the total branched-chain 2-oxo acid dehydrogenase activity in the body was calculated as only about 1% whereas liver contributed over 90%, again suggesting the inter-organ relationships for whole body BCAA metabolism outlined above.

Studies using different muscle preparations however have resulted in largely different values for muscle branched-chain 2-oxo acid dehydrogenase capacity. Shinnick & Harper (1976) calculated dehydrogenase activity in diaphragm muscle homogenates as 0.47 μmol CO₂ produced h⁻¹ g of tissue⁻¹. Studies using incubations of quarter diaphragms with 0.5 mM-L-[¹⁴C]-leucine as substrate have revealed that approximately 2.6 μmol ¹⁴CO₂ are produced h⁻¹ g of tissue⁻¹ (Chang & Goldberg, 1978b). Therefore the rate of oxidative decarboxylation observed in the tissue incubations is greater than the rate of enzyme activity measured in cell-free homogenates using saturating substrate concentrations and hence supposedly representing estimates of maximum metabolic
capacity. Clearly, as these discrepancies exist between the assayed enzyme capacity and enzyme activity in the intact cell the conclusions concerning total body BCAA metabolism derived from branched-chain 2-oxo acid dehydrogenase measurements in various tissues are open to question. Furthermore, the activity of branched-chain 2-oxo acid dehydrogenase from a number of tissues has been shown to be affected by regulatory factors which effect marked variations in the measured activity level (Wohlhueter & Harper, 1970; Buse et al., 1975; Pettit et al., 1978 Van Hinsbergh et al., 1979).

It is possible that the extraction procedures and the assay conditions can cause alterations in the measured branched-chain 2-oxo acid dehydrogenase activity. In fact it has been pointed out that the degree of activation of dehydrogenase extracted from various tissues of the rat varies considerably. Activation is near maximal in preparations from kidney and liver, whereas the dehydrogenase from heart and skeletal muscle is almost completely inactivated (Odessey, 1980). The comparison of branched-chain 2-oxo acid dehydrogenase activities from different tissues cannot therefore be made unless the state of activation or inactivation of the enzyme from each tissue is taken into account. These findings suggest that muscle may play a greater role in the oxidation of BCAAs than was suggested by Shinnick & Harper (1976).

Species differences in the metabolism of BCAAs have been suggested by the studies of Khatra et al. (1977a). These workers showed that in rat over 70% of the total body content
of branched-chain 2-oxo acid dehydrogenase activity was located in the liver and, although the specific activity of the enzyme in muscle was only 1 - 2% of the specific activity in the liver, muscle accounted for 10% of whole body branched-chain 2-oxo acid dehydrogenase activity. In man, the specific activity of the enzyme in liver was only about 5% of that found in rat liver, whereas the specific activity of the enzyme in human muscle was similar to that in rat muscle. Consequently the distribution of the dehydrogenase activity in man was different from that found for the rat, with liver only comprising 30%, and muscle over 60%, of the measured whole body activity of the enzyme. This suggests that muscle is an important site of BCAA metabolism in man, more so than in the rat. An alternative explanation is that in liver preparations from man, in contrast to those from the rat, the dehydrogenase is considerably inactivated. However, in agreement with the former interpretation is the finding that branched-chain 2-oxo acid transfer between muscle and liver in man is small compared to the rat (Elia & Livesey, 1981).

Three isoenzymes of the branched-chain aminotransferase have been reported and designated types I, II, and III (Ichihara et al., 1975). Type II which is found only in the liver, is specific for leucine (Goto et al., 1977) whereas types I and III are capable of transaminating all three BCAAs. Whereas the Km's of isoenzymes I and III for leucine are approximately 2.5 - 4.3 mM, isoenzyme II differs in that its Km for leucine is very much higher, approximately 25 mM (Ichihara et al., 1975). Enzymes
I and III have very similar Km values for the three BCAAs but their tissue distribution and chromatographic and immunochemical properties differ. Isoenzyme III is found in the brain, ovary and placenta and predominates in fetal and cancerous tissue (Ichihara et al., 1975). Isoenzyme I however, is the only iso-enzyme present in kidney, muscle, heart, spleen, lung, gut, adipose tissue and lactating mammary gland. Hence the transamination of BCAAs in muscle is believed to be catalysed by an aminotransferase (Type I) which accepts all three BCAAs (leucine, valine and isoleucine) as substrates (Ichihara et al., 1975). Similarly, in muscle, a single branched-chain 2-oxo acid dehydrogenase complex is believed to accept all three BCAAs as substrates (Connelly et al., 1968; Odessey & Goldberg, 1979).

It appears to be generally accepted that the remaining steps in the pathways for further catabolism of BCAAs by muscle are those which have been established for their catabolism in liver (Meister, 1965; Rodwell, 1969) as shown for the catabolism of leucine and valine in Fig. 1.2. Although it has been reported by McGarry & Foster (1969) that 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) lyase, the enzyme which catalyses the cleavage of HMG-CoA, was not found in homogenates of rat skeletal muscle, evidence to the contrary has been presented. Van Hinsbergh et al. (1979) studied the oxidation of 4-methyl-2-oxopentanoate by intact rat skeletal muscle preparations and found that a large proportion of the $^{14}$CO$_2$ released from [U-$^{14}$C]-4-methyl-2-oxopentanoate was derived other than from the C-1 position. For this further oxidation of 4-methyl-2-oxopentanoate carbon to
Accepted Routes for the Catabolism of Valine and Leucine
(FIG. 1.2 Continued)

LEUCINE

\[
\text{H}_3\text{C-CH-CH}_{2}-\text{O-}\text{NH}_3^+ \quad \text{transamination, 2-oxoglutarate as acceptor} \quad \text{H}_3\text{C-CH}_{2}-\text{C-}\text{COO}^- \quad \text{oxidative decarboxylation}
\]

\[
\text{H}_3\text{C-CH}_{2}-\text{C-}\text{S-CoA} \quad \text{partially \beta-oxidation}
\]

\[
\text{H}_3\text{C-CH}_{2}-\text{C-}\text{S-CoA} \quad \text{biotin ATP}
\]

\[
\text{ATP} \rightarrow \text{Acetoacetyl-CoA} \rightarrow \text{Acetyl CoA}
\]

\[
\text{H}_3\text{C-CH}_{2}-\text{C-}\text{S-CoA} \quad \text{3-Hydroxy-3-methylglutaryl-CoA}
\]

\[
\text{Acetyl CoA} \quad \text{aldol cleavage}
\]

\[
\text{H}_3\text{C-CH}_{2}-\text{C-}\text{S-CoA} \quad \text{acetoacetate}
\]
CO\textsubscript{2} to occur by the catabolic route shown in Fig. 1.2, the carbon chain must first be cleaved and such a pathway would require the presence of 3-hydroxy-3-methylglutaryl-CoA lyase.

Valine carbon is also believed to be metabolised in muscle by the route established in liver. Support for this has come from the reports that an intermediate of that pathway, 3-hydroxyisobutyrate is released from isolated rat hindquarters perfused with valine (Spydevold, 1979) and that when isolated hindquarters of rats starved for 48 h are perfused with [U-\textsuperscript{14}C]-valine, \textsuperscript{14}C-labelled tricarboxylic acid cycle intermediates are found in muscle.

1.3.2 Branched-Chain Amino Acids and the Regulation of Protein Turnover in Skeletal Muscle

Branched-chain amino acids, and leucine in particular, appear to be able to influence rates of protein synthesis and degradation in muscle. The effects of adding glucose, amino acids and the BCAAs on the synthesis and breakdown of protein in diaphragm preparations from fed and starved rats have been studied (Fulks et al., 1975). Diaphragms from both fed and starved-rats showed increased synthesis and decreased breakdown of protein when amino acids were added at 5-fold normal or normal plasma concentrations. These changes could be entirely accounted for by the BCAAs alone. Similarly, Buse & Reid (1975) observed that when a mixture of all three BCAAs (each at 3 mM) was added to the incubation medium, \textsuperscript{14}C-lysine incorporation into protein was stimulated by 20%. In fact, leucine alone at
0.5 mM stimulated incorporation by 25%, whereas 0.5 mM-valine or isoleucine alone had little effect. These effects of leucine are of interest because they were demonstrated with leucine concentrations which are within the range over which blood leucine concentrations change in the fed and fasted states (Adibi, 1976), and because they only occur in muscle. In liver or fibroblasts, BCAAs have no such effect (Goldberg & Tischler, 1981).

The two effects of leucine i.e. stimulation of protein synthesis and inhibition of protein breakdown apparently involve different mechanisms. Experiments using a presumed inhibitor of leucine transamination, D,L-cycloserine, have shown that protein synthesis is stimulated when leucine is added to muscle incubations containing cycloserine to the same extent as in the absence of cycloserine. However, this transaminase inhibitor completely blocks the decrease in protein breakdown in muscle caused by leucine (Goldberg & Tischler, 1981). The inhibition of protein breakdown therefore requires leucine catabolism while increased protein synthesis does not. In support of the above suggestion, it has been shown that incubating muscle with the 2-oxo acid derived from leucine, 4-methyl-2-oxopentanoate, did not stimulate protein synthesis, but, like leucine, did inhibit protein breakdown (Goldberg & Tischler, 1981). How these effects are mediated is at present unknown.
1.3.3. Branched-Chain Amino Acids and Alanine Release from Muscle

As noted above BCAA release by muscle is less than would be expected from their relative frequency in muscle proteins (Ruderman & Berger, 1974) and in contrast with most other amino acids, that are largely metabolised in the liver, the principal tissue initiating BCAA metabolism in the body is muscle (Adibi, 1976). In vitro studies have shown that BCAAs are very effective in stimulating alanine formation and release by muscle (Ruderman & Berger, 1974; Odessey et al., 1974; Garber et al., 1976b). The greater release of alanine by the isolated muscle of starved rats compared with fed rats when BCAAs are added to the medium, seems related to the enhanced muscle oxidation of BCAAs in starvation (Odessey et al., 1974; Hutson et al., 1980). The in vivo rate of oxidation of leucine has been observed to increase when rats are starved for 4 days compared with fed controls (Meikle & Klain, 1972). Human patients undergoing weight reduction by fasting also showed a marked increase in the rate of leucine oxidation in vivo soon after starting to fast, which continued as long as caloric deprivation was maintained (Adibi, 1980).

Plasma concentrations of BCAAs in humans are greatly affected by alterations in dietary status. In response to fasting in man the most dramatic and prompt changes in plasma concentrations of amino acids are those of the BCAAs and alanine. Starvation, even for only one day, increases the concentrations of all three BCAAs in the plasma of healthy human subjects (Adibi,
The levels of these BCAAs reach a peak value by the second day of starvation and remain elevated during the first week. Resumption of a regular diet during the first week of starvation or prolongation of starvation to 2 weeks lowers the concentrations of BCAAs to basal levels. The direction of change in plasma concentrations of alanine during starvation is the opposite to that of the BCAAs (Adibi, 1976). Thus, in a situation of enhanced gluconeogenesis when hepatic extraction of alanine is increased and muscle release of alanine is increased, plasma concentrations of the BCAAs are increased - again suggesting a connection between the BCAAs and alanine release by muscle. Dietary induced changes in plasma concentrations of BCAAs in rats are similar to those described in man. Starvation increases the concentrations of all three BCAAs in rat plasma, although the development of hyperbranched-chain aminoacidemia is not as rapid in the rat as it is in man (Adibi, 1971). In adult rats the plasma concentrations of BCAAs become elevated after 6 days of starvation, while in man they are increased after 1 day of fasting (Adibi, 1976). The source of these amino acids has not been defined although they may come in part from the liver which releases BCAAs during starvation in rats (Mallette et al., 1969b; Bloxham, 1972) and man (Marliss et al., 1971).

Some investigators have maintained that under physiological conditions the relationship involving branched-chain amino acids and muscle alanine formation is confined to transamination, glucose being the main source of pyruvate for alanine synthesis in muscle (Chang & Goldberg, 1978a,b; Felig & Koivisto, 1979).
Patients with hereditary enzymatic defects in the oxidative decarboxylation of BCAAs (maple syrup urine disease) have low serum alanine levels (Haymond et al., 1973; 1978). Studies of alanine fluxes in these patients have indicated that BCAA catabolism is an important rate-limiting event for alanine production in vivo (Haymond et al., 1978), and therefore suggest that the involvement of BCAAs with muscle alanine release is dependent on metabolism of BCAAs beyond the transamination step.

Thus although it has been suggested that glucose is the principal source of pyruvate for alanine synthesis in muscle and that BCAA involvement in alanine formation is restricted to transamination, whatever the origin of pyruvate, the above findings demonstrate a relationship between alanine release and BCAA oxidation by muscle.

It has been shown that muscle is capable of fine control of the level of tricarboxylic acid cycle intermediates in order to maintain optimal oxidative conditions in various physiological situations, heart and skeletal muscle both having an increased content of tricarboxylic acid cycle intermediates in fasting or diabetic animals (Davis et al., 1980). Challenging glucose-perfused rat hearts with a fatty acid or oxaloacetate (Williamson, 1965; Bowman, 1966; Randle et al., 1970; Davis & Bremer, 1973) also results in an elevation in the level of endogenous intermediates of the tricarboxylic acid cycle. A similar but smaller effect has also been seen with perfused skeletal muscle (Spydevold et al., 1976) and it was suggested that muscle was capable of replenishing tricarboxylic acid cycle intermediates by carboxylation reactions.
Indeed it has been shown that CO$_2$ can be continuously fixed into carboxylic acids in skeletal muscle and that acetate and pyruvate stimulate this process (Lee & Davis, 1979).

The metabolism of BCAAs results in the formation of 2-carbon units from leucine, but isoleucine and valine are converted to 4-carbon units that may replenish the tricarboxylic acid cycle intermediate pools (Spydevold et al., 1976; Lee & Davis, 1979).

If valine and isoleucine do indeed contribute 4-carbon units to the tricarboxylic acid cycle which are subsequently used for alanine formation, then mechanisms must exist for the efflux of cycle intermediates to form 3-carbon compounds. Lee & Davis (1979) have suggested that such mechanisms are present in skeletal muscle. Muscle decarboxylation reactions result in the conversion of 4-carbon skeletons to the 3-carbon compound pyruvate and hence by transamination to alanine, showing a means whereby the carbon of amino acids can form the carbon skeleton of alanine released by the muscle. The possible existence of such a pathway therefore implies that in some situations alanine released from muscle and subsequently used as a precursor for hepatic gluconeogenesis may be protein-derived and hence capable of making a net contribution to the total glucose pool.

The above scheme would therefore provide mechanisms for control of the level of the endogenous pool of cycle intermediates, net synthesis of a 3-carbon glucose precursor from a non-carbohydrate source and amino nitrogen release from muscle during
net protein breakdown.

Possible alternative routes for alanine synthesis from valine in muscle are shown in Fig. 1.3. The routes differ by the enzymes which decarboxylate 4-carbon intermediates of the tricarboxylic acid cycle and supply pyruvate for alanine formation.

Route (a) is via malic enzyme (either NAD- or NADP-specific);
Route (b) is via "oxaloacetate decarboxylase"; and
Route (c) involves the enzymes phosphoenolpyruvate carboxy-kinase (PEPCK) and pyruvate kinase.

Skeletal muscle has been reported to contain appreciable amounts of malic enzyme (Nolte et al., 1972; Lee & Davis, 1979) and PEPCK activities (Opie & Newsholme, 1967; Newsholme & Williams, 1978).

1.4 AIMS OF THE PRESENT STUDY

The main aims of the present study were therefore to firstly establish that BCAAs stimulate alanine release from muscle, and then to clarify the possible relationship between BCAA metabolism and muscle alanine release and the possible sources of pyruvate involved in de novo alanine formation in different nutritional states.

An attempt was made to determine whether BCAAs are capable of contributing carbon for de novo pyruvate and hence alanine formation in muscle and to elucidate the pathway(s) by which this could occur.

The Chapters which follow describe and discuss the
Alternative Routes of Alanine Synthesis in Muscle

Valine → 3-methyl → 2-oxobutyrate → succinyl-CoA

2-oxoglutarate → Glutamate → succinate

Alanine → pyruvate → malate → fumarate

Pyruvate → oxaloacetate

Phosphoenolpyruvate → alanine
results of studies on alanine formation and BCAA metabolism in vitro in various physiological states and the measurement of relevant enzyme activities in muscle extracts, as well as investigating the subcellular distribution of some of these enzymes.
CHAPTER TWO

MATERIALS AND METHODS
MATERIALS AND METHODS

2.1 CHEMICALS

Sodium $^{14}$C bicarbonate (0.1 mCi.mmol$^{-1}$), L-$[1-^{14}$C]-
leucine ($\sim$ 50 mCi.mmol$^{-1}$), L-$[U-^{14}$C]-leucine ($\geq$ 300 mCi.mmol$^{-1}$),
L-$[1-^{14}$C]-valine (40 - 60 mCi.mmol$^{-1}$) and L-$[U-^{14}$C]-valine
($\geq$ 250 mCi.mmol$^{-1}$) were obtained from Amersham International
(formerly The Radiochemical Centre), Amersham, Bucks., U.K.

L-alanine dehydrogenase (EC 1.4.1.1), glutamate dehydrogenase
(EC 1.4.1.2), lactate dehydrogenase (EC 1.1.1.27), malate
dehydrogenase (EC 1.1.1.37), pyruvate kinase (EC 2.7.1.40),
hexokinase (EC 2.7.1.1), glucose 6-phosphate dehydrogenase
(EC 1.1.1.49), adenosine 5'-diphosphate (disodium salt),
$\beta$-nicotinamide-adenine dinucleotide (NAD), $\beta$-nicotinamide-
adename dinucleotide, reduced (NADH) (disodium salt), phospho-
enolpyruvate (sodium salt), oxaloacetic acid, 2-oxo-glutarate,
streptozotocin (N-D-glucosyl-(2)-N'-nitrosomethylurea),
dithiothreitol, antimycin A and glucose test combination kit
(glucose oxidase (EC 1.1.3.4)-peroxidase (EC 1.11.1.7) method)
were obtained from Boehringer Corporation Ltd., Lewes, Sussex,
U.K.

Glutaminase (EC 3.5.1.2), adenosine 5'-triphosphate
(disodium salt), inosine 5'-diphosphate (sodium salt), inosine
5'-triphosphate (sodium salt), acetyl coenzyme A (sodium salt),
pyridoxal 5-phosphate, cycloheximide, 5,5'-dithiobis-(2-nitro-
benzoic acid) (DTNB), tartronic acid (hydroxymalonic acid),
aminooxyacetic acid, 4-methyl-2-oxopentanoic acid ($\alpha$-ketoiso-
caproic acid), 3-methyl-2-oxobutanoic acid (α-ketoisovaleric acid), 1-nitroso-2-naphthol, tyrosine standard solution (L-tyrosine, 0.025 mg/ml, in 0.2M trichloroacetic acid), L-leucine, Tris [tris(hydroxymethyl)aminomethane], Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] and "Dowex"-50W (H⁺ form) cation exchange resin (8% cross linked, 200 - 400 dry mesh) were obtained from Sigma Chemical Company Ltd., Poole, Dorset, U.K.

β-nicotinamide-adenine dinucleotide phosphate (NADP) was obtained from Cambrian Chemicals, Croydon, Surrey, U.K.

2,5-Diphenyloxazole (PPO) was obtained from Packard Instruments Company Inc., Downers Grove, Ill., U.S.A. and 1,4-di-(2-(α-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) from Fisons Scientific Apparatus, Loughborough, Leics., U.K.

Synperonic NX supplied by Durham Chemicals Distributors Ltd., Birtley, Tyne and Wear, U.K., and hyamine 10X (hydroxide) by Intertechnique, Uxbridge, Middx., U.K.

The following generous gifts were received:

3-mercaptopicolinic acid from Dr. H.L. Saunders (SK & F Laboratories, Philadelphia, Pa., U.S.A.), L-cycloserine from Dr. D.H. Williamson (Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford, U.K.); hypoglycin A from Dr. E.A. Kean (University of the West Indies, Kingston, Jamaica);

3-aminopicolinic acid from Dr. C.H. Reynold (Wellcome Foundation Ltd., Dartford, Kent, U.K.); L-2-amino-4-methoxy-trans-but-3-enoic acid from Dr. W.E. Scott (Hoffman-La Roche Inc., Nutley, N.J., U.S.A.) and clofibric acid from Dr. G.G. Gibson (University
of Surrey, Guildford, Surrey, U.K.

L-valine, insulin (crystalline, bovine), agidex powder (amyloglucosidase (EC 3.2.1.3) preparation), dichloroacetic acid, hydrazine hydrate, ninhydrin reagent spray and other inorganic chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K.

2.2 TREATMENT OF ANIMALS

Male rats of a Wistar Albino strain, bred in and obtained from the Animal Unit of the University of Surrey (Director: Mr. P. Scobie-Trumper) were maintained in conditions of 12 hours light, 12 hours darkness at 21 ± 2°C, were allowed free access to water at all times and were fed (Spratt's Laboratory Diet No. 1 pellets (Table 2.1), Spratt's Patent Ltd., Barking, London, U.K.) ad libitum unless otherwise stated.

2.2.1 Dietary Supplements

Some rats were weaned onto a powdered diet (Spratt's Laboratory Diet No. 2 (Table 2.1), Spratt's Patent Ltd., Barking, London, U.K.). After 4 days on the powdered diet, rats were transferred to powdered diet supplemented with L-valine (5 % w/w) or L-leucine (5 % w/w) and remained fed ad libitum on the diets for approximately 21 days until sacrifice. Controls were maintained on the unsupplemented powdered diet throughout.

2.2.2 Streptozotocin Treatment

Rats, which had been fasted for 24 h, were injected
### TABLE 2.1 Composition of Spratt's Laboratory Diets

**Average Calculated Composition of Laboratory Animal Diets**

<table>
<thead>
<tr>
<th>Chemical Composition</th>
<th>Laboratory Diet 1 (pelleted)</th>
<th>Laboratory Diet 2 (powdered)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
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<td>8.8</td>
</tr>
<tr>
<td>Ether Extract (%)</td>
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<td>3.5</td>
</tr>
<tr>
<td>Crude Protein (%)</td>
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<td>21.5</td>
</tr>
<tr>
<td>Crude Fibre (%)</td>
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<td>2.7</td>
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<tr>
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<td>17.2</td>
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<tr>
<td>Digestible Energy (MJ/kg)</td>
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<td>14.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vitamin and Mineral Composition</th>
<th>Laboratory Diet 1 (pelleted)</th>
<th>Laboratory Diet 2 (powdered)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine (%)</td>
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<td>1.1</td>
</tr>
<tr>
<td>Methionine (%)</td>
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<td>0.39</td>
</tr>
<tr>
<td>Calcium (%)</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Phosphorus (%)</td>
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</tr>
<tr>
<td>Vitamin A (i.u./kg)</td>
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<td>11,000</td>
</tr>
<tr>
<td>Vitamin D₃ (i.u./kg)</td>
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<td>1,200</td>
</tr>
<tr>
<td>α-tocopherol (i.u./kg)</td>
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<td>24</td>
</tr>
<tr>
<td>Vitamin K₃ (mg/kg)</td>
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</tr>
<tr>
<td>Riboflavin (mg/kg)</td>
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<tr>
<td>Pyridoxine (mg/kg)</td>
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</tr>
<tr>
<td>Pantothenic Acid (mg/kg)</td>
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<tr>
<td>Nicotinic Acid (mg/kg)</td>
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<td>Folic Acid (mg/kg)</td>
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</tr>
<tr>
<td>Choline Chloride (mg/kg)</td>
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<td>Manganese (mg/kg)</td>
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<td>Iron (mg/kg)</td>
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<tr>
<td>Iodine (mg/kg)</td>
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<tr>
<td>Copper (mg/kg)</td>
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</tr>
<tr>
<td>Cobalt (mg/kg)</td>
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</tr>
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</table>
intraperitoneally with streptozotocin (100 mg in 0.01 M Na citrate/kg body weight) and then allowed to feed ad libitum. Two days after injection, rat urine was checked for the presence of glucose using Bili-Labstix (Ames Co., Stoke Poges, Slough, Bucks., U.K.). If the glucose concentration found in the urine was greater than 28 mmol l⁻¹ the animals were designated "streptozotocin-diabetic" and sacrificed by decapitation. Trunk blood was collected and blood glucose concentration measured. Experimental parameters measured on tissues from the animals were only accepted as characteristic of "streptozotocin-diabetic" animals when blood glucose levels in fed animals were > 15 mM. Control rats were injected with equivalent volumes of 0.01 M Na citrate.

2.2.3 Triiodothyronine Treatment

Rats were given intraperitoneal injections of L-triiodothyronine (L-T³), dissolved in 0.9 % NaCl-5 mM NaOH, at a dose of 250 μg L-T³/kg body weight daily for 6 days (Carter et al., 1981). Control animals were injected over the same time period with equivalent volumes of diluent.

2.3 IN VITRO SKELETAL MUSCLE PREPARATIONS AND INCUBATIONS

2.3.1 Cut Hemi-diaphragm Preparations and Incubations

Hemi-diaphragm preparations were obtained from rats weighing 65 - 100 g. Above this weight the flat diaphragm becomes too thick to ensure that cells within the muscle are
exposed to concentrations of nutrients similar to those found in the incubation medium, and that the entire muscle is adequately oxygenated (Goldberg et al., 1975).

Rats were killed by cervical dislocation and the diaphragms rapidly dissected free of the rib cage. After cutting through the skin on the ventral surface, the lower edge of the diaphragm was freed by cutting the several ligamentous attachments of the liver. The oesophagus and vena cava were severed below the diaphragm. The pleural cavity was exposed by snipping the diaphragm from the ribs close to the costal border. Pericardial membranes were severed above the diaphragm and the diaphragm carefully trimmed free from the ring of ribs to which the muscle was attached.

The area of the diaphragm through which passed the oesophagus and vena cava is too thick to allow adequate oxygenation of all fibres at 37°C (Goldberg et al., 1975) and was therefore cut away and discarded.

The remaining tissue was gently blotted, dried and bisected down the central tendon to yield hemi-diaphragms. After rapidly weighing the hemi-diaphragms they were promptly placed in individual 10 ml flasks containing 2 ml of unsupplemented Krebs & Henseleit (1932) saline at room temperature. The Krebs-bicarbonate buffer had been equilibrated with 95% \( O_2 \), 5% \( CO_2 \) and contained 119 mM-NaCl, 5 mM-KCl, 3 mM-CaCl\(_2\), 1 mM-MgSO\(_4\), 1 mM-KH\(_2\)PO\(_4\) and 25 mM-NaHCO\(_3\) at pH 7.4. The flasks were gassed with \( O_2/CO_2 \) (95%/5%) for 1 min, tightly stoppered and incubated for 30 min at 37°C in a shaking incubator (Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.) at 50 cycles min\(^{-1}\).
At the end of the 30 min pre-incubation period, the hemidiaphragms were removed from the flasks, blotted dry, and transferred into flasks containing fresh Krebs-Henseleit saline and substrates or other additions as indicated. After gassing and sealing the flasks, they were incubated for 2 h at 37°C in the shaking incubator. In preliminary experiments it was shown that re-gassing the flasks after incubation for 1 h had no significant effect on the parameters measured.

2.3.2 Intact Soleus and Extensor Digitorum Longus Muscle Preparations and Incubations

Preparations of intact soleus and extensor digitorum longus (EDL) muscles were obtained from rats weighing 50 - 70 g. Both of these leg muscles are cylindrical and in rats weighing more than 70 g the muscles are of sufficient thickness to cause problems of diffusion of nutrients into the incubated muscle (Goldberg et al., 1975).

Soleus and EDL muscles are useful for studies comparing muscle types since they contain substantially different proportions of muscle fibre type. Approximately 80 - 90% of the fibres of the soleus muscle are of the red slow-twitch variety whereas the EDL muscle contains primarily fast-twitch fibres (approximately 50% white and 50% fast-twitch red fibres) and very few slow ones (Close, 1975; Maizels et al., 1977).

Procedures for the dissection of soleus and EDL muscles were basically as described by Maizels et al., (1977).

Rats were killed by cervical dislocation and both hind-
limbs removed. Soleus and EDL muscles were dissected from both limbs thus yielding four muscle preparations per animal. After removing the skin, hind legs were anchored to the dissection board with dissection pins. The Achilles tendon was exposed and tied. The tendon was cut distal to the tie and the soleus and gastrocnemius (which also inserts into the Achilles tendon) muscles pulled away by traction to expose the proximal tendon of the soleus. By a combination of blunt and sharp dissection, the gastrocnemius muscle was peeled away from the soleus. A second tie was then placed around the proximal tendon of the soleus and the tendon cut proximal to the tie. The freed soleus was then extended and tied to a stainless-steel frame and weighed. The frame and ties had been pre-weighed and hence muscle weight was obtained by difference.

After removal of the soleus, legs were turned and pinned to the dissection board. The tendon of the EDL was exposed, tied and cut distal to the tie. The EDL was exposed by combining traction and sharp dissection until its proximal tendon, attached to the patella, was exposed. The proximal tendon was tied and cut proximal to the tie. The isolated EDL was then secured to a stainless-steel frame and weighed as for the soleus.

Stainless-steel frames were employed to maintain the muscles under slight tension during the incubation procedures. After weighing, and trimming away excess cotton ties, muscles and frames were placed into polystyrene tubes (LP3, 10.5 mm x 63.5 mm) containing 1.5 ml of Krebs-Henseleit saline at 37°C.
and the tubes were stoppered, connected to the gassing apparatus which allowed continuous gassing of the media with 95% O₂, 5% CO₂ and agitation of the tissue, (Fig. 2.1) and incubated for 30 min at 37°C.

After the 30 min pre-incubation period, frames and muscles were transferred to 1.5 ml of fresh medium containing any appropriate addition(s), and incubated for 2 h at 37°C with continuous gassing.

2.3.3 Processing of Samples

At the end of the incubation period the muscle preparation was removed and the incubation medium deproteinised by adding ice-cold 20% (w/v) perchloric acid (HClO₄) (0.1 ml/ml medium) final concentration 0.18M). The acidified medium was centrifuged at 4°C and a known volume of the supernatant was neutralised with KOH. Further centrifugation at 4°C precipitated KClO₄ and yielded a neutralised supernatant which was decanted and stored on ice prior to determination of metabolites within 3 hours.

When muscle tyrosine release was measured, 0.25 ml of incubation medium was added to 0.25 ml of ice-cold 0.6M trichloroacetic acid (CCl₃COOH), centrifuged at 4°C and 0.4 ml of the resulting supernatant used for tyrosine determination.

When muscle content of metabolites was measured, on removal from the incubation medium the muscle preparation was quickly blotted and dropped into liquid N₂. The muscle was powdered with a mortar and pestle pre-cooled with liquid N₂,
FIG. 2.1  Gassing Apparatus for Intact Leg Muscle Preparations

- Incubation Medium
- Portex Tubing
- Stainless Steel Frame
- Soleus or Extensor digitorum longus maintained under slight tension
- LP3 Tube
- Needle (25G)
- 1 ml syringe barrel
- Water Bath
- 37°C
allowed to thaw into 1 ml 0.27M-perchloric acid (HClO₄) and homogenised using a ground-glass homogeniser and pestle. Neutralised extracts were prepared as for the incubation media.

A paired experimental design was used in every set of tissue incubation experiments e.g. one hemi-diaphragm from each rat was incubated in control medium and its pair in experimental medium. Similarly, soleus or EDL muscle from one leg was incubated in control medium while the corresponding muscle from the other hindleg was incubated in test medium.

Control flasks and tubes which did not contain muscle preparations but which contained the same incubation media as those used experimentally were simultaneously incubated in all studies. These control incubation media were treated identically to media in which muscle had been incubated and the resulting neutralised supernatants served as "blanks" in metabolite assays.

2.4 METABOLITE ASSAYS

Spectrophotometry was carried out using a Gilford Model 250 Spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio, U.S.A.) fitted with a model 245i automatic cuvette programmer, a constant temperature cell housing, and a Servoscribe RE 542-20 Chart recorder. 10 mm light-path silica cuvettes were used, either 3 ml or 1 ml volume.

A Perkin-Elmer 3000 Fluorescence Spectrometer (Perkin-Elmer Ltd., Beaconsfield, Bucks., U.K.) was used for fluorimetric measurements.
Alanine was determined enzymatically on aliquots of neutralised samples by the method of Williamson (1974). The reaction observed was catalysed by the enzyme L-alanine dehydrogenase:

\[ \text{L-alanine} + \text{NAD}^+ + \text{H}_2\text{O} \leftrightarrow \text{pyruvate} + \text{NADH} + \text{H}^+ \]

Hydrazine served to trap pyruvate in this system and the resulting increase in NADH was measured by the increase in absorbance at 340 nm after incubation at 30°C for 60 min, by which time the reaction was complete. In some cases NADH was measured fluorimetrically rather than spectrophotometrically. The excitation wavelength was 340 nm and the emission wavelength was 460 nm (Taegtmeyer et al., 1977).

It has been shown that some commercial preparations of alanine dehydrogenase are contaminated to varying extents with activity towards BCAAs and thus the presence of BCAAs in the assay for alanine can give falsely high values (Lund & Baveral, 1978). Although some slight activity towards BCAAs was observed with alanine dehydrogenase, at no time were any of the enzyme preparations contaminated to the extent observed by Lund & Baveral (1978). Neutralised extracts of incubation media, incubated without muscle, served as assay blanks in the present studies, hence correcting for any interference.

Glutamate in neutralised samples was determined spectrophotometrically using glutamate dehydrogenase as described by Bernt & Bergmeyer, (1974). To assay glutamine, an aliquot of sample was first treated with glutaminase to covert glutamine to glutamate (Lund, 1974) and the total glutamate in the sample was
then measured. Glutamine was then calculated as the difference between the basal and glutaminase-treated samples.

Lactate and pyruvate were determined spectrophotometrically using lactate dehydrogenase by the methods of Hohorst (1963) and Bucher et al., (1965), respectively, and malate was measured spectrophotometrically using malate dehydrogenase by the method of Gutmann & Wahlefeld, (1974).

Tyrosine was measured by a modification of the method of Wong et al., (1964). Tyrosine reacts with 1-nitroso-2-naphthol, forming a red colour. This coloured complex is unstable, but when heated in the presence of nitric acid forms a stable yellow compound which is fluorescent. The fluorescence was measured with an excitation wavelength of 440 nm and emission wavelength of 535 nm, and values for tyrosine obtained from a standard curve (prepared with L-tyrosine) which was linear over the range in which test values lay.

Adenosine 5'-triphosphate in neutralised tissue extracts was determined using hexokinase and glucose 6-phosphate dehydrogenase (Lamprecht & Trautschold, 1974).

Glucose in neutralised samples was measured spectrophotometrically with hexokinase and glucose 6-phosphate dehydrogenase by the method of Slein (1963) or by the method of Werner et al., (1970) using glucose oxidase (EC 1.1.3.4) and peroxidase (EC 1.11.1.7). Blood glucose was determined by the above two methods after deproteinisation with HClO₄ (0.24M, final concentration).

Muscle glycogen was isolated as described by Cowgill
and Pardee (1957). The muscle preparation was blotted dry and
digested in 30 % (w/v) KOH in a boiling-water bath for 30 min.
The digest was then cooled to 40 - 50°C and 95 % (w/v) ethanol
added. After thorough stirring the mixture was incubated at
80 - 90°C for 5 min, cooled to room temperature, centrifuged
and the supernatant discarded. Glycogen was then redissolved
in 0.05M-sodium acetate buffer, pH 4.5. Glycogen concentration
was determined after enzymic hydrolysis with Agidex, a preparation
of amyloglucosidase, as described by Vernon & Walker (1970).
Glycogen-derived glucose was estimated as described above.

2.5 CENTRIFUGATION AND FRACTIONATION

2.5.1 Crude Extracts of Muscle

"Crude extracts" of diaphragm, soleus and EDL muscles
were prepared by homogenising freshly dissected muscles,
using a ground-glass homogeniser and motor-driven pestle, in
9 volumes of ice-cold extraction medium consisting of 100 mM-
Tris/HCl, 1 mM-EDTA, 5 mM-MgCl₂ and 20 mM-2-mercaptoethanol at
a final pH of 7.5 (Crabtree et al., 1972). The 10 % (w/v)
homogenates were centrifuged at 600 g for 10 min at 4°C in
a Beckman J-6 centrifuge (Beckman-RIIC Ltd., High Wycombe,
Bucks., U.K.). The resulting supernatants were termed "crude
extracts".
2.5.2 Subcellular Fractionation of Muscle

Subcellular fractionation of muscle was attempted by the methods described below and each fraction obtained was sonicated at 0 - 4°C (4 x 15 s at 5A using a Soniprobe type 1130/1A, Dawe Instruments Ltd., London, U.K.) prior to assaying for enzyme activities.

Method 1 - Rat muscle was minced in buffer as described by Crabtree et al. (1972). The tissue was homogenised using an all-glass homogeniser and the volume of homogenate adjusted to be ten times the weight of the tissue. The homogenate (H) was centrifuged at 800 g for 10 min, the supernatant (S₁) decanted and the pellet (N) washed and resuspended in buffer. The 800 g supernatant (S₁) was centrifuged at 20,000 g for 10 min. The pellet was then washed, resuspended in buffer and denoted the mitochondrial pellet (MT) and the supernatant (S₂) centrifuged at 100,000 g for 60 min. The resulting supernatant was called the cytosolic fraction (C) and the pellet when rinsed and resuspended in buffer, denoted as the microsomal suspension (MC).

Method 2 - the method of Odessy & Goldberg (1979). Rat muscle was minced and washed at 0 - 4°C in buffer A, consisting of 0.25 M-sucrose, 50 mM-KCl, 5 mM-MgCl₂, 5 mM-EGTA and 50 mM-Tris/HCl, pH 7.8. The tissue was homogenised using a motor-driven all-glass homogeniser and the volume of homogenate adjusted to give a final concentration of 100 mg tissue/ml. The homogenate (H) was centrifuged at 800 g for 5 min, the supernatant (S₁) decanted and the pellet re-extracted
Method 1

Homogenate (H) → Pellet + Supernatant ($S_1$)

$800 \, g \times 10 \, min$

Pellet washed, resuspended (N)

Supernatant (N)

Homogenate (H) → Pellet + Supernatant (S$_1$)

$20,000 \, g \times 10 \, min$

Pellet washed, resuspended (MT)

Supernatant (MT)

Homogenate (H) → Pellet + Supernatant (S$_2$)

$100,000 \, g \times 60 \, min$

Pellet washed, resuspended (C)

Supernatant (C)

FIG. 2.2 Subcellular Fractionation of Muscle by Method 1

(Crabtree et al., 1972)
Method 2

Homogenate (H) → 800 g x 5 min → Supernatant ($S_1$) + Pellet

Supernatant ($S_1$) + Pellet → resuspended → 800 g x 5 min → Supernatant ($S_0$) + Pellet

10,000 g x 10 min → Supernatant ($S_0$) + Pellet

Supernatant ($S_0$) + Pellet → resuspended → washed → resuspended → (N)

Pellet + Supernatant (discarded)

combined, washed, resuspended → 100,000 g x 60 min → (MT)

Supernatant ($S_2$) + Pellet

washed, resuspended → (MC)

FIG. 2.3 Subcellular Fractionation of Muscle by Method 2

(Odessey & Goldberg, 1979)
in the above buffer and recentrifuged at 800 g for 5 min. Both 800 g supernatants were then centrifuged at 10,000 g for 10 min. The 10,000 g supernatant of the second 800 g fraction (S₀) was discarded. The 10,000 g pellets from both fractions were combined, washed, resuspended in buffer B (0.25 M-sucrose, 50 mM-KCl, 10 mM-MgCl₂, 0.1 mM-EDTA and 50 mM-Tris/HCl, pH 7.8) and denoted the mitochondrial suspension (MT). The 10,000 g supernatant (S₂) of the first 800 g fraction was centrifuged for 60 min at 100,000 g and the resulting supernatant denoted the cytosolic fraction (C) and the pellet, when rinsed and resuspended in buffer B, denoted as the microsomal suspension (MG).

**Method 3** - the method of Ernster & Nordenbrand (1967). Immediately after excision, rat muscle samples were quickly weighed and immersed in ice-cold 0.15 M-KCl. The tissue was cut with scissors into small pieces and rinsed with several portions of 0.15 M-KCl. The minced tissue was then rinsed with Chappell-Perry medium, consisting of 0.1 M-KCl, 0.05 M-Tris/HCl, pH 7.4, 1 mM-ATP (Na salt), 5 mM-MgSO₄ and 1 mM-EDTA (Chappell & Perry, 1954), and suspended in 4 volumes of the same medium. Homogenisation was then carried out, at 0 - 4°C, with a relatively loose-fitting all-glass Potter-Elvehjem homogeniser and the homogenate diluted with Chappell-Perry medium to a volume of 10 times the initial weight of the muscle. This homogenate (H) was centrifuged at 600 g for 7 min. The supernatant was decanted into a new tube and recentrifuged as before. The resulting supernatant (S₁) was decanted and centrifuged at 14,000 g for
Method 3

FIG. 2.4  Subcellular Fractionation of Muscle by Method 3
(Ernster & Nordenbrand, 1967)
10 min in an MSE SS50 Superspeed (MSE Scientific Instruments, Crawley, Sussex, U.K.). The pellet was resuspended in Chappell-Perry medium and recentrifuged at 14,000 g for 10 min. The supernatant from this second 14,000 g x 10 min spin was discarded and the pellet (MT) resuspended in Chappell-Perry medium.

The supernatant \((S_2)\) from the first 14,000 g x 10 min spin was centrifuged at 100,000 g for 60 min in an MSE SS50 yielding the cytosolic fraction \((C)\) (supernatant) and the microsomal pellet \((MC)\). The surface of the tightly packed microsomal pellet was rinsed twice with Chappell-Perry medium to remove any remaining cytosolic fraction and the microsomal pellet resuspended in Chappell-Perry medium.

Muscle was removed, cut into small pieces and gently homogenised with 9 volumes of medium, consisting of 20 mM-Hepes, 300 mM-mannitol, 1 mM-EDTA and 0.1 % (w/v) bovine serum albumin at pH 7.4, in a glass homogeniser with a loose-fitting Teflon pestle. The homogenate (H) was filtered through two layers of muslin and then centrifuged at 600 g for 5 min. The 600 g pellet (N) was washed twice with the above medium before resuspension in the medium and the 600 g supernatant (S₁) was centrifuged at 5,000 g for 20 min. The pellet was washed twice in medium before resuspension in that medium and denoted the mitochondrial suspension (MT). The 5,000 g supernatant (S₂) was centrifuged at 100,000 g for 1 h. The resulting pellet was washed twice in medium before resuspension and then termed the microsomal suspension (MG). The 100,000 g supernatant was called the cytosolic fraction (C).

2.5.3 Fractional Extraction of Muscle

The fractional extraction procedure employed for muscle was essentially as described by Taylor et al. (1978).

Freshly dissected muscle was rinsed with a medium consisting of 150 mM-KCl, 10 mM-Hepes (4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid), 2 mM-EGTA and 2 % (w/v) bovine serum albumin (essentially fatty acid free) at pH 7.2, and finely
Method 4

Homogenate (H) → 600 g x 5 min

Pellet + Supernatant (S₁)

washed, resuspended

(N) Pellet + Supernatant (S₂)

washed, resuspended

(NT) Pellet + Supernatant (c)

washed, resuspended

(MC)

FIG. 2.5 Subcellular Fractionation of Muscle by Method 4
(Surholt & Newsholme, 1981)
chopped with scissors. Two portions of chopped tissue were removed for the preparation of whole homogenates (see below). The remainder, in 20 volumes (volume A) of the above KCl-based medium, was transferred to a glass homogenising tube and gently disrupted manually with about 30 strokes of a very loose-fitting Teflon pestle. The resulting muscle suspension was transferred to a small glass beaker and stirred continuously for 30 min at 4°C. The suspension was then centrifuged at 30,000 g for 15 min in a Beckman L5-65B Ultracentrifuge. The resulting supernatant was assayed for enzyme activities. The sediment was resuspended in the original volume (volume A) of the above KCl-based medium and the entire procedure of stirring, centrifuging and enzyme activity determinations repeated. After 3 extractions of the tissue with the KG1-based medium the sediment was extracted once with volume A of 100 mM-potassium phosphate, pH 7.2. The sediment from this final extraction was thoroughly homogenised in 0.5 x volume A of 100 mM-Tris/HCl containing 1 mM-EDTA, 5 mM-MgCl$_2$, 20 mM-2-mercaptoethanol and 2.4 mM-sodium deoxycholate at pH 7.4 with a ground-glass homogeniser and was then assayed for enzyme activities without further treatment.

Enzyme activities were determined in two whole homogenate preparations. Both were prepared by ground-glass homogenisation, one in the KCl-based medium including 2.4 mM-sodium deoxycholate and the other in the above Tris-based medium.

2.6 ENZYME ASSAYS

All enzyme assays were performed at 30°C unless otherwise
Phosphoenolpyruvate carboxykinase (PEPCK) (EC 4.1.1.32) activity was assayed by four methods.

**Method A** was based on the radioactive incorporation method of Chang & Lane (1966). The assay medium contained 100 mM-imidazole/HCl, pH 6.6, 3 mM-phosphoenolpyruvate (Na salt), 2 mM-IDP (Na salt), 2 mM-MnCl₂, 2 mM-dithiothreitol, 2.5 mM-NADH (Na salt), 50 mM-NaH¹⁴CO₃ (40 mCi. mole⁻¹), 2.5 µg antimycin A and 12 units of malate dehydrogenase (in glycerol) in a final volume of 1 ml. Phosphoenolpyruvate was omitted from controls. The assay was terminated after 15 min by adding 1 ml of 2 M-HCl. Carbon dioxide was bubbled through the acidified assay solution for 10 min to drive off ᵣ¹⁴CO₂ not incorporated into acid-stable products. A 0.5 ml aliquot was removed, 4 ml of scintillation cocktail (5 g PPO, 0.2 g dimethyl-POPOP/litre toluene: Synperonic NX (2:1 v/v)) added, and counted in a LKB 1210 Utrabeta Liquid Scintillation Counter (LKB-Produkter AB, Bromma, Sweden).

Units of enzyme activity for this method are defined as μmoles of ᵣ¹⁴CO₂ fixed/min.

**Method B** was the continuous spectrophotometric assay for PEPCK activity as described by Opie & Newsholme (1967). The assay medium consisted of 66 mM-Tris/HCl, pH 7.5, 1 mM-MnCl₂, 1 mM-phosphoenolpyruvate (Na salt), 0.16 mM-NADH (Na salt), 1.54 mM-IDP (Na salt), 17 mM-NaHCO₃ and 12 units of malate dehydrogenase (in glycerol) in a final volume of 1 ml. NaHCO₃ was omitted from controls.
Method C was also a continuous spectrophotometric assay in the direction of oxaloacetate formation and was based on the method described by Bentle & Lardy (1976). The assay medium contained 50 mM-Hepes-NaOH, pH 6.5, 1 mM-MnCl₂, 2 mM-phosphoenolpyruvate (Na salt), 0.25 mM-NADH (Na salt), 1 mM-IDP (Na salt), 50 mM-NaHCO₃, 1 mM-dithiothreitol, 2.5 μg antimycin A and 7.5 units of malate dehydrogenase (in glycerol), in a final volume of 1 ml. Phosphoenolpyruvate was omitted from controls.

Method D measured the PEPCK reaction in the direction of phosphoenolpyruvate formation and was also described by Bentle & Lardy (1976), based on the method of Seubert & Huth (1965).

Phosphoenolpyruvate was formed in medium containing 50 mM-Hepes-NaOH, 2 mM-ITP, 3 mM-MgCl₂ and 1.5 mM-oxaloacetate at pH 7.5 in a volume of 1 ml. After 5 min, the reaction was stopped by adding 10 - 15 mg KBH₄ and the mixture was placed on ice. After a further 5 min, the mixture was acidified with 0.4 ml 6% (w/v) perchloric acid. The samples were then neutralised, centrifuged at 4°C and aliquots of supernatant removed for phosphoenolpyruvate determination. Phosphoenolpyruvate was determined in medium containing 70 mM-Tris/HCl, 0.15 mM-NADH, 1.67 mM-ADP, 4.2 mM-MgSO₄, 33 mM-KCl and 5.5 units of lactate dehydrogenase and 4 units of pyruvate kinase at pH 7.6 in a final volume of 1 ml.

The assay of pyruvate kinase (EC 2.7.1.40) activity was based on that described by Zammit et al. (1978). The assay medium for the measurement of maximal activities contained
160 mM-triethanolamine/HCl, pH 7.35, 10 mM-MgCl₂, 80 mM-KCl, 0.17 mM-NADH, 5 mM-ADP, 2 mM-phosphoenolpyruvate, 2.5 µg antimycin A and 25 µg of lactate dehydrogenase in a final volume of 1 ml. Phosphoenolpyruvate was omitted from controls.

Lactate dehydrogenase (EC 1.1.1.27) activity was determined by the method of Kornberg (1955). The assay medium contained 33 mM-potassium phosphate, pH 7.4, 0.33 mM-sodium pyruvate and 0.1 mM-NADH in 3 ml. Pyruvate was omitted from blanks.

NADP-dependent malate dehydrogenase,"malic enzyme" (EC 1.1.1.40) activity was assayed by the method of Swierczynski et al. (1980), the assay mixture containing 50 mM-Tris/HCl, pH 7.4, 1 mM-MnCl₂, 10 mM-L-malate (omitted from blanks), 0.5 mM-NADP and 2 µg antimycin A. NADP-dependent "malic enzyme" was also determined by the method of Nagel et al. (1980). The assay medium contained 50 mM-Tris/HCl, pH 7.3, 10 mM-L-malate (omitted from blanks), 2 mM-MnSO₄, 0.1 mM-dithiothreitol and 0.5 mM-NADP.

The activity of NAD(P)-dependent "malic enzyme", with either NAD or NADP as cofactor, was measured as described by Nagel et al. (1980) in medium containing 50 mM-Tris/HCl, pH 7.3, 10 mM-L-malate (omitted from blanks), 2 mM-MnSO₄, 0.1 mM-dithiothreitol, 5 mM-sodium fumarate and either 0.5 mM-NAD or 0.5 mM-NADP.

Oxaloacetate decarboxylase (EC 4.1.1.3) activity was assayed by measuring oxaloacetate consumption and pyruvate production by the methods described by Dean & Bartley (1973).

Branched-chain aminotransferase (EC 2.6.1.42) activity was assayed as follows. The assay mixture, containing 100 mM-potassium phosphate buffer, pH 8.4, 15 mM-2-oxoglutarate, 0.1 mM-pyridoxal phosphate and 3 mM-L-[1-14C]-leucine or valine (167 mCi.mole⁻¹) in a final volume of 1 ml, was incubated in 10 ml conical flasks containing glass centre wells and stoppered with rubber serum caps. In the glass centre wells were placed gelatin capsules (Parke, Davis & Company, Hounslow, London, U.K.) which in turn contained 1 cm x 2 cm glass fibre paper wicks (Whatman GF/C) and 0.2 ml hyamine 10X (hydroxide). The assay mixture was warmed for 5 min at 37°C in a shaking water bath. The tissue preparation was then added and the reaction terminated after 10 min by the injection of 0.5 ml 0.5 M-H₂SO₄ through the serum caps into the assay mixture. The flasks were then placed on ice and when cool 1 ml of 30 % (w/v) H₂O₂ was injected to decarboxylate the 2-oxoacids produced by transamination. The flasks were then gently shaken for 1 h at 20°C. The 14CO₂ evolved was absorbed by the hyamine-soaked glass fibre paper wick in the gelatin capsule. The capsule was lifted out by forceps at the end of the 1 h period and immersed in 4 ml scintillation cocktail (see above) + 0.3 ml distilled water. Tissue blanks contained acid inactivated tissue samples.

The activity of succinate dehydrogenase (EC 1.3.99.1) was assayed as follows. 0.25 ml 1.25 % (w/v) 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) in 0.2 M-
potassium phosphate buffer, pH 7.0 and 0.25 ml 0.3 M-Na succinate and diluted tissue sample were incubated in a 1 ml volume for 10 min at 37°C. 0.25 ml 0.3 M-Na malonate, a competitive inhibitor of succinate dehydrogenase, replaced Na succinate in the assay blanks. The reaction was stopped by the addition of 6 ml of a mixture of 10% (w/v) trichloroacetic acid: 96% (v/v) ethanol: ethyl acetate (2:13:20) by volume. The mixtures were then centrifuged at approximately 500 g for 5 min and the absorbance of the supernatants measured at 490 nm. Activity was calculated assuming that reduced INT has an extinction coefficient of $20 \times 10^3$ l.mol$^{-1}$cm$^{-1}$.

Alanine aminotransferase (EC 2.6.1.2) activity was assayed by the method of Segal et al. (1962) in a volume of 3 ml. The assay volume contained 100 mM-Tris/HCl, pH 8.0, 6.6 mM-2-oxoglutarate, 0.1 mM-NADH, 33 mM-L-alanine (omitted from blanks) and 25 μg lactate dehydrogenase.

Aspartate aminotransferase (EC 2.6.1.1) activity was assayed by the method of Herzfeld & Greengard (1971) in a volume of 1 ml. The assay mixture contained 100 mM-Tris/HCl, pH 7.6, 6.6 mM-2-oxoglutarate, 0.2 mM-NADH, 80 mM-L-aspartate (omitted from blanks) and 5 units of malate dehydrogenase.

Glutamate dehydrogenase (EC 1.4.1.2) activity was assayed in a volume of 1 ml containing 50 mM-potassium phosphate, pH 7.5, 60 mM-NH$_4$Cl, 3 mM-2-oxoglutarate (omitted from blanks) 0.15 mM-NADH, 1 mM-EDTA, 1.6 mM-ADP and 2 μg antimycin A, based on the method of Williamson et al. (1967b) which was in turn a modification of the method of Schmidt (1963) assayed in the
presence of NADH and 1.6 mM-ADP which activated the enzyme (Tomkins et al., 1963).

Citrate synthase (EC 4.1.3.7) activity was determined by the method of Alp et al. (1976). The 1 ml assay volume contained 50 mM-Tris/HCl, pH 8.1, 0.2 mM-DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)), 0.1 mM-acetyl-CoA and 0.5 mM-oxaloacetate (omitted from blanks). The assay for citrate synthase is based on the reaction of CoASH with Ellman's reagent (DTNB) (Ellman, 1959; Srere et al., 1963).

\[
\text{CoASH} + O_2N-S-S-CO_2^- \\
\downarrow \\
O_2N-S-CoA + S-CO_2^- + H^+ 
\]

The mercaptide ion absorbs light at 412 nm. Thus when acetyl-CoA and oxaloacetate react in the presence of citrate synthase and DTNB the rate of CoASH formation can be followed spectrophotometrically. Activity was calculated assuming a molar extinction coefficient of \(13.6 \times 10^3 \text{1.mol}^{-1}\text{cm}^{-1}\) for the mercaptide ion (Srere et al., 1963).
2.7 OXIDATION AND TRANSAMINATION OF BRANCHED-CHAIN AMINO ACIDS IN VITRO

Branched-chain amino acid oxidation was assessed by the release of $^{14}\text{CO}_2$ from muscle incubated in the presence of $^{14}\text{C}$-labelled valine or leucine. L-$[1^{-14}\text{C}]$-valine, L-$[U^{-14}\text{C}]$-valine, L-$[1^{-14}\text{C}]$-leucine and L-$[U^{-14}\text{C}]$-leucine were employed in these studies. Muscle preparations previously pre-incubated as described (Section 2.3) were incubated at $37^\circ\text{C}$ in a shaking incubator under an atmosphere of 95% $\text{O}_2$:5% $\text{CO}_2$ in narrow necked (14/23) 25 ml conical flasks. The incubation media contained either L-$[1^{-14}\text{C}]$ or L-$[U^{-14}\text{C}]$-valine or leucine (67 mCi.mole$^{-1}$). The flasks were stoppered with self-sealing caps fitted with polyethylene hanging centre wells (Kontes Scientific Glassware, Vineland, N.J., U.S.A.). After incubation for 105 min, 0.2 ml hyamine 10X (hydroxide) was injected through the caps into the centre wells to trap $^{14}\text{CO}_2$ evolved. 15 min later (i.e. after 2 h incubation), the caps were opened, muscle removed from the flasks, the caps rapidly replaced and 1 ml of 0.5 M-$\text{H}_2\text{SO}_4$ injected through the caps into the incubation medium. The $^{14}\text{CO}_2$ driven from the incubation medium by the acidification was collected during 1 h shaking at $37^\circ\text{C}$. The caps were then removed from the flasks and the hyamine taken for radioactivity counting. Hyamine was removed from each well by Pasteur pipette and the wells rinsed with 0.3 ml distilled $\text{H}_2\text{O}$ which was then combined with the hyamine. 4 ml of scintillation cocktail (see Section 2.6) was added and radioactivity counted.

In experiments using L-$[1^{-14}\text{C}]$-leucine or valine, branched-
chain 2-oxo acids released during the incubation can be measured by decarboxylation of the 2-oxo acids with $\text{H}_2\text{O}_2$ and measuring the $^{14}\text{CO}_2$ evolved. The relevant flasks were therefore re-stoppered with fresh cups of hyamine 10X (0.2 ml) and 1 ml of 30% (w/v) $\text{H}_2\text{O}_2$ injected into the media. The $^{14}\text{CO}_2$ evolved was collected for a further hour with shaking at $37^\circ\text{C}$ and the hyamine removed for radioactivity counting as above. Thus a value for branched-chain 2-oxo acid release into the media was obtained which, when added to the oxidation of $1^{14}\text{C}$ observed, gave a measure of the total transamination of the amino acid and also provides information on the branched-chain 2-oxo acid dehydrogenase (EC 1.2.4.4) activity of the muscle preparation. Comparison of the rates of $^{14}\text{CO}_2$ production for L-[1-$^{14}\text{C}$] or L-[U-$^{14}\text{C}$]-amino acids provided information on to what extent the branched-chain amino acid carbon skeleton was oxidised by the muscle preparations (see Chapter 3).
2.8 CHROMATOGRAPHIC PROCEDURES

After 2 h incubation and removal of tissues, the incubation media for muscle preparations incubated as described in Section 2.7 were acidified with 20% (w/v) HClO₄ (0.1 ml/ml medium). The flasks were shaken at 37°C for 1 h and the hyamine 10X counted as previously. The acidified media were centrifuged and the supernatants neutralised as in Section 2.3.3. A 2.5 ml aliquot of deproteinised, neutralised extract was then applied to a Dowex 50W H⁺-form column (1 x 4 cm) and washed through with 20 ml deionised H₂O. The amino acids absorbed on the column were eluted with 35 ml 1.5 M-HCl (Spydevold, 1976). The first 20 ml through the column and the next 35 ml were collected. Both these samples were lyophilised and the residue taken up in 3 ml deionised H₂O. These samples were further lyophilised, then each taken up in 200 µl deionised H₂O. 20 µl of a sample derived from the 1.5 M-HCl wash-through were spotted in one corner of a cellulose thin-layer chromatography plate (pre-coated 20 x 20 cm, layer thickness, 0.1 mm; Merck 5716 supplied by BDH Chemicals, Poole, Dorset, U.K.) for 2-dimensional separation by electrophoresis and thin-layer chromatography. 10 µl of an amino acid carrier mixture was applied on top of the experimental sample spot. Electrophoresis was carried out at pH 1.9 (2.5% (v/v) formic acid; 7.8% (v/v) acetic acid in deionised H₂O (Walker & Bark, 1966)) in an electrophoresis tank (Chemlab Instruments Ltd., Ilford, Essex, U.K.) supplied by an Electrophoresis Power Supply Type 264 (Medical & Biological Instrumentation Ltd., Ashford, Kent, U.K.) at 450V potential.
(<10mA) for 1.5 h. Each plate was then thoroughly dried, turned through 90°, and developed by thin-layer chromatography. The solvent system in the second direction was butan-1-ol/acetic acid/water (3/1/1 by volume), (Walker & Bark, 1966). Spots corresponding to amino acids were visualised by spraying with ninhydrin reagent (BDM Chemicals, Poole, Dorset, U.K.) scraped from the plates and counted for radioactivity. Spots were identified by their Rf values on chromatography and relative mobilities on electrophoresis (Katz & Lewis, 1966; Chudzik & Klein, 1968).
CHAPTER THREE

ALANINE RELEASE AND BRANCHED-CHAIN AMINO ACID METABOLISM IN MUSCLE IN VITRO
3.1 INTRODUCTION

Since the studies of Odessey et al., (1974) it has been known that adding certain amino acids to the incubation medium of in vitro muscle preparations stimulates the release of alanine from those preparations. As noted in Chapter 1 there is controversy about the source of pyruvate for alanine synthesis in muscle. Although some investigators have maintained that under physiological conditions glucose is the main source (Odessey et al., 1974; Chang & Goldberg, 1978b), others have implicated amino acids as the primary precursors for pyruvate in the muscle (Goldstein & Newsholme, 1976; Garber et al., 1976b). Whatever the origin of pyruvate there appears to be a relationship between alanine release and branched-chain amino acid oxidation by the muscle. The greater release of alanine by isolated muscle of fasted rats compared with fed rats when branched-chain amino acids are added to the medium seems related to the enhancement of the muscle oxidation of branched-chain amino acids by starvation (Odessey et al., 1974). Patients with hereditary enzymatic defects in the oxidative decarboxylation of branched-chain amino acids (Maple Syrup Urine Disease) have low serum alanine levels (Haymond et al., 1973;1978). Studies of alanine fluxes in these patients have indicated that branched-chain amino acid catabolism is an important rate-limiting event for alanine production in vivo (Haymond et al., 1978).
The main aim of the experiments described in this chapter was to study the interrelationship between muscle alanine release and branched-chain amino acid metabolism by examining the incubation conditions necessary for alanine release by muscle, by studying the extent of branched-chain amino acid metabolism and its involvement with alanine production by muscle, and by comparing the release of other metabolites from muscle with the release of alanine. Incubations of rat hemi-diaphragm and intact soleus and extensor digitorum longus (EDL) preparations were employed. The muscles were taken from fed and fasted animals in order to observe how muscle amino acid metabolism and production in vitro varies in different nutritional states in which adaptations of the enzymes involved have occurred.

3.2 RESULTS AND DISCUSSION

3.2.1 The Muscle Preparation

The diaphragm is a thin sheet of muscle fibres which can be rapidly dissected from the rat. The muscle is composed of three distinct regions: two larger lateral portions, which are referred to as hemi-diaphragms and a smaller dorsal segment adjacent to the spinal cord, which is too thick to be used for tissue incubations, through which passes the oesophagus, aorta and vena cava. In the experiments reported here, the small dorsal triangular segment of muscle has been discarded and the responses of the contralateral hemi-diaphragms compared in a paired experimental design. This muscle preparation offers the
advantage of comparisons between muscle segments from the same animal, thereby reducing problems caused by biological variability. Young growing rats weighing 60 - 100 g or less were used. Diaphragms of rats of this size are thin enough to avoid problems of diffusion of nutrients between the medium and the incubated muscle (Goldberg et al., 1975).

Goldberg et al. (1975) reported that the cut hemi-diaphragm preparations may leak intracellular components during the first half hour after the muscle is trimmed away from the ribs and bisected. However, after this initial leakage, these reporters found the preparation to be stable by several criteria, including the tissue content of ATP and inorganic phosphate which did not fall significantly during a subsequent 90 min incubation. They also showed that the leakage of total creatine and proteins is about 10 times more rapid during the first 30 min than afterwards. They observed that the ratio of phospho-creatine:total creatine and ATP:total creatine remained constant during a subsequent incubation period suggesting that the majority of cells in the tissue preparation maintain their energy reserves. In addition, intracellular concentrations of several amino acids (e.g. phenylalanine and tyrosine) remained constant during the incubation. They therefore recommend a 30 min pre-incubation when using hemi-diaphragm preparations in studies involving measurements of the incubation medium and this was employed in the present study. Following the pre-incubation period, Goldberg et al.; (1975) found that rates of glucose oxidation, amino acid oxidation, protein synthesis,
protein degradation and nucleic acid synthesis were all linear for at least 2 hours. Hemi-diaphragm ATP content was found not to fall significantly in the present study during the 2 h incubation period. Using paired hemi-diaphragms from 48 h fasted rats, the tissue concentration of ATP was found to be $2.9 \pm 0.3 (3) \mu$moles/g after the initial 30 min pre-incubation and $2.4 \pm 0.5 (3) \mu$moles/g after a further 2 h incubation, indicating that the tissue preparations remained viable throughout the incubation period.

With regard to muscle fibre type, diaphragm is a mixture in which red fibres predominate (60%) but white and intermediate fibres each make up 20% of the total (Goldberg et al., 1975). For studies involving the comparison of muscle types, preparations of soleus and EDL muscles are useful. They are of similar size and shape but contain substantially different proportions of fibre types. 80 - 90% of the fibres of the "red" soleus muscle are of the slow twitch variety whereas the "white" EDL muscle contains mainly fast twitch fibres and very few slow ones. Both muscles are cylindrical and so a limitation on their usefulness is imposed by size. In rats heavier than 70 g, the muscles become too thick to allow adequate diffusion of nutrients, oxygen and precursors into the muscle. Therefore, in this study, the animals from which soleus and EDL preparations were removed for incubation weighed no more than 70 g.

The soleus and EDL muscle preparations used in this study do not involve cutting the muscle. The intact muscle preparations were held on frames under slight tension during
incubation (Maisels et al., 1977). Stretched muscle has been shown to maintain higher concentrations of high-energy phosphates at the end of an incubation period than unstretched muscles (Seider et al., 1980). Maisels et al. (1977) have shown these preparations to maintain phosphocreatine and ATP concentrations, and constant rates of glucose uptake and lactate release throughout a 2 h incubation.

3.2.2 Effect of Amino Acid Substrates on Muscle Alanine Release

In Vitro

Alanine can be formed de novo in skeletal muscle by the transfer of amino groups to pyruvate. The direct transamination of pyruvate in muscle is largely restricted to glutamate (Rowsell, 1956; Rowsell & Corbett, 1958) and is catalysed by alanine aminotransferase. If the amino groups for alanine synthesis are derived from other amino acids, transamination with 2-oxoglutarate must occur to form glutamate for transamination via alanine aminotransferase. As transamination of 2-oxoglutarate in muscle has only been found to occur to any great extent with aspartate and valine, isoleucine and leucine (the branched-chain amino acids) (Krebs, 1975), these four amino acids and glutamate were expected to be the principal amino group donors for alanine formation.

Following a 30 min pre-incubation period in the absence of exogenous substrates, hemi-diaphragms were incubated for a further 2 h in the presence of the individual amino acids. Table 3.1 shows that the branched-chain amino acids were indeed
### TABLE 3.1 Rates of Metabolite Release By Hemi-Diaphragms From a) Fed and b) 48 h-Fasted Rats

Values represent the means of measurements from different rats and are given together with S.E.M. and the number of observations in parentheses. Statistical analysis (Student's t-test) was carried out on paired observations and differences with respect to incubations in the absence of any additions are shown by:  *p < 0.05, **p < 0.01, ***p < 0.001.

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>Alanine</th>
<th>Pyruvate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>1.48 ± 0.14 (13)</td>
<td>0.86 ± 0.10 (13)</td>
<td>6.43 ± 0.70 (13)</td>
</tr>
<tr>
<td>3 mM-Valine</td>
<td>2.47 ± 0.15 (13)**</td>
<td>0.85 ± 0.13 (13)</td>
<td>6.53 ± 0.48 (13)</td>
</tr>
<tr>
<td>10 mM-Glucose</td>
<td>1.89 ± 0.13 (12)**</td>
<td>2.18 ± 0.17 (12)**</td>
<td>16.50 ± 0.74 (12)**</td>
</tr>
<tr>
<td>10 mM-Glucose + 3 mM-Valine</td>
<td>3.05 ± 0.19 (5)**</td>
<td>2.62 ± 0.08 (5)**</td>
<td>18.50 ± 0.31 (5)**</td>
</tr>
<tr>
<td>3 mM-Leucine</td>
<td>2.57 ± 0.17 (5)**</td>
<td>0.38 ± 0.08 (5)**</td>
<td>7.44 ± 1.23 (5)</td>
</tr>
<tr>
<td>3 mM-Glutamate</td>
<td>3.59 ± 0.09 (4)**</td>
<td>0.37 ± 0.06 (4)**</td>
<td>7.20 ± 0.83 (4)</td>
</tr>
</tbody>
</table>
(TABLE 3.1 Continued)

b) **48 h-FASTED**

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>Alanine</th>
<th>Pyruvate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>1.06 ± 0.12 (12)</td>
<td>1.43 ± 0.10 (12)</td>
<td>5.11 ± 0.35 (12)</td>
</tr>
<tr>
<td>3 mM-Valine</td>
<td>1.93 ± 0.15 (7)***</td>
<td>1.19 ± 0.13 (7)*</td>
<td>4.64 ± 0.42 (7)</td>
</tr>
<tr>
<td>10 mM-Glucose</td>
<td>1.67 ± 0.20 (8)***</td>
<td>4.97 ± 0.34 (8)***</td>
<td>30.4 ± 2.13 (8)***</td>
</tr>
<tr>
<td>10 mM-Glucose + 3 mM-Valine</td>
<td>3.21 ± 0.29 (5)***</td>
<td>3.94 ± 0.22 (5)***</td>
<td>22.4 ± 1.91 (5)***</td>
</tr>
<tr>
<td>3 mM-Leucine</td>
<td>2.07 ± 0.11 (4)***</td>
<td>0.35 ± 0.07 (4)***</td>
<td>4.49 ± 0.41 (4)</td>
</tr>
<tr>
<td>3 mM-Glutamate</td>
<td>3.25 ± 0.33 (4)***</td>
<td>0.79 ± 0.11 (4)***</td>
<td>7.26 ± 0.95 (4)</td>
</tr>
</tbody>
</table>
found to stimulate alanine release from muscle. Valine and leucine both significantly increased alanine release from diaphragm preparations from fed or 48 h-starved rats. The presence of 3 mM-valine or 3 mM-leucine caused a 67 % and a 74 % increase, respectively, in alanine release from fed rat diaphragm and a 82 % and a 95 % increase from 48 h-starved rat diaphragm.

The presence of 3 mM-glutamate, which is the immediate donor of amino groups for the transamination of pyruvate to alanine, increased alanine release to a greater extent than 3 mM-valine or 3 mM-leucine from muscle of both fed (143 %) and fasted (207 %) rats.

The finding that valine and leucine appreciably increased the release of alanine by hemi-diaphragm muscle preparations in vitro was in agreement with earlier observations that alanine release by diaphragm preparations is stimulated by a mixture of valine, leucine and isoleucine together at physiological concentrations (Odessey et al., 1974) or by isoleucine alone (Goldstein & Newsholme, 1976). A mixture of other plasma amino acids (omitting the branched-chain amino acids) failed to increase alanine output by diaphragm muscle, even at amino acid concentrations which were 5-times the normal plasma levels (Odessey et al., 1974). 10 mM-substrate concentrations of branched-chain and other amino acids increased alanine output by the isolated perfused hindquarter (Ruderman & Lund, 1972; Ruderman & Berger, 1974) and by an epitrochlaris muscle preparation in vitro (Garber et al., 1976b). These observations, together
with those reported here, support the enzymic data that the branched-chain amino acids are, together with aspartate and glutamate, the major amino nitrogen donors for alanine formation in muscle tissues (Krebs, 1975).

The addition of 10 mM-glucose to the incubation medium increased alanine, pyruvate and lactate release by hemi-diaphragms from fed and fasted rats (Table 3.1). With fed rats, the stimulation of lactate and pyruvate release by glucose, presumably reflecting increased glycolysis, was more than doubled (256% of lactate plus pyruvate release in glucose-free medium), whereas alanine release was increased by only 28%. Similarly, with fasted rats, lactate and pyruvate release was increased more than five-fold (540% of lactate + pyruvate release in glucose-free medium), whereas alanine release was increased by only 58%. The rate of lactate and pyruvate release in the presence of 10 mM-glucose with fasted rats was about double that with fed rats, whereas increased alanine release was only marginally greater with fasted rats compared to fed rats in these experiments. These findings suggest a limitation of alanine production by muscle under the above circumstances which is not governed by the rate of glycolysis.

The addition of 10 mM-glucose and 3 mM-valine together increased alanine release to a greater extent than the sum of the rates with each substrate separately: +2.15 as against +1.48 for the sum of the separate rates with fasted rats (in μmol/2 h/g tissue). This effect of valine and glucose together on alanine release was greater with fasted rats (145% of the sum of the
separate rates) than with fed rats (112%). With glucose as the sole added substrate the provision of amino groups was the factor limiting alanine release. With valine alone as substrate, the provision of pyruvate was limiting. Therefore the addition of substrate amounts of both glucose and valine synergistically increased the rate of alanine release which was seen to depend on both the supply of donor amino groups and pyruvate.

In the absence of glucose in the incubation media, the stimulation of alanine release by valine, leucine and glutamate, was accompanied by a decrease in pyruvate release, except in the case of stimulation of alanine release by valine from hemi-diaphragms from fed rats. This observation agreed with a role for valine, leucine and glutamate in providing the amino groups for transamination of pyruvate to form alanine. However, the decrease in pyruvate release was only equivalent to the increase in alanine release in the case of leucine with fasted rats. In the other cases, the decrease in pyruvate release was less than was expected on the basis of increased alanine release. This raises the possibility that these substrates may themselves contribute carbon for pyruvate formation for alanine release.

When hemi-diaphragms were incubated in the presence of the 2-oxo acids of valine and leucine, i.e. 3-methyl-2-oxo-butanoate and 4-methyl-2-oxopentanoate, the rates of release of alanine by the muscle preparations were markedly decreased (Table 3.2). This was to be expected as the provision of 3 mM-2-oxo acids would displace the coupled transaminations towards branched-chain amino acid and 2-oxoglutarate formation. The
TABLE 3.2 Effect of 4-Methyl-2-Oxopentanoate and 3-Methyl-2-Oxobutanoate on the Rate of Metabolite Release by Hemi-Diaphragms From: a) Fed, and b) 48 h-Fasted Rats

Values represent the means of measurements from different rats and are given together with S.E.M. and the number of observations in parenthesis. Statistical analysis (Student's t-test) was carried out on paired observations and differences with respect to incubations in the absence of any additions are shown by: * p < 0.05, ** p < 0.01, *** p < 0.001.

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>Alanine (μmol/2 h/g tissue)</th>
<th>Pyruvate (μmol/2 h/g tissue)</th>
<th>Lactate (μmol/2 h/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>1.47 ± 0.11 (8)</td>
<td>0.91 ± 0.13 (8)</td>
<td>9.06 ± 1.14 (8)</td>
</tr>
<tr>
<td>3 mM-4-methyl-2-oxopentanoate</td>
<td>0.94 ± 0.13 (4)</td>
<td>1.00 ± 0.09 (4)*</td>
<td>16.72 ± 2.18 (4)*</td>
</tr>
<tr>
<td>3 mM-3-methyl-2-oxobutanoate</td>
<td>0.78 ± 0.06 (4)*</td>
<td>1.37 ± 0.31 (4)**</td>
<td>9.28 ± 1.40 (4)</td>
</tr>
</tbody>
</table>
(TABLE 3.2 Continued)

b) 48 h-FASTED

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>Alanine</th>
<th>Metabolite Formation</th>
<th>(μmol/2 h/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>0.81 ± 0.08 (14)</td>
<td>1.03 ± 0.13 (14)</td>
<td>6.42 ± 0.45 (14)</td>
</tr>
<tr>
<td>3 mM-4-methyl-2-oxopenatanoate</td>
<td>0.43 ± 0.05 (7)*</td>
<td>0.86 ± 0.08 (7)</td>
<td>7.93 ± 0.38 (7)</td>
</tr>
<tr>
<td>3 mM-3-methyl-2-oxobutanoate</td>
<td>0.40 ± 0.11 (7)***</td>
<td>2.00 ± 0.14 (7)***</td>
<td>10.16 ± 1.80 (7)*</td>
</tr>
</tbody>
</table>
resulting lowered glutamate concentration would thus displace
the alanine aminotransferase reaction towards pyruvate formation.

However, in the case of 3-methyl-2-oxobutanoate (keto-
valine) and tissue from 48 h-fasted rats, the decrease in alanine
release observed was much less than the resulting increase in
pyruvate (and lactate) release.

Among the possible explanations for the above observations
are inhibition of intracellular pyruvate oxidation by the
branched-chain 2-oxo acids (either supplied exogenously, Table
3.2, or formed by the transamination of the branched-chain amino
acids, Table 3.1) (Dreyfus & Prensky, 1967; Kanzaki et al., 1969;
Bowden et al., 1971) or pyruvate formation from the added amino
acids (Goldstein & Newsholme, 1976) or 2-oxo acids.

3.2.3 A Non-Glycolytic Source of Pyruvate for Alanine Formation?

The amounts of lactate and pyruvate released from hemi-
diaphragms were substantial when the preparations were incubated
in unsupplemented Krebs-bicarbonate medium, even when the
preparations were taken from 48 h-starved rats. This implies
that the carbon for the lactate and pyruvate release is derived
from endogenous sources. Muscle glycogen is a logical suggestion as pyruvate can be provided directly via glycogenolysis and glycolysis. Indeed muscle glycogen has been suggested as the source in vivo of the muscle-derived lactate, pyruvate (and perhaps alanine) which may give rise to glucose formation via the circulation and hepatic or renal gluconeogenesis during short-term starvation (Sugden et al., 1976). Goldstein and Newsholme (1976), however, reported that hemi-diaphragm preparations showed no statistically significant difference in glycogen content before or after a 60 min period of incubation with glutamate as the only added substrate. Their results were obtained with tissue taken from fed rats, suggesting that in the fed state the carbon of the lactate, pyruvate and alanine released cannot be derived from net muscle glycogenolysis.

Similarly, other workers have shown an increased rate of alanine release and a lower rate of net glycogen breakdown when hemi-diaphragms from fed rats were incubated in the presence of 3 mM-glutamate compared with no addition to the incubation medium (Odedra & Palmer, 1981). If the carbon for alanine formation had been derived exclusively from glycogen an increased rate of net glycogen breakdown would have been expected when alanine release was stimulated in the presence of glutamate.

However, Table 3.3 shows that hemi-diaphragm preparations from fed rats in the present study do in fact show a decrease in glycogen content during a 2 h incubation period. The difference observed between glycogen content at the start and end of the 2 h was substantial (5.5 μmol glucosyl units/g tissue). This amount
<table>
<thead>
<tr>
<th></th>
<th>Glycogen content (µmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a) FED</strong></td>
<td></td>
</tr>
<tr>
<td>30 min pre-incubation</td>
<td>9.58 ± 1.97 (4)</td>
</tr>
<tr>
<td>30 min pre-incubation + 2 h incubation</td>
<td>4.11 ± 2.00 (4)***</td>
</tr>
<tr>
<td><strong>b) 48 h-FASTED</strong></td>
<td></td>
</tr>
<tr>
<td>30 min pre-incubation</td>
<td>0.94 ± 0.24 (4)</td>
</tr>
<tr>
<td>30 min pre-incubation + 2 h incubation</td>
<td>0.05 ± 0.05 (4)**</td>
</tr>
</tbody>
</table>

Hemi-diaphragms were pre-incubated for 30 min in unsupplemented Krebs-bicarbonate medium. One of each pair was then taken for glycogen determination. The other was incubated in fresh medium for a further 2 h before glycogen determination.

Values represent the means of measurements from different rats and are given together with S.E.M. and the number of observations in parentheses. Statistical analysis (Student's t-test) was carried out on paired observations and differences are shown by:

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. 
of 6-carbon units is sufficient to provide the carbon for the lactate, pyruvate and alanine (3-carbon compounds) released by fed muscle preparations incubated in the absence of glucose from the medium. The decrease in glycogen content during the 2 h incubation period found with diaphragm preparations from 48 h-fasted rats (0.89 μmol glucosyl units/g tissue) would, however, not be sufficient to supply the carbon for the lactate, pyruvate and alanine release. These results suggest that in certain circumstances muscle is capable of producing pyruvate by pathways other than glycolysis. It is possible that in this case the carbon of the lactate, pyruvate and alanine released by the tissue may be derived from the carbon skeletons of amino acids, arising from muscle proteolysis.

The pathways may therefore exist in muscle to provide net pyruvate for alanine formation. Such pathways are at variance with the proposal that alanine released from skeletal muscle forms part of a glucose-alanine cycle (Mallette et al., 1969; Felig et al., 1970) in which circulating glucose taken up by skeletal muscle serves by glycolysis as the source of pyruvate for alanine formation, which has received strong support (Chang & Goldberg, 1978a, b). This pathway does not allow for the provision of net carbon for new body glucose synthesis during fasting. However, pyruvate derived from muscle protein breakdown would make a net contribution via alanine and gluconeogenesis.

The lack of a direct relationship between the rate of muscle alanine release and glycolytic pyruvate production was also suggested by the effects of starvation on metabolite release
by hemi-diaphragms incubated in the presence of 10 mM-glucose or 10 mM-glucose and 3 mM-valine (Fig. 3.1). In both media alanine release was significantly decreased (p < 0.01) by 24 h of starvation, but was significantly increased (p < 0.01) as starvation progressed for longer time periods. In contrast, both pyruvate and lactate release from the muscle preparations increased progressively with starvation and did not show the reduction at 24 h observed with alanine. Interestingly, this pattern of alanine release during progressive starvation correlates with protein degradation (Li & Goldberg, 1976).

3.2.3.1 Effect of Insulin on Muscle Alanine Release

Table 3.1 showed that when the intracellular provision of pyruvate was increased by incubating muscle preparations in the presence of glucose, the release of alanine, pyruvate, and lactate were stimulated. The effect on alanine release of a further stimulation of glycolysis from glucose by insulin is shown in Table 3.4. Insulin significantly stimulated glucose uptake by the muscle preparations. It also stimulated pyruvate and lactate release by approximately 50% from muscle from fed rats and about 100% from muscle from 48 h-starved rats, but significantly decreased alanine release by about 25% from hemi-diaphragms from fed rats and decreased alanine release (although not significantly) from hemi-diaphragms from starved rats. Thus, the presence of insulin, which stimulated glucose metabolism sufficiently to almost double lactate and pyruvate release, actually lowered alanine release. These results are in agreement
TABLE 3.4  Effect of Insulin (50 munits/ml) on the Rate of Metabolite Release and Glucose Uptake by Hemi-Diaphragms From a) Fed, and b) 48 h-Fasted Rats

Values represent the means of measurements from different rats and are given together with S.E.M. and the number of observations in parentheses. Statistical analysis (Student's t-test) was carried out on paired observations and differences with respect to incubations in the presence of 10 mM-glucose are shown by:

* p < 0.05, ** p < 0.01, *** p < 0.001.

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>Alanine</th>
<th>Pyruvate</th>
<th>Lactate</th>
<th>Glucose Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM-Glucose</td>
<td>2.29 ± 0.14 (4)</td>
<td>1.28 ± 0.13 (4)</td>
<td>14.9 ± 1.2 (4)</td>
<td>18.9 ± 6.6 (4)</td>
</tr>
<tr>
<td>10 mM-Glucose + 50 munits/ml of insulin</td>
<td>1.68 ± 0.18 (4)**</td>
<td>1.78 ± 0.34 (4)*</td>
<td>21.4 ± 1.2 (4)**</td>
<td>53.4 ± 8.1 (4)**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>Alanine</th>
<th>Pyruvate</th>
<th>Lactate</th>
<th>Glucose Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM-Glucose</td>
<td>2.50 ± 0.28 (4)</td>
<td>4.68 ± 0.60 (4)</td>
<td>29.6 ± 3.4 (4)</td>
<td>22.0 ± 7.1 (4)</td>
</tr>
<tr>
<td>10 mM-Glucose + 50 munits/ml of insulin</td>
<td>2.08 ± 0.42 (4)***</td>
<td>10.19 ± 0.87 (4)***</td>
<td>59.4 ± 7.0 (4)***</td>
<td>65.7 ± 3.9 (4)***</td>
</tr>
</tbody>
</table>
with those of other workers (Ruderman & Berger, 1974; Odessey et al., 1974).

The level of insulin is, however, probably one of the most important factors regulating protein balance in skeletal muscle (Cahill et al., 1972). After food intake, the elevated plasma level of insulin promotes the net uptake of amino acids by muscle and their incorporation into protein, while on fasting, the fall in insulin leads to a net release of amino acids from muscle (Felig, 1975; Ruderman, 1975). It is possible that the reduction in alanine release by insulin observed in the present study may have been in part due to the actions of the hormone in not only promoting the incorporation of amino acids into proteins but also in inhibiting protein degradation (Fulks et al., 1975; Rannels et al., 1975; Li & Goldberg, 1976; Hansen et al., 1977). For these reasons the effect of insulin on muscle alanine release was re-investigated using different experimental conditions. 3 mM-valine was present in all incubations together with 5 mM-glucose in order to provide adequate amino groups (i.e. glutamate) and pyruvate for alanine formation. A measure of the rate of net protein breakdown was also obtained by measuring the rate of tyrosine release from the hemi-diaphragms. Tyrosine was measured because this amino acid is not appreciably synthesised nor catabolised by muscle (Fulks et al., 1975). As a result its production by isolated muscle must reflect net protein breakdown. Thus the effect of insulin on the degree of protein degradation observed from the hemi-diaphragms could be taken into account when considering alanine release. Some
experiments were also conducted in the presence of cycloheximide, an inhibitor of protein synthesis (Stanners, 1966; Pestka, 1971). The reasoning behind this addition was that cycloheximide would inhibit the incorporation of alanine into muscle proteins and hence the stimulatory effect of insulin on this process would be negated. A better estimation of the effect of insulin on hemi-diaphragm alanine production could thus be made. Cycloheximide was added to the incubation medium at a concentration (0.5 mM) used by other workers to inhibit protein synthesis in incubated muscle preparations (Goldberg et al., 1980). Insulin was again found to substantially increase the rate of glucose uptake by hemi-diaphragm preparations (by 68% in the absence of cycloheximide, and by 58% in the presence of cycloheximide) (Table 3.5). The rates of pyruvate and lactate release were also substantially increased by the presence of insulin (71% and 58% respectively with cycloheximide present). Under these experimental conditions insulin was not observed to cause a decrease in the rate of alanine release from the muscle preparations but gave a slight (although statistically insignificant) increase (11% with cycloheximide absent, and 10% with cycloheximide present). Other workers have reported that 0.1 mM-cycloheximide has no effect on alanine release by rat epitrochlearis muscle preparations (Karl et al., 1976). In the present study the alanine release when 0.5 mM-cycloheximide was present was slightly, (although not significantly) greater than that observed when it was omitted from the incubations. This was to be expected as the reincorporation of alanine into muscle proteins would be
TABLE 3.5  Effect of Insulin (50 munits/ml) on the Rate of Metabolite Release and Glucose Uptake by Hemi-Diaphragms From 48 h-Fasted Rats

Values represent the means of measurements from different rats and are given together with S.E.M. and the number of observations in parentheses.

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>Metabolite Release and Glucose Uptake (μmol/2 h/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alanine</td>
</tr>
<tr>
<td>5 mM-glucose + 3 mM-valine</td>
<td>2.68 ± 0.11 (4)</td>
</tr>
<tr>
<td>5 mM-glucose + 3 mM-valine</td>
<td>2.98 ± 0.16 (4)</td>
</tr>
<tr>
<td>+ 50 munits of insulin</td>
<td></td>
</tr>
<tr>
<td>5 mM-glucose + 3 mM-valine</td>
<td>2.97 ± 0.28 (4)</td>
</tr>
<tr>
<td>+ 0.5 mM-cycloheximide</td>
<td></td>
</tr>
<tr>
<td>5 mM-glucose + 3 mM-valine</td>
<td>3.27 ± 0.11 (4)</td>
</tr>
<tr>
<td>+ 0.5 mM-cycloheximide + 50 munits/ml of insulin</td>
<td></td>
</tr>
</tbody>
</table>
blocked. The increase, however, was unaffected by the presence of insulin. The rate of protein degradation, as measured by tyrosine release, was not significantly different in the presence or absence of insulin. Other workers have also observed no effect of insulin on amino acid release from rat muscle incubated in vitro (Garber et al., 1976a, 1976b; Karl et al., 1976). In those studies the concentration of insulin (when added) was varied over a wide range of concentrations (10 μunits/ml to 100 m units/ml) producing increases in the rates of glucose uptake and pyruvate release but no significant effect on alanine release or the rate of release of any of the amino acids studied (Garber et al., 1976a). Fulks et al. (1975) on the other hand, have reported that insulin (added at 100 munits/ml) significantly decreased the rate of tyrosine released by diaphragms of 2 day starved rats in vitro. Their results are expressed as percentage changes in comparison with tyrosine released from diaphragm pieces incubated in unsupplemented medium. The addition of insulin (100 munits/ml) to unsupplemented medium caused a significant (15%) decrease in tyrosine release. The addition of 10 mM-glucose caused a 10% decrease in tyrosine release and when 10 mM-glucose was added together with insulin (0.1 units/ml) a 19% decrease in tyrosine release was observed. Both these results were statistically significant when compared with the release of tyrosine from diaphragm preparations incubated in unsupplemented medium. However, those investigators made no study of whether insulin caused a significant reduction in tyrosine release from muscle incubated in the presence of glucose.
Their findings are therefore not directly comparable with those of Garber and coworkers or of the present study. In the present experiment, it appears therefore that decreased degradation of muscle protein and increased incorporation of alanine into muscle protein by insulin can be discounted as reasons for the alanine release in the presence of insulin to appear low in comparison with the increased release of lactate and pyruvate observed in the presence of insulin.

This experiment, as in the previous insulin experiments of this study and those of Garber et al. (1976a), failed to confirm a correlation between the rates of glucose uptake, pyruvate production and alanine release and these findings were contrary to the concept of the glucose-alanine cycle.

Other evidence that glucose metabolism and amino acid release are functionally independent processes in skeletal muscle has been presented by investigators who employed inhibitors of glycolysis. Garber et al. (1976a) and Goldstein & Newsholme (1976) have reported that inhibiting glycolysis with iodoacetate and fluoride did not decrease the rate of alanine synthesis and release by preparations of skeletal muscle. However conflicting results have been reported by Chang & Goldberg (1978b) who found a decreased rate of alanine release in the presence of iodoacetate or fluoride. They also commented that besides affecting glycolysis these compounds could increase the rate of net protein breakdown (as observed for sodium fluoride by Goldstein & Newsholme, 1976). Chang & Goldberg (1978b) found that 10 mM-sodium fluoride increased the rate of net protein breakdown
by over 70% and that 0.2 mM-iodoacetate accelerated proteolysis by about 90%. These findings suggest that the supposedly unaltered alanine releases observed in the presence of those inhibitors in the studies of Goldstein & Newsholme (1976) and Garber and coworkers (1976a) may have been the result of decreased alanine formation and increased release of alanine derived from protein breakdown.

However, although the glucose-alanine cycle probably functions as proposed, under some conditions the source of pyruvate for alanine formation in muscle may not always be from glycolysis. The experiments in the present study in which alanine release from muscle taken from 48 h-starved rats was stimulated by BCAAs as sole added substrate, while muscle glycogen levels were very low, strongly suggest (as was concluded by Garber et al., 1976b and Goldstein & Newsholme, 1976) that amino acids can be a source of pyruvate for alanine formation in some cases.

Chang & Goldberg (1978a) have, however, stated that the formation of the carbon skeleton of alanine from amino acids is of little or no quantitative importance and that the glucose-alanine cycle as originally proposed, adequately explains their data for alanine formation. They also state that some of their data appear to show net synthesis of glutamine from BCAAs and succinate but discount net alanine production from these precursors. It is possible that their conclusions may not be justified. The muscle incubations they used were carried out in the presence of glucose and their data show that glycolysis was extremely rapid. Maizels et al. (1977) have shown that the rate of
glycolysis in such muscle preparations is many times greater than in resting muscle \textit{in vivo} or in perfused hindquarter so that accumulation of lactate and pyruvate is rapid and substantial. This situation would lead to a decrease in the observed amount of amino acid-derived carbon forming alanine because of the large pool of glycolytically-derived lactate and pyruvate present in these muscle preparations.

To summarise: the proposed glucose-alanine cycle cannot explain some of the experimental findings of this present work. It is suggested from the results of this study that amino acids are potential sources of pyruvate for alanine formation in skeletal muscle and may be of particular importance in situations when the glycolytic rate in muscle is low.

3.2.4 Branched-Chain Amino Acid Metabolism by Rat Hemi-Diaphragm Preparations

The capacity for alanine production by muscle increases during starvation (Fig. 3.1). Similarly, starvation is accompanied by increased branched-chain amino acid metabolism by muscle (Goldberg & Odessy, 1972; Buse et al., 1976; Paul & Adibi, 1976). This section describes experiments on the effect of starvation on the metabolism of the BCAAs, valine and leucine by rat hemi-diaphragm preparations \textit{in vitro}.

Transamination of a BCAA results in the formation of the corresponding branched-chain 2-oxo acid (BCOA) with the next step of metabolism being decarboxylation at the C1 position of the carbon chain by BCOA dehydrogenase(s). The total
FIG. 3.1 Changes in Metabolite Release by Hemi-Diaphragm During Starvation

Each column represents mean ± S.E.M. using tissues from at least 4 different animals.

Additions to □ 10 mM-glucose
medium: □ 10 mM-glucose + 3 mM-valine

ALANINE

PYRUVATE

LACTATE

Rate of Release (μmol/2 h/g of tissue)

0 1 2 3 4 5 6 7

0 24 48 72

Hours Fasted
transamination of the BCAA by muscle can therefore be determined by measuring the sum of the CO₂ released from the C1 position and the amount of BCOA produced and released by the muscle.

By using BCAAs radio-labelled in the C1 position only, the ¹⁴CO₂ released by the action of BOAA dehydrogenases can be collected and measured. The 2-oxo acids can be determined by making use of the fact that H₂O₂ treatment of 2-oxo acids will specifically decarboxylate at the C1 position (Odessey & Goldberg, 1979). The BCOA derived from [1-¹⁴C]-BCAAs will therefore release ¹⁴CO₂ when treated with H₂O₂, which can be collected and counted to measure the amount of the BCOA present.

Total transamination of valine (Fig. 3.2) by hemi-diaphragm preparations was therefore measured as the sum of ¹⁴CO₂ and ¹⁴C-ketovaline (3-methyl-2-oxobutyrate) produced from [1-¹⁴C]-valine. In addition, an assessment of the branched-chain 2-oxo acid dehydrogenase activity was made from the ¹⁴CO₂ collected during incubations with [1-¹⁴C]-branched-chain amino acids (Odessey & Goldberg, 1979).

Values for the rate of oxidation of [1-¹⁴C]-BCAAs obtained by measuring ¹⁴CO₂ production tell us little of the extent to which the carbon skeletons of leucine and valine are oxidised by the hemi-diaphragm muscle preparations. To explore this aspect, one hemi-diaphragm from each pair was incubated in the presence of [1-¹⁴C]-BCAA and the other hemi-diaphragm in the presence of [U-¹⁴C]-BCAA. If both labelled species of the amino acid, i.e. [1-¹⁴C]- and [U-¹⁴C]-, are added to their respective incubation media at the same specific radioactivity
FIG. 3.2  Effect of Starvation on the Rate of Transamination of 3 mM-Valine by Rat Hemi-Diaphragm Preparations Incubated a) in the Absence of Glucose, and b) in the Presence of 5 mM-Glucose

Each value represents the mean ± S.E.M. of at least 4 observations.

a)

Rate of valine transamination (μmol/2 h/g tissue)  

<table>
<thead>
<tr>
<th>Hours Fasted</th>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.0</td>
<td>4.0</td>
<td>2.0</td>
<td>0</td>
</tr>
</tbody>
</table>

b)

Rate of valine transamination (μmol/2 h/g tissue)  

<table>
<thead>
<tr>
<th>Hours Fasted</th>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.0</td>
<td>4.0</td>
<td>2.0</td>
<td>0</td>
</tr>
</tbody>
</table>
the ratio of:
\[
\frac{^{14}C_2 \text{ released from } [U-^{14}C]-\text{amino acid}}{^{14}C_2 \text{ released from } [1-^{14}C]-\text{amino acid}}
\]

will give a measure of what proportion of the amino acid carbon is oxidised by hemi-diaphragm preparations. If all the BCAA carbon skeletons which are decarboxylated in the C1 position are completely oxidised to CO₂ the expected ratio would be 1.0. If, however, decarboxylation of C1 is the only source of \(^{14}C_2\) released from the carbon skeleton, the expected ratio would be 0.20 for valine (which has a five carbon skeleton) and 0.17 for leucine (which has a six carbon skeleton).

Thus, by incubating one of a pair of hemi-diaphragms in the presence of L-[1-\(^{14}C\)]-amino acid and the other with L-[U-\(^{14}C\)] amino acid the following information can be obtained:

1. \([1-^{14}C]\)-amino acid oxidation, giving an indication of the branched-chain 2-oxo acid dehydrogenase activity of the tissue;

2. branched-chain 2-oxo acid formation and release;

3. total transamination of the amino acid (the sum of (1) and (2) above);

4. \([U-^{14}C]\)-amino acid oxidation, giving an indication (when compared to (1) above) of the extent to which the amino acid carbon skeleton is oxidised.

Fig. 3.2 shows the effect of starvation on the rate of transamination of valine incubated in the presence or absence of 5 mM-glucose. Branched-chain 2-oxo acid dehydrogenase activity
was assessed from the decarboxylation of $[1-^{14}C]$-valine and is shown in Fig. 3.3 and 3-methyl-2-oxobutyrate release is shown in Fig. 3.4. With valine as substrate both branched-chain aminotransferase and branched-chain 2-oxo acid dehydrogenase activities increased with starvation. The increase in release of $^{14}CO_2$ from $[1-^{14}C]$-branched-chain amino acids by muscle preparations during starvation has been reported previously (Goldberg & Odessey, 1972; Adibi et al., 1974). Similarly, other workers have found that the rates of total transamination (sum of BCOA released and BCOA oxidised) were higher by hind-quarter preparations from 3 day-starved rats than by fed controls and the percentage of BCOA formed which was oxidised was also higher (Zapalowski et al., 1981), in agreement with the findings of the present study (Fig. 3.5).

It might be concluded that the activity of the dehydrogenase was increased because a greater proportion of the 2-oxo acids formed were decarboxylated. However, as it is well-known that as branched-chain amino acid concentration is increased a greater proportion of the 2-oxo acids formed are oxidised (Hutson et al., 1978; 1980; Spydevold, 1979), it is possible that this effect of starvation could be the result of an increased intracellular concentration of branched-chain amino acids. The increased oxidation could result from activation of the dehydrogenase by branched-chain amino acids (Khatra et al., 1977b; Roberts & Sokatch, 1978; Frick et al., 1981) or by the 2-oxo acids themselves (Waymack et al., 1980; Hughes & Halestrap, 1981). Alternatively, Zapalowski et al., (1981) have suggested that if
FIG. 3.3  Effect of Starvation on the Rate of Decarboxylation of 3 mM-[1-¹⁴C]-Valine by Rat Hemi-Diaphragm Preparations Incubated, a) in the Absence of Glucose, and b) in the Presence of 5 mM-Glucose

Each column represents mean ± S.E.M. using tissues from at least 4 different animals.

a)

Rate of 3 mM-[1-¹⁴C]-valine decarboxylation (µmol/2 h/g tissue)

<table>
<thead>
<tr>
<th>Hours Fasted</th>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

b)

Rate of 3 mM-[1-¹⁴C]-valine decarboxylation (µmol/2 h/g tissue)

<table>
<thead>
<tr>
<th>Hours Fasted</th>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>
FIG. 3.4 Effect of Starvation on the Rate of $[^{14}C]3$-Methyl-2-oxobutyrate Release From Rat Hemi-Diaphragms

Incubated in 3 mM-$[^{14}C]$-Valine a) in the Absence of Glucose and b) in the Presence of 5 mM-Glucose

Each value represents the mean ± S.E.M. of at least 4 observations.

a)

Rate of 3-methyl-2-oxobutyrate release ($\mu$mol/2 h/g tissue)

<table>
<thead>
<tr>
<th>Hours Fasted</th>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate</td>
<td>1.0</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

b)

Rate of 3-methyl-2-oxobutyrate release ($\mu$mol/2 h/g tissue)

<table>
<thead>
<tr>
<th>Hours Fasted</th>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate</td>
<td>1.4</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
</tr>
</tbody>
</table>
FIG. 3.5  Effect of Starvation on the Percentages of $[1^{-14}C]$-Valine Transaminated by Rat Hemi-Diaphragm Preparations Which are Released as $[1^{-14}C]$-3-Methyl-2-oxobutyrate.

Hemi-Diaphragms Incubated with 3 mM-$[1^{-14}C]$-Valine

a) in the Absence of Glucose and b) in the Presence of 5 mM-Glucose

Each column represents the means ± S.E.M. of observations from tissues from at least 4 different animals.

a)

\[ \% \text{ of } [1^{-14}C]\text{-valine} \]
\[ \text{transaminated} \]
\[ \text{which is released as } [1^{-14}C]\text{-3-} \]
\[ \text{methyl-2-oxobutyrate} \]

\begin{center}
\begin{tabular}{c|c|c|c|c}
\hline
         & 0 & 24 & 48 & 72 \\
\hline
Hours Fasted & 40 & 20 & 0 & 0 \\
\hline
\end{tabular}
\end{center}

b)

\[ \% \text{ of } [1^{-14}C]\text{-valine} \]
\[ \text{transaminated} \]
\[ \text{which is released as } [1^{-14}C]\text{-3-} \]
\[ \text{methyl-2-oxobutyrate} \]

\begin{center}
\begin{tabular}{c|c|c|c|c}
\hline
         & 0 & 24 & 48 & 72 \\
\hline
Hours Fasted & 50 & 25 & 25 & 25 \\
\hline
\end{tabular}
\end{center}
it is assumed that transport of BGOA into the mitochondria is limited when BCAA concentrations are low (as suggested by the hindquarter perfusion studies of Hutson et al., 1978; 1980) then another explanation exists. A carrier for the efflux of BGOA from the cells may become saturated before that for transport into mitochondria. As a result the rate at which BGOA cross the mitochondrial membrane would continue to increase, and hence a greater percentage would be oxidised, as their concentration increased.

The effect of starvation on the rate of 3 mM-leucine metabolism by rat hemi-diaphragm preparations is shown in Figs. 3.6 - 3.9. Differences were observed between the effect of starvation on leucine and valine metabolism. There was a \( p < 0.01 \) significant reduction in leucine transamination by tissue from 24 h-fasted rats, (tissue incubated both in the absence and presence of glucose), after which the rate of transamination increased as starvation progressed and the percentage BGOA formed that was oxidised, progressively increased (Fig. 3.9). This initial decline in leucine metabolism with fasting is difficult to compare with other reports of starvation. Paul & Adibi (1976) reported that starvation increased the rate of \( \alpha \)-decarboxylation of leucine but used homogenates of muscle taken from 5-day fasted rats. Similarly, the small increase in \( ^{14} \text{CO}_2 \) production from \([1-^{14}\text{C}]-\text{leucine}\) reported by Hutson et al. (1980) was comparing 3-day starved rats with fed controls.

It is interesting to note the rates of \( ^{14} \text{CO}_2 \) release observed from \([1-^{14}\text{C}]-\text{leucine}\) or \([1-^{14}\text{C}]-\text{valine}\) in the present
FIG. 3.6  Effect of Starvation on the Rate of Transamination of 3 mM-Leucine by Rat Hemi-Diaphragm Preparations Incubated a) in the Absence of Glucose and, b) in the Presence of 5 mM-Glucose

Each value represents the mean ± S.E.M. of at least 4 observations.

a)

Rate of leucine transamination (μmol/2 h/g tissue)

b)
FIG. 3.7  Effect of Starvation on the Rate of Decarboxylation of 3 mM-[1-14C] Leucine by Rat Hemi-Diaphragm Preparations Incubated, a) in the Absence of Glucose and b) in the Presence of 5 mM-Glucose

Each value represents the mean ± S.E.M. of at least 4 observations.

a)  
Rate of 3 mM-[1-14C] leucine decarboxylation (μmol/2 h/g tissue)

b)  
Rate of 3 mM-[1-14C] leucine decarboxylation (μmol/2 h/g tissue)
FIG. 3.8 Effect of Starvation on the Rate of [1-\textsuperscript{14}C]-4-Methyl-2-oxopentanoate Release From Rat Hemi-Diaphragms Incubated in 3 mM-[1-\textsuperscript{14}C]-Leucine a) in the Absence of Glucose and b) in the Presence of 5 mM-Glucose

Each value represents the mean ± S.E.M. of at least 4 observations.

a)

Rate of 4-methyl-2-oxopentanoate release (\(\mu\text{mol}/2\text{ h/g tissue}\))

<table>
<thead>
<tr>
<th>Hours Fasted</th>
<th>Rate of Release ((\mu\text{mol}/2\text{ h/g tissue}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td>24</td>
<td>1.2</td>
</tr>
<tr>
<td>48</td>
<td>1.2</td>
</tr>
<tr>
<td>72</td>
<td>1.2</td>
</tr>
</tbody>
</table>

b)

Rate of 4-methyl-2-oxopentanoate release (\(\mu\text{mol}/2\text{ h/g tissue}\))

<table>
<thead>
<tr>
<th>Hours Fasted</th>
<th>Rate of Release ((\mu\text{mol}/2\text{ h/g tissue}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.6</td>
</tr>
<tr>
<td>24</td>
<td>1.2</td>
</tr>
<tr>
<td>48</td>
<td>1.2</td>
</tr>
<tr>
<td>72</td>
<td>0.6</td>
</tr>
</tbody>
</table>
Effect of Starvation on the Percentages of $[1^{-14}C]$-Leucine Transaminated by Rat Hemi-Diaphragm Preparations Which are Released as $[1^{-14}C]$-4-Methyl-2-oxopentanoate. Hemi-Diaphragms Incubated with 3 mM-$[1^{-14}C]$-Leucine

a) in the Absence of Glucose and b) in the Presence of 5 mM-Glucose

Each value represents the mean ± S.E.M. of at least 4 observations.
experiments using hemi-diaphragm preparations. The rate of 4-methyl-2-oxopentanoate decarboxylation by diaphragm muscle homogenates under so-called optimal conditions has been reported as being 0.47 \( \mu \text{mol CO}_2/\text{h/g tissue} \) (Shinnick & Harper, 1976). However, the observed \(^{14}\text{CO}_2\) production from leucine by hemi-diaphragm from fed rats in the present experiment was 2.8 \( \mu \text{mol CO}_2/\text{h/g tissue} \). This suggests that on homogenisation there is a substantial loss of branched-chain 2-oxo acid dehydrogenase activity.

The extent of branched-chain amino acid metabolism by muscle was studied in order to ascertain whether BCAAs could provide the carbon for de novo alanine synthesis by muscle. Previous workers have suggested that muscle BCAA metabolism is restricted to 2-oxo acid decarboxylation (Paul & Adibi, 1976).

It has been suggested, in view of the reported distribution of activities of the first two enzymes of BCAA metabolism between the liver and muscle of the rat, i.e. rat muscle contains relatively high activity of branched-chain aminotransferase but low BCOA dehydrogenase activity, whereas rat liver contains low branched-chain aminotransferase activity but high BCOA dehydrogenase activity (Shinnick & Harper, 1976), that the metabolism of BCAAs in vivo involves cooperation between both tissues. It is proposed that rat muscle transaminates BCAAs and then releases a large proportion of the resulting 2-oxo acids into the circulation from which they are removed and oxidised by the liver (Livesey & Lund, 1980; Hutson et al., 1980). Human muscle, in contrast, does not release such a large proportion of the BCOAs formed.
from the transamination of BGAAs. The BGOAs appear to be more important respiratory fuels for human muscle than for rat muscle (Elia & Livesey, 1981). To assess the extent to which the carbon chain of valine was oxidised in rat muscle under the conditions of the present study, a comparison was made of the amount of $^{14}$C$_2$O$_2$ released from [U-$^{14}$C]-valine and [1-$^{14}$C]-valine.

The rates of oxidation of [U-$^{14}$C]-valine during fasting in hemi-diaphragm preparations are shown in Fig. 3.10 and the ratios of $^{14}$C$_2$O$_2$ release from [U-$^{14}$C]-valine / $^{14}$C$_2$O$_2$ release from [1-$^{14}$C]-valine are shown in Fig. 3.11. The ratios obtained at all times were intermediate between 0.2 and 1.0 indicating that oxidation did not only involve decarboxylation by branched-chain 2-oxo acid dehydrogenase but also that the valine carbon skeleton was not completely oxidised. Despite the increased metabolism of valine with starvation in the presence of 5 mM-glucose, the ratio decreased with starvation. This indicates that as starvation progresses the valine carbon is being less completely oxidised, allowing for the provision of valine-derived carbon as a potential source of pyruvate for alanine synthesis.

The effect of starvation on the oxidation of 3 mM-[U-$^{14}$C]-leucine by hemi-diaphragm preparations is shown in Fig. 3.12. Paul & Adibi (1976) using muscle homogenates found that the source of $^{14}$C$_2$O$_2$ released from the oxidation of leucine by skeletal muscle was mainly the carboxyl group of leucine. According to the established pathways for leucine oxidation (Meister, 1965), isovaleryl-CoA, the product of leucine de-
FIG. 3.10 Effect of Starvation on the Rate of Oxidation of
3 mM-[^14]C]-Valine by Rat Hemi-Diaphragm Preparations
Incubated a) in the Absence of Glucose, and b) in the
Presence of 5 mM-Glucose

Each value represents the mean ± S.E.M. of at least 4 observations.

a)

Rate of oxidation of 3 mM-[^14]C]-valine
(μmol/2 h/ g tissue)

b)

Rate of oxidation of 3 mM-[^14]C]-valine
(μmol/2 h/ g tissue)
FIG. 3.11 Effect of Starvation on the Ratio of $^{14}$CO₂ Released From 3 mM-[U-$^{14}$C]-Valine to $^{14}$CO₂ From 3 mM-[1-$^{14}$C]-Valine by Paired Rat Hemi-Diaphragm Preparations Incubated a) in the Absence of Glucose and, b) in the Presence of 5 mM-Glucose

Each column represents the means ± S.E.M. of observations from tissues from at least 4 different animals.

a)

Ratio of $^{14}$CO₂ release from $^{14}$CO₂ release from $[^{14}$C]$^{-}$valine $[^{14}$C]$^{-}$valine

<table>
<thead>
<tr>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Hours Fasted

b)

Ratio of $^{14}$CO₂ release from $^{14}$CO₂ release from $[^{14}$C]$^{-}$valine $[^{14}$C]$^{-}$valine

<table>
<thead>
<tr>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Hours Fasted
FIG. 3.12 Effect of Starvation on the Rate of Oxidation of 3 mM-[U-\textsuperscript{14}C]-leucine by Rat Hemi-Diaphragm Preparations Incubated a) in the Absence of Glucose and, b) in the Presence of 5 mM-Glucose

Each value represents the mean ± S.E.M. of at least 4 observations.

a) Rate of oxidation of 3 mM-[U-\textsuperscript{14}C]-leucine (\(\mu\text{mol}/2\text{ h/g tissue}\))

b) Rate of oxidation of 3 mM-[U-\textsuperscript{14}C]-leucine (\(\mu\text{mol}/2\text{ h/g tissue}\))
carboxylation, through a series of reactions is converted to 3-hydroxy-3-methyl glutaryl CoA (HMG-CoA); there is no release of CO$_2$ from these conversions. The cleavage of HMG-CoA to acetoacetate and acetyl-CoA and their subsequent oxidation by the tricarboxylic acid cycle is necessary for the production of additional CO$_2$ molecules from leucine. McGarry and Foster (1969) have reported that HMG-CoA is not present in the skeletal muscle of rats. Therefore the lack of HMG-CoA lyase could account for the failure of the muscle homogenates, observed by Paul & Adibi (1976) to release CO$_2$ from leucine other than its carboxyl group. The results of this study, however, do not agree with the conclusions of Paul and Adibi (1976).

Fig. 3.13 shows the ratios of $^{14}$CO$_2$ release from [U-$^{14}$C]-leucine to [1-$^{14}$C]-leucine. As for valine, the ratios obtained indicate that muscle can metabolise leucine beyond the decarboxylation step catalysed by branched-chain 2-oxo acid dehydrogenase, resulting in the release of further CO$_2$. This either indicates that HMG-CoA lyase activity is in fact present in muscle or that leucine is not metabolised by the conventionally accepted route.

The finding in the present study that muscle is capable of oxidising the carbon skeleton of BCAAs beyond the initial decarboxylation step receives support from the work of Veerkamp and Wagenmakers (1981). They compared the ratio of $^{14}$CO$_2$ released from [U-$^{14}$C] to [1-$^{14}$C]-4-methyl-2-oxopentanoate and 3-methyl-2-oxobutyrate by homogenates of diaphragm and by hemi-diaphragm preparations. Their results for the ratios were: 4-methyl-2-oxopentanoate, homogenate $0.14 \pm 0.01$ and hemi-
FIG. 3.13 Effect of Starvation on the Ratio of $^{14}\text{CO}_2$ Released From 3 mM-[U-$^{14}$C]-Leucine to $^{14}\text{CO}_2$ From 3 mM-[1-$^{14}$C]-Leucine by Paired Rat Hemi-Diaphragm Preparations Incubated a) in the Absence of Glucose and, b) in the Presence of 5 mM-Glucose

Each column represents the means ± S.E.M. of observations from tissues from at least 4 different animals.

a)

b)
and 3-methyl-2-oxobutyrate, homogenate
0.21 ± 0.03 and hemi-diaphragm 0.46 ± 0.09. Similar results
were also reported when comparing homogenates with intact
Other workers have reported contradictory findings for the
further metabolism of leucine. In agreement with Paul & Adibi
(1976), Van Hinsbergh et al. (1979) found that \(^{14}\text{CO}_2\) was only
liberated from \([\text{U-}^{14}\text{C}]\)-leucine by \(\alpha\)-decarboxylation of 4-methyl-
2-oxopentanoate with homogenates and mitochondria of quadriceps
muscle. However, these workers found that in intact muscle
4-methyl-2-oxopentanoate oxidation proceeds more to completeness.
This was also observed by Odessey and Goldberg (1972) and Buse
et al. (1975). The discrepancy might be explained by loss of
enzyme activity or by alterations in the mitochondrial environ­
ment during homogenisation. However, appreciable oxidation of
leucine (25 - 60 % of total \(\text{CO}_2\) produced) has been reported
by some investigators beyond the 4-methyl-2-oxopentanoate
dehydrogenase step, using homogenates of quadriceps and gastro-
cnemius muscles (Dohm et al., 1976). Perhaps the conditions
involved in some muscle homogenate preparations have an inhibitory
effect on branched-chain 2-oxo acid dehydrogenase activity. On
comparing the results of the present study with those of workers
who used muscle homogenates (Paul & Adibi, 1976; Shinnick &
Harper, 1976; Van Hinsbergh et al., 1979), not only does the
diaphragm preparation exhibit a substantially higher rate of
\(^{14}\text{CO}_2\) release from \([1-^{14}\text{C}]\)-BCAA than homogenates but also the
metabolism of the BCAA carbon chain is more complete than with
homogenates. Obviously, initial decarboxylation of BCAA is obligatory before metabolism can proceed further and so it appears that in some homogenate preparations the α-decarboxylating dehydrogenase activity is limiting for further metabolism.

Early studies of Johnson & Connelly (1972) suggested that the branched-chain 2-oxo acid dehydrogenase complex may be subject to inter-conversion by a phosphorylation-dephosphorylation mechanism. Pettit et al. (1978) did not obtain evidence for such interconversion with the enzyme purified from kidney. However, Parker and Randle (1978) found ATP to inactivate the rat heart enzyme and Odessey (1980) and Paul and Adibi (1982) made the same observation with branched-chain 2-oxo acid dehydrogenase from rat muscle. Likewise, other workers have presented evidence that the branched-chain 2-oxo acid dehydrogenase of various tissues may well be subject to regulation by covalent modification (Gubler & Malquist, 1979; Odessey & Goldberg, 1979).

Parker and Randle (1978) reported that the oxidation of 4-methyl-2-oxopentanoate by extracts of heart mitochondria can be greatly increased by incubating isolated mitochondria without an oxidisable substrate. This finding was confirmed by other workers (Sans et al., 1980a) who also found that hearts perfused without other substrates oxidise $[1-^{14}\text{C}]-\text{leucine}$ at very rapid rates and that extracts of mitochondria prepared from such hearts also oxidise $[1-^{14}\text{C}]-4\text{-methyl-2-oxopentanoate}$ at very rapid rates. On the other hand, they found that hearts perfused with glucose, pyruvate, β-hydroxybutyrate, acetoacetate
and palmitate have a greatly reduced capacity to oxidise [1-\(^{14}\)C]-leucine to \(^{14}\)CO\(_2\) and that extracts of mitochondria prepared from such rats oxidise 4-methyl-2-oxopentanoate at a much reduced rate (Sans et al., 1980a). The results are interesting when compared with the present study. The hemi-diaphragm preparations were pre-incubated in substrate-free incubation media which may have promoted activation of the decarboxylating dehydrogenase. However, the presence of 5 mM-glucose in the subsequent experimental incubation media did not cause a sizeable decrease in [1-\(^{14}\)C]-BCAA oxidation compared to glucose-free media (Fig. 3.7).

It is also possible that the rate of \(^{14}\)CO\(_2\) production from [1-\(^{14}\)C]-labelled BCAAs may depend not only on the transamination or decarboxylation reactions but also on the further oxidation route. Reactions distal to oxidative decarboxylation may influence \(^{14}\)CO\(_2\) production by accumulation of intermediates (Tanaka et al., 1976; Rhead et al., 1981). The finding of the lower ratios of release from [U-\(^{14}\)C]- to [1-\(^{14}\)C]-BCAAs with muscle homogenates may possibly be due to loss of activity of the enzymes catalysing reactions beyond 2-oxo acid decarboxylation, or by alterations in the mitochondrial environment during homogenisation (Krebs & Lund, 1977).

The metabolism of BCAAs by muscle under the conditions used in this study makes available substantial amounts of carbon to the muscle which could possibly be used for pyruvate formation, resulting in \textit{de novo} synthesis of alanine derived from BCAA carbon. The maximum potential provision of 3-carbon or 4-carbon
units to muscle in this way was calculated, on the assumption that carbon not oxidised to $^{14}\text{CO}_2$ or released in the form of branched-chain 2-oxo acids was available to the muscle. These calculated values will be overestimates because, for example, Spydevold (1979) studying valine metabolism has reported that muscle is capable of releasing not only 3-methyl-2-oxobutyrate but also 3-hydroxyisobutyrate. Nevertheless, by comparing rates of $[1-^{14}\text{C}]$- and $[\text{U}^{14}\text{C}]$-oxidation it is possible to calculate the amount of carbon made available to muscle from measurements of the decarboxylation of the 2-oxo acid and the amount of the remaining carbon which is then oxidised. For example, in the case of hemi-diaphragm preparations from fed rats incubated in the presence of 3 mM-valine, valine oxidation measured as $^{14}\text{CO}_2$ release from $[1-^{14}\text{C}]$-valine = $2.49 \pm 0.07 \mu\text{mol/2 h/g tissue}$ and from $[\text{U}^{14}\text{C}]$-valine = $1.40 \pm 0.06 \mu\text{mol/2 h/g tissue}$, where both the $[1-^{14}\text{C}]$-valine and $[\text{U}^{14}\text{C}]$-valine had been present at the same specific radioactivity. Thus, $5 \times 1.40 = 7.0 \mu\text{mol}$ of carbon were oxidised/2 h/g tissue and $4 \times 2.49 = 9.96 \mu\text{mol}$ of carbon were made available by decarboxylation of the 2-oxo acid/2 h/g tissue. Of the 7.0 $\mu\text{mol}$ of carbon oxidised/2 h/g tissue, 2.49 $\mu\text{mol}$ were from the initial 1-C oxidation. Hence, 4.51 $\mu\text{mol}$ of carbon were oxidised from positions 2,3,4 and 5. The remaining BCAA derived carbon is therefore $9.96 - 4.51 \mu\text{mol}$ carbon atoms = $5.45 \mu\text{mol}$ C atom i.e. $1.82 \mu\text{mol}$ of 3C units or $1.36 \mu\text{mol}$ of 4C units. The values for the different periods of starvation studied are shown in Table 3.6. As starvation proceeds BCAA makes available
TABLE 3.6 Potential Amount of 3 or 4-Carbon Units Made Available to Muscle From the Carbon Skeleton of Valine and Leucine During Starvation: a) When Incubated in the Absence of Glucose and b) When Incubated in the Presence of 5 mM-Glucose

<table>
<thead>
<tr>
<th>Nutritional State</th>
<th>Carbon Units Made Available (μmol/2 h/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>From Valine</td>
</tr>
<tr>
<td></td>
<td>From Leucine</td>
</tr>
<tr>
<td>Starvation Time (h)</td>
<td>3C units</td>
</tr>
<tr>
<td>0</td>
<td>1.82</td>
</tr>
<tr>
<td>24</td>
<td>1.83</td>
</tr>
<tr>
<td>48</td>
<td>3.30</td>
</tr>
<tr>
<td>72</td>
<td>2.55</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Nutritional State</th>
<th>Carbon Units Made Available (μmol/2 h/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>From Valine</td>
</tr>
<tr>
<td></td>
<td>From Leucine</td>
</tr>
<tr>
<td>Starvation Time (h)</td>
<td>3C units</td>
</tr>
<tr>
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<td>1.0</td>
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<td>2.0</td>
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<td>48</td>
<td>3.20</td>
</tr>
<tr>
<td>72</td>
<td>4.52</td>
</tr>
</tbody>
</table>
substantial amounts of non-oxidised carbon to the muscle. The potential amount of four-carbon units available from valine metabolism is more than sufficient to account for the valine stimulated alanine release observed at all stages of starvation (c.f. Fig. 3.1). The pathway for the provision of pyruvate from valine would require the withdrawal of carbon from the tricarboxylic acid cycle and will be discussed in more detail later. The possible involvement of leucine as a direct supply of carbon for pyruvate production is much more dubious as the accepted route of leucine metabolism would make such a pathway of leucine carbon impossible. This aspect was also considered by Garber et al., (1976b). They suggested that as leucine metabolism had been characterised in liver but not in skeletal muscle, that possibly leucine metabolism in skeletal muscle may proceed via a route which would permit the provision of net pyruvate. Leech et al. (1979) have also encountered this apparent puzzle. Although acknowledging that leucine is generally accepted as being entirely ketogenic these workers reported that alanine synthesis from dogfish muscle proceeded as rapidly with leucine as in the presence of any other substrate even in the absence of glucose. However, alternative explanations can be proposed to account for leucine stimulation of alanine formation in the absence of glucose. Garber et al. (1976b) presented no direct evidence for leucine carbon contributing directly to alanine formation. The source of the pyruvate in incubations without added glucose could be from other amino acids made available by muscle protein breakdown and leucine has been
reported to inhibit the oxidation of pyruvate in skeletal muscle during fasting (Chang & Goldberg, 1978c). Also, the concentration of the added leucine (and in particular with the high leucine concentration - 10 mM - used by Garber and coworkers) would stimulate the transamination of leucine and 2-oxoglutarate towards 4-methyl-2-oxopentanoate and glutamate formation. The subsequent increased amount of glutamate present would drive the transamination of pyruvate to increase alanine formation. Thus in the experiments performed without added glucose, involving muscle from 48 h-starved rats in which glycogen breakdown is not believed to be the source of pyruvate for leucine-stimulated alanine release, it is possible that pyruvate is formed from other amino acids. The inhibition of pyruvate oxidation by leucine observed under these circumstances would therefore enable substantial transamination with glutamate resulting in alanine production.

3.2.4.1 Effect of Glucose on Branched-Chain Amino Acid Metabolism by Rat Hemi-Diaphragm Preparations

As mentioned above, leucine has been reported to inhibit the oxidation of glucose and pyruvate in skeletal muscle during fasting (Chang & Goldberg, 1978c). In heart, however, both glucose and pyruvate are inhibitory to leucine oxidation (Buse et al., 1972; Sans et al., 1980b).

A summary of the effect of the presence of 5 mM-glucose in the incubations of the present study on leucine and valine metabolism is shown in Table 3.7. Interestingly, whereas in
TABLE 3.7  Effect of 5 mM-Glucose on Branched-Chain Amino Acid Oxidation, Transamination and Branched-Chain 2-Oxo Acid Release by Rat Hemi-Diaphragm Preparations

Results are mean differences obtained from measurements on tissue from at least four different animals incubated in the absence of glucose and tissue from at least four different animals in the presence of 5 mM-glucose.  - denotes that values are lower when 5 mM-glucose is present while + indicates an increase when 5 mM-glucose is present.

a) tissue incubated in 3 mM-valine

<table>
<thead>
<tr>
<th>Period of Starvation (h)</th>
<th>[1-¹³C]- valine oxidation (µmol/2 h g tissue)</th>
<th>[U-¹³C]-valine oxidation (µmol/2 h g tissue)</th>
<th>Ratio ¹³CO₂ release [U-¹³C]-valine [¹³C]-valine</th>
<th>Transamination 3-methyl-2-oxobutyrate release (µmol/2 h g tiss.)</th>
<th>Mean Differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-0.67 (26.9 %)</td>
<td>-0.18 (12.9 %)</td>
<td>+0.14</td>
<td>-0.67 (19.4 %) +0.16 (16.5 %)</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>-0.42 (15.3 %)</td>
<td>-0.52 (31.7 %)</td>
<td>-0.12</td>
<td>+0.58 (18.3 %) +0.88 (20.4 %)</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>-0.23 (5.9 %)</td>
<td>-0.17 (8.7 %)</td>
<td>-0.02</td>
<td>+0.01 (0.2 %) +0.23 (3.6 %)</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>+0.74 (20 %)</td>
<td>-0.44 (20 %)</td>
<td>-0.21</td>
<td>+1.12 (27.3 %) +0.38 (9.5 %)</td>
<td></td>
</tr>
</tbody>
</table>
(TABLE 3.7 Continued)

b) tissue incubated in 3 mM-leucine

| Period of Starvation (h) | \([1^{14}C]\)-leucine oxidation (\(\mu\)mol/2 h/g tissue) | \([U^{14}C]\)-leucine oxidation (\(\mu\)mol/2 h/g tissue) | Ratio \(\frac{14^{14}CO_2}{\text{release}}\) \([U^{14}C]\)-leucine \(\frac{[1^{14}C]\text{-leucine}}{[1^{14}C]\text{-leucine}}\) (\(\mu\)mol/2 h/g tiss.) | Transamination \(4\)-methyl-
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>(\pm 0.50) (8.7%)</td>
<td>+0.74 (21.7%)</td>
<td>+0.07</td>
<td>+0.55 (8.9%)</td>
</tr>
<tr>
<td>24</td>
<td>+0.47 (16.3%)</td>
<td>+0.61 (34%)</td>
<td>+0.09</td>
<td>+0.67 (19.2%)</td>
</tr>
<tr>
<td>48</td>
<td>+0.52 (11.1%)</td>
<td>+1.00 (40.5%)</td>
<td>+0.19</td>
<td>+0.33 (5.9%)</td>
</tr>
<tr>
<td>72</td>
<td>+1.73 (29%)</td>
<td>+0.57 (19.1%)</td>
<td>-0.03</td>
<td>+1.11 (16.3%)</td>
</tr>
</tbody>
</table>
the presence of glucose as a potential substrate leads to a sizeable decrease in amino acid oxidation (Sans et al., 1980b) the presence of glucose was found to increase the oxidation of leucine by rat hemi-diaphragm preparations without altering the response to fasting i.e. the decrease at 24 h starvation was still observed.

The presence of glucose may stimulate leucine catabolism in hemi-diaphragm preparations by making pyruvate available for transamination to alanine. The subsequent lowering of the level of glutamate will permit more leucine to be transaminated and hence more 4-methyl-2-oxopentanoate will be made available for oxidation.

Although Chang & Goldberg (1978c) have reported that leucine is the most effective of the BCAAs at inhibiting pyruvate oxidation, they also found that 3 mM-isoleucine can inhibit pyruvate oxidation by diaphragms from fasted rats. In the present study the presence of glucose caused a small decrease in the metabolism of valine by hemi-diaphragms from fed rats (Table 3.7). However, as starvation progressed the inhibitory effect of glucose on valine metabolism, as measured by release of $\text{^{14}CO}_2$ from [1-$\text{^{14}C}$]-valine decreased until at 72 h starvation the presence of glucose caused stimulation of valine oxidation. Valine was shown by Chang & Goldberg (1978c) not to be as effective an inhibitor of glucose and pyruvate oxidation as leucine. However, Table 3.8 shows that when hemi-diaphragms are incubated with valine in the presence of glucose there appears to be a sparing of glucose in the fasted state. The
TABLE 3.8 Effect of 3 mM-Valine on Lactate, Pyruvate and Alanine Release by Hemi-Diaphragms Incubated in the Presence of 10 mM-Glucose

Values represent the means of measurements from different rats and are given together with S.E.M. and the number of observations in parentheses. Statistical analysis (Student's t-test) was carried out on paired observations and differences are shown by:

* p < 0.05, ** p < 0.01, ***p < 0.001.

a) FED

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>Alanine</th>
<th>Pyruvate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM-Glucose</td>
<td>1.88 ± 0.12</td>
<td>1.74 ± 0.26</td>
<td>15.84 ± 1.11</td>
</tr>
<tr>
<td></td>
<td>(12)</td>
<td>(12)</td>
<td>(12)</td>
</tr>
<tr>
<td>10 mM-Glucose + 3 mM-Valine</td>
<td>3.00 ± 0.10</td>
<td>1.70 ± 0.21</td>
<td>16.95 ± 0.64</td>
</tr>
<tr>
<td></td>
<td>(12)**</td>
<td>(12)</td>
<td>(12)</td>
</tr>
</tbody>
</table>

b) 48 h-FASTED

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>Alanine</th>
<th>Pyruvate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM-Glucose</td>
<td>1.96 ± 0.15</td>
<td>5.72 ± 0.47</td>
<td>32.84 ± 2.39</td>
</tr>
<tr>
<td></td>
<td>(8)</td>
<td>(8)</td>
<td>(8)</td>
</tr>
<tr>
<td>10 mM-Glucose + 3 mM-Valine</td>
<td>3.34 ± 0.28</td>
<td>3.58 ± 0.25</td>
<td>23.46 ± 1.45</td>
</tr>
<tr>
<td></td>
<td>(8)**</td>
<td>(8)**</td>
<td>(8)**</td>
</tr>
</tbody>
</table>
amounts of pyruvate and lactate released by muscle from 48 h-starved rats were significantly lower when 3 mM-valine was present. While the results of the present study suggest that 3 mM-valine may decrease glucose utilisation by hemi-diaphragms prepared from 48 h-fasted rats it is doubtful whether the effect is on pyruvate oxidation alone (Chang & Goldberg, 1978c). A decrease in pyruvate oxidation would presumably cause an increase in lactate and pyruvate release by the muscle preparation, whereas significant decreases of pyruvate and lactate release were observed.

All the BCAAs may therefore exert an inhibitory effect on glucose oxidation by muscle from starved rats. The present experiments uphold the finding of other workers that skeletal muscle differs from heart muscle with respect to BCAA and glucose metabolism. Differences were also observed in experiments reported in Chapter 4 (4.2.2.1).

The *in vitro* observations that leucine inhibition of glucose oxidation occurs with muscle from starved animals but not from fed animals (Chang & Goldberg, 1978c) may explain the paradoxical effects of intravenously administered leucine before and after 4 weeks of starvation in man, (Sherwin, 1978). Initially leucine infusion decreased plasma glucose concentration possibly as a consequence of leucine stimulated insulin secretion (Milner, 1970; Andersson *et al.*, 1977), whereas after fasting it increased plasma glucose concentration. Studies of the rates of endogenous glucose production and utilisation in these human subjects revealed that the increases are largely due to a reduction in
glucose utilisation when leucine was infused. Apparently, the inhibition of gluconeogenesis by leucine, as shown in vitro (Greenberg & Reaven, 1966), is not involved in the effect of leucine on plasma glucose concentration in vivo in fasted man.

Apart from its action on muscle, leucine has been found to inhibit glucose oxidation by brain slices in vitro (Palaiologos et al., 1979). The site of this inhibition appears to be at the level of pyruvate oxidation (Chang & Goldberg, 1978c). In fact, the branched-chain 2-oxo acid inhibition of pyruvate uptake and oxidation by brain mitochondria has been suggested as a biochemical basis of mental retardation in patients with Maple Syrup Urine Disease (Halestrap et al., 1974).

3.2.4.2 Effect of Insulin on Branched-Chain Amino Acid Metabolism by Rat Hemi-Diaphragm Preparations

Table 3.9 shows the results of experiments comparing the oxidation of 3 mM-valine by hemi-diaphragm preparations from 48 h-fasted rats incubated both in the presence and absence of insulin (50 munits/ml incubation medium). The presence of insulin stimulated the decarboxylation of $[^1_{-14}C]$-valine by over 30\% while decreasing 3-methyl-2-oxobutyrate release by over 40\%.

The percentages of 3-methyl-2-oxobutyrate formed by transamination of 3 mM-$[^1_{-14}C]$-valine which were released from the muscle or decarboxylated were 21.9 ± 2.6 and 78.1 ± 2.6 respectively in the absence of insulin but 11.0 ± 2.8 and 89.0 ± 2.8 respectively in the presence of insulin. The increase in valine carbon oxidation stimulated by insulin was not however confined to
TABLE 3.9  Effect of Insulin (50 munits/ml Incubation Medium) on the Metabolism of 3 mM-Valine by Hemi-Diaphragm Preparations From 48 h-Starved Rats

Results shown are the means of at least four observations ± S.E.M. The control values were measured at the same time as those where insulin is present.

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>+INSULIN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(μmol/2 h/g tissue)</td>
<td></td>
</tr>
<tr>
<td>Release of $^{14}$CO₂ from $[1^{-14}C]$-Valine</td>
<td>3.83 ± 0.21</td>
<td>5.02 ± 0.20</td>
</tr>
<tr>
<td>Release of 3-methyl-2-oxobutyrate</td>
<td>1.07 ± 0.10</td>
<td>0.62 ± 0.15</td>
</tr>
<tr>
<td>Total transamination of $[1^{-14}C]$-valine</td>
<td>4.90 ± 0.10</td>
<td>5.64 ± 0.05</td>
</tr>
<tr>
<td>Release of $^{14}$CO₂ from $[U^{-14}C]$-valine</td>
<td>1.71 ± 0.01</td>
<td>3.04 ± 0.02</td>
</tr>
<tr>
<td>Ratio of $^{14}$CO₂ released from $[U^{-14}C]$-[1^{-14}C]-valine</td>
<td>0.45 ± 0.02</td>
<td>0.61 ± 0.03</td>
</tr>
</tbody>
</table>
decarboxylation at the C1 position. The \(^{14}\text{CO}_2\) released from 3 mM-[U-\(^{14}\text{C}\)]-valine was increased by 78\% in the presence of insulin and the ratio of \(^{14}\text{CO}_2\) released from [U-\(^{14}\text{C}\)]-valine to [1-\(^{14}\text{C}\)]-valine was increased by 36\% indicating that in the presence of insulin a much higher proportion of the valine carbon chain was oxidised. Thus although no sizeable difference in the rate of transamination was observed with insulin, the extent of oxidation of the resulting carbon skeleton was increased. This suggests that insulin has an effect other than simply by increasing the transport of amino acids into the muscle. Using rat hindquarter preparations Hutson et al. (1980) found that when insulin was present decarboxylation rates of leucine were 4- to 5-fold greater in hindquarters from starved versus fed rats. These workers used 0.2 mM-leucine in their experiments. It is possible that insulin stimulation of leucine transport into the muscle was contributing to the stimulation of decarboxylation observed. However, at the high concentration of valine employed in the present study it is unlikely that this factor contributes greatly to insulin stimulation of BGAA oxidation. The above findings are consistent with the suggestion that the carbon skeleton of valine may be made available for the formation of pyruvate and hence alanine in skeletal muscle. With increased oxidation of the carbon chain, less carbon would be available for alanine synthesis and so in the presence of insulin less alanine would be expected to be derived from valine carbon. An increase in the proportion of valine oxidised by the perfused rat hind-limb preparation in the presence of insulin was also
observed by Zapalowski et al. (1981).

A situation in which muscle alanine release has been reported to be increased is diabetes (Ruderman & Berger, 1974; Karl et al., 1976). It was of interest therefore to see whether muscle from diabetic animals made more BCAA carbon available to the muscle than muscle from normal fed animals. Chemical diabetes was induced with streptozotocin. This compound has diabetogenic activity (Junod et al., 1967) which is apparently the result of irreversible damage to the insulin secreting beta cells of the pancreas without affecting glucagon secretion by the alpha cells. The destruction of the beta cells produces permanent diabetes in experimental animals (Brosky & Logothetopoulus, 1969; Chang & Schneider, 1971; Rossini et al., 1977). Rats made diabetic by a single intraperitoneal injection of streptozotocin (100 mg/kg body weight) were used 2 days after injection in the fed state. The use of 48 h-starved streptozotocin-induced diabetic rats was not possible as death resulted when animals were starved for longer than 24 h. The results shown in Table 3.10 therefore compare the oxidation of 3 mM-valine by tissues from fed streptozotocin-induced rats with those from fed controls. No appreciable difference between the tissues was observed. Karl et al. (1976) have measured the activity of branched-chain aminotransferase in muscle from streptozotocin-diabetic rats and found it to be no different from activity in muscle taken from control animals. However, other workers using lower BCAA concentrations have observed that in fed streptozotocin-induced diabetic animals, a much higher percentage of the branched-chain 2-oxo acid
TABLE 3.10  Effect of Streptozotocin-Induced Diabetes on the
Metabolism of 3 mM-Valine by Hemi-Diaphragm Preparations
From Fed Rats

Results shown are the means of at least four observations ± S.E.M.
Control values were measured at the same time as those using
tissue from diabetic rats.

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>STREPTOZOTOCIN DIABETIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(μmol/2 h/g tissue)</td>
<td></td>
</tr>
<tr>
<td>Release of $^{14}$CO₂ from $[1-^{14}C]$-valine</td>
<td>$3.10 \pm 0.25$</td>
<td>$3.09 \pm 0.26$</td>
</tr>
<tr>
<td>Release of 3-methyl-2-oxobutyrate</td>
<td>$0.49 \pm 0.30$</td>
<td>$0.60 \pm 0.04$</td>
</tr>
<tr>
<td>Total transamination of $[1-^{14}C]$-valine</td>
<td>$3.60 \pm 0.56$</td>
<td>$3.69 \pm 0.40$</td>
</tr>
<tr>
<td>Release of $^{14}$CO₂ from $[U-^{14}C]$-valine</td>
<td>$1.46 \pm 0.08$</td>
<td>$1.57 \pm 0.05$</td>
</tr>
<tr>
<td>Ratio of $^{14}$CO₂ released from $[U-^{14}C]$-valine</td>
<td>$0.47 \pm 0.02$</td>
<td>$0.51 \pm 0.05$</td>
</tr>
</tbody>
</table>
| Blood glucose (mM)       | $6.72 \pm 0.06$   | $20.6 \pm 1.5$
dehydrogenase of muscle exists in the active form, leading to a
greater release of $^{14}\text{C}_2\text{O}_2$ from $[1^{-14}\text{C}]$-leucine than in controls
(Paul & Adibi, 1982). It is difficult to compare my results
with this other study however, since totally different muscle
preparations were employed, Paul & Adibi, (1982) using isolated
mitochondria.

3.2.5 Glutamine Release by Skeletal Muscle

The other amino acid released by skeletal muscle in
quantities which far exceed its relative abundance in muscle
proteins, is glutamine (Ruderman & Lund, 1972). Branched-chain
amino acids have been observed to stimulate the release of
 glutamine by muscle (Garber et al., 1976b) and it has been
suggested that BCAAs may also serve as sources of carbon for the
 glutamine released (Chang & Goldberg, 1978a). Provided that
 alanine was derived from BCAA metabolism it would therefore be
expected that alanine and glutamine release by muscle would
follow the same pattern. Alanine production from rat skeletal
muscle has been shown to be increased by starvation (Fig. 3.1
and Blackshear et al., 1974; MacDonald et al., 1976; Goodman
et al., 1978) and diabetes (Karl et al., 1976). These conditions,
however, appear to elicit no change or a reduction in glutamine
release by skeletal muscle (MacDonald et al., 1976; Karl
et al., 1976). There therefore seems to be a closer relation-
ship between muscle BCAA metabolism and alanine release than
with glutamine release. Fig. 3.14 shows the release of alanine
and glutamine by muscles from 48 h-starved rats incubated in
FIG. 3.14  Alanine and Glutamine Release by Muscles From 48 h-Starved Rats

Each column represents the mean ± S.E.M. of at least 4 observations. Statistically significant differences (by paired t-test) between metabolite release in the absence and presence of 3 mM-valine are shown by: * p < 0.05; ** p < 0.01; *** p < 0.001.

- Krebs-Ringer medium
- Krebs-Ringer + 3 mM-valine
the absence and presence of 3 mM-valine. Valine was found to stimulate both alanine and glutamine release by hemi-diaphragm preparations. However, although the increase in alanine release from soleus and EDL muscles stimulated by valine was substantial, the valine-stimulated increase in glutamine release was negligible from both muscles.

The above findings call into question whether glutamine and alanine release from muscle bear a direct relationship to one another and agree with the suggestion that alanine transports most of the extra nitrogen generated when the catabolism of amino acids in muscle is increased during starvation (see Chapter 1, 1.2.1).

3.2.6 Summary

Branched-chain amino acids stimulate the release of alanine from muscle and the rate of alanine release is not necessarily related to the rate of pyruvate formation via glycolysis.

Although it has been suggested that rat muscle releases most of the carbon skeleton of BCAA as the corresponding 2-oxo acid after transamination, due to a restriction on further metabolism by low branched-chain 2-oxo acid dehydrogenase activity, the present study has established in incubations with L-[1-\textsuperscript{14}C]-valine and L-[1-\textsuperscript{14}C]-leucine (in the presence or absence of 5 mM-glucose), that of the valine and leucine which is transaminated at least 60% and 80% respectively is further decarboxylated. Experiments employing both L-[1-\textsuperscript{14}C]- and
L-[U-\(^{14}\text{C}\)]-BCAA have shown that muscle metabolises BCAA carbon chains beyond the decarboxylation step of the 2-oxo acid dehydrogenase, but not to complete oxidation. The amount of the BCAA-derived carbon not oxidised is sufficient to account for the observed synthesis de novo of alanine by muscle.

Starvation, a situation where increased alanine release is observed is also characterised by increased muscle availability of BCAA carbon. The extent to which this occurs in different nutritional states in the intact animal, however, remains in question, although BCAAs may prove to be a useful source of alanine carbon during starvation when muscle glycogen stores have been depleted.
CHAPTER FOUR

EFFECT OF METABOLIC INHIBITORS ON MUSCLE METABOLISM IN VITRO
4.1 INTRODUCTION

The previous chapter suggested that the carbon skeleton of alanine, formed and released by muscle, may be derived in some situations from branched-chain amino acids (BCAAs). The experiments described in this chapter were conducted with a view to providing more details of the interrelationship between the metabolism of BCAAs and alanine formation in skeletal muscle. Use was made of several compounds known to act as specific inhibitors of various metabolic reactions involved in the proposed pathway of conversion of BCAA carbon to alanine.

4.2 RESULTS AND DISCUSSION

4.2.1 De Novo Alanine Synthesis Involving Transamination of Pyruvate

Alanine released by skeletal muscle can be derived from two possible sources, degradation of muscle proteins or de novo synthesis by transamination of pyruvate. Evidence that the BCAA-stimulation of alanine release was largely the result of increased de novo synthesis of alanine, rather than the result of enhanced proteolytic release of alanine residues from muscle proteins, was obtained by using the aminotransferase inhibitors, aminooxyacetate and L-cycloserine. L-cycloserine is an inhibitor of cytoplasmic transamination and particularly of alanine aminotransferase (Wong et al., 1973; Williamson et al., 1974). Amino-
oxyacetate is a less specific aminotransferase inhibitor (Rognstad & Katz, 1970) that affects both mitochondrial and cytoplasmic transaminations (Longshaw et al., 1972; Williamson et al., 1974). The increase in alanine release from rat hemidiaphragm preparations observed in the presence of 3 mM-valine was inhibited 60% by 5 mM-cycloserine (Table 4.1). The results obtained using 1 mM-aminooxyacetate were comparable with those found with 5 mM-cycloserine (Table 4.1 + Fig. 4.1). Other workers have made similar observations with other muscle preparations in vitro (Ruderman & Berger, 1974; Garber et al., 1976a; Taegtmayer et al., 1977) and with the hepatectomised rat in vivo (Blackshear et al., 1975).

Further evidence that valine stimulation of alanine release is the result of de novo alanine formation by transamination of pyruvate in muscle comes from experiments involving dichloroacetate. The presence of dichloroacetate causes the activation of the enzyme pyruvate dehydrogenase in tissue preparations. This effect was first demonstrated by Whitehouse & Randle (1973) on pyruvate dehydrogenase of rat heart. Using purified heart enzyme preparations these workers found that dichloroacetate inhibits the ATP-dependent phosphorylation and inactivation of pyruvate dehydrogenase by pyruvate dehydrogenase kinase (Whitehouse et al., 1974). Dichloroacetate therefore activates pyruvate dehydrogenase by exerting its effect on the kinase rather than by binding directly to the pyruvate decarboxylase component of the complex.

The effect of the presence of 10 mM-dichloroacetate on
TABLE 4.1  Effect of L-Cycloserine (5 mM) and Aminoxyacetate (1 mM) on the Rate of Alanine Release by Hemi-Diaphragms from a) Fed, and b) 48 h-Starved Rats

Values represent the means of measurements from different rats and are given together with S.E.M. and the number of observations in parentheses.

a) Fed

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>Control</th>
<th>+ 5 mM- Cycloserine</th>
<th>+ 1 mM- Aminoxyacetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.48 ± 0.14 (13)</td>
<td>2.10 ± 0.10 (4)</td>
<td>1.93 ± 0.09 (12)</td>
</tr>
<tr>
<td>3 mM-Valine</td>
<td>2.47 ± 0.15 (13)</td>
<td>2.51 ± 0.17 (4)</td>
<td>2.18 ± 0.15 (4)</td>
</tr>
<tr>
<td>Mean Difference</td>
<td>+0.99</td>
<td>+0.41</td>
<td>+0.25</td>
</tr>
</tbody>
</table>

b) 48 h-Starved

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>Control</th>
<th>+ 1 mM- Aminoxyacetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.06 ± 0.12 (12)</td>
<td>1.48 ± 0.07 (4)</td>
</tr>
<tr>
<td>3 mM-Valine</td>
<td>1.93 ± 0.15 (7)</td>
<td>1.43 ± 0.12 (4)</td>
</tr>
<tr>
<td>Mean Difference</td>
<td>+0.87</td>
<td>-0.05</td>
</tr>
</tbody>
</table>
FIG. 4.1  Effect of Aminoxyacetate on 3 mM-Valine-Stimulated Alanine Formation by Hemi-Diaphragm

Each column represents the means of measurements of alanine release from tissues prepared from at least 4 animals and are expressed as a percentage of the alanine released by control tissues incubated in the absence of added valine.
valine-stimulated alanine production is shown in Fig. 4.2. In both the fed and fasted state dichloroacetate inhibits valine-stimulated alanine release by the hemi-diaphragm preparations and greatly decreases the release of lactate and pyruvate into the incubation medium. Similar results have been reported by Goodman et al., (1978). These workers found that when 1 mM-dichloroacetate was present in media perfusing hindquarters of fed, fasted or streptozotocin-induced diabetic rats, releases of alanine, pyruvate and lactate from the hindquarters were significantly reduced. The low amounts of lactate and pyruvate released are due to the stimulation of pyruvate oxidation by the dichloroacetate activation of pyruvate dehydrogenase. These results show that valine-stimulated alanine release is affected by the availability of pyruvate and provide further evidence that the valine-stimulated increase in alanine release is not due merely to increased alanine release from muscle proteins but to de novo alanine formation via transamination of pyruvate.

4.2.2 Effect of Inhibition of Branched-Chain Amino Acid Metabolism on Alanine Release

4.2.2.1 Dichloroacetate

The pyruvate for alanine formation has been suggested to be partly derived from the carbon of valine in certain circumstances (e.g. in moderate starvation). Alterations in the metabolism of BCAAs would therefore be expected to produce corresponding changes in muscle alanine production.
FIG. 4.2  Effect of Dichloroacetate on Alanine, Pyruvate and Lactate Release From Hemi-Diaphragms From a) Fed Rats, and b) 48 h-Starved Rats

Values represent the means ± S.E.M. of at least 4 observations.

- □ no addition
- □ 3 mM-valine
- □ 10 mM-dichloroacetate
- □ 10 mM-dichloroacetate + 3 mM-valine

a)

b)
Dichloroacetate increases leucine oxidation by both liver (Crabb & Harris, 1978; Harris et al., 1978) and heart preparations (Sans et al., 1980a; 1980b). The dichloroacetate stimulation of leucine oxidation by liver is a consequence of the ability of this tissue to dechlorinate dichloroacetate to form glyoxylate (Harris et al., 1978; Demangre et al., 1978). Glyoxylate then increases leucine catabolism by accepting amino groups by leucine transamination (Harris et al., 1978). The stimulation of BCAA metabolism by dichloroacetate in liver is therefore due to a stimulation of the initial transamination. No evidence has been found for branched-chain 2-oxo acid dehydrogenase activation in liver by dichloroacetate (Crabb et al., 1981).

In heart the mechanism of stimulation of BCAA metabolism by dichloroacetate differs. Dehalogenation of dichloroacetate has not been demonstrated in mammalian tissues other than liver (Crabb et al., 1981) and the mechanism of stimulation in the heart is considered to involve activation of the branched-chain 2-oxo acid dehydrogenase complex (Sans et al., 1980a; 1980b). As there is considerable evidence that the above complex of heart (and skeletal muscle) is regulated by covalent modification by phosphorylation–dephosphorylation (Parker & Randle, 1978; 1980; Odessey, 1980; Harris & Keezer, 1981), it is tempting to suggest that dichloroacetate causes activation of the heart branched-chain 2-oxo acid dehydrogenase complex in a similar manner to which it activates pyruvate dehydrogenase i.e. by inhibiting the dehydrogenase kinase.
However, dichloroacetate has not been found to stimulate BCAA metabolism in skeletal muscle. In response to dichloroacetate, muscle from fasted rats releases less alanine and more BCAAs (Goodman et al., 1978). An increase in BCAA release also occurs during dichloroacetate infusion into intact rats (Blakeshear et al., 1975). An inhibitory effect of dichloroacetate on the oxidation of leucine by hemi-diaphragms prepared from 48 h-starved rats is shown in Table 4.2. Dichloroacetate therefore inhibits BCAA metabolism in skeletal muscle, in contrast to its effect in the heart.

Possible reasons for the inhibitory effect of dichloroacetate on skeletal muscle BCAA metabolism include: impaired transamination due to a shortage of pyruvate to regenerate 2-oxo-glutarate via alanine formation; decreased oxidation of the 2-oxo acids due to either an inhibitory effect of dichloroacetate on the muscle dehydrogenase or to decreased amounts of available CoASH. CoASH is necessary for further metabolism of the BCAAs, but dichloroacetate stimulation of pyruvate oxidation to acetyl-CoA will lower the available CoASH levels.

The total amount of BCOA formed during a tissue incubation employing [1-\(^{14}\)C]-BCAA can be calculated as the sum of the [1-\(^{14}\)C]-BCOA released into the incubation medium and the \(^{14}\)CO\(_2\) given off during the experiment due to decarboxylation of the BCOA.

The percentage of 4-methyl-2-oxopentanoate formed from leucine which was oxidised (decarboxylated) (Table 4.2) was similar whether dichloroacetate was present or absent (82.0 % and 80.7 % respectively in the absence of glucose; and 86.4 % and 88.3 % respectively in the presence of glucose). This suggests
TABLE 4.2  Effect of 10 mM-Dichloroacetate on the Metabolism of 3 mM-[\textsuperscript{1-14}C]-Leucine by Hemi-Diaphragms Prepared From 48 h-Starved Rats

One of each pair of hemi-diaphragms was incubated in the presence of dichloroacetate and the other in its absence. Values shown are the means ± S.E.M. of 3 observations.

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>3 mM-L-[\textsuperscript{1-14}C]-Leucine Oxidation (μmol/2 h/g of muscle)</th>
<th>4-methyl-2-oxopenatanoate Release</th>
<th>Transamination of 3 mM-L-[\textsuperscript{1-14}C]-Leucine (μmol/2 h/g of muscle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>5.44 ± 0.32</td>
<td>1.30 ± 0.17</td>
<td>6.74 ± 0.26</td>
</tr>
<tr>
<td>5 mM-Glucose</td>
<td>6.33 ± 0.36</td>
<td>0.84 ± 0.16</td>
<td>7.17 ± 0.45</td>
</tr>
<tr>
<td>10 mM-Dichloroacetate</td>
<td>2.77 ± 0.03</td>
<td>0.61 ± 0.08</td>
<td>3.38 ± 0.09</td>
</tr>
<tr>
<td>5 mM-Glucose + 10 mM-Dichloroacetate</td>
<td>2.28 ± 0.17</td>
<td>0.36 ± 0.10</td>
<td>2.64 ± 0.27</td>
</tr>
</tbody>
</table>
that dichloroacetate did not have its effect by inhibiting branched-chain 2-oxo acid dehydrogenase. Results in agreement with this suggestion are shown in Table 4.3. In experiments comparing the effect of 10 mM-dichloroacetate on the metabolism of 3 mM-\([1-^{14}C]\)-leucine and 3 mM-\([U-^{14}C]\)-leucine, it was again found that dichloroacetate lowered the transamination of leucine but had no appreciable effect on the percentage of 4-methyl-2-oxopenatanoate formed which was oxidised. On comparing \([1-^{14}C]\)- with \([U-^{14}C]\)-leucine metabolism it was noted that the presence of dichloroacetate does not alter the extent to which the carbon skeleton of leucine is oxidised to CO\(_2\) by hemi-diaphragm preparations. Dichloroacetate therefore appears to inhibit BCAA metabolism in muscle by attenuating transamination, possibly due to a decreased availability of pyruvate as noted above. It greatly reduced the total transamination of leucine both in the presence or absence of glucose, which shows that the decreased transamination observed was not merely due to the muscle preparation having an inadequate glycolytic source of pyruvate. Dichloroacetate may therefore be considered to inhibit the proposed pathway of alanine formation from BCAA carbon by two means as a consequence of stimulating pyruvate oxidation. The decreased pyruvate levels cause a decrease in BCAA transamination and, in addition, the oxidation of pyruvate formed from the BCAA carbon will be increased. Both these effects may contribute towards the decrease in valine-stimulated alanine release observed in the presence of dichloroacetate (Fig. 4.2).
TABLE 4.3  Effect of 10 mM-Dichloroacetate on the Metabolism of 3 mM-[1-¹⁴C]-Leucine and 3 mM-[U-¹⁴C]-Leucine by Hemi-Diaphragms Prepared From 48 h-Starved Rats

Paired hemi-diaphragm preparations were used for all incubations, one incubated with [¹⁴C]-leucine, the other with [U-¹⁴C]-leucine. Values shown are the means ± S.E.M. of 3 observations.

**Incubation Additions**

<table>
<thead>
<tr>
<th></th>
<th>5 mM-Glucose</th>
<th>5 mM-Glucose +10 mM-dichloroacetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mM-[1-¹⁴C]-leucine oxidation (µmol/2 h/g tissue)</td>
<td>6.50 ± 0.72</td>
<td>2.45 ± 0.08</td>
</tr>
<tr>
<td>4-methyl-2-oxopentanoate release (µmol/2 h/g tissue)</td>
<td>1.05 ± 0.03</td>
<td>0.53 ± 0.10</td>
</tr>
<tr>
<td>Transamination of 3 mM-[1-¹⁴C]-leucine (µmol/2 h/g tissue)</td>
<td>7.55 ± 0.73</td>
<td>2.98 ± 0.02</td>
</tr>
<tr>
<td>3 mM-[U-¹⁴C]-leucine oxidation (µmol/2 h/g tissue)</td>
<td>3.20 ± 0.46</td>
<td>1.24 ± 0.09</td>
</tr>
<tr>
<td>Ratio [U-¹⁴C]-leucine oxidation [1-¹⁴C]</td>
<td>0.49 ± 0.08</td>
<td>0.51 ± 0.05</td>
</tr>
</tbody>
</table>
4.2.2.2 Hypoglycin

Hypoglycin (L-2-amino-3-methylenecyclopropylpropionic acid) is present in the unripe arillus of the Jamaican ackee fruit and its ingestion causes a disease characterised biochemically by severe hypoglycaemia and disturbances of carbohydrate and lipid metabolism (Sherratt & Osmundsen, 1976; Billington et al., 1978). Hypoglycin is transaminated to methylenecyclopropylpyruvate, which is oxidatively decarboxylated to methylenecyclopropylacetyl-CoA (Kean & Rainford, 1973; Sherratt & Osmundsen, 1976). Methylenecyclopropylacetyl-CoA has been proposed as the active hypoglycaemic metabolite owing to its inhibition of hepatic gluconeogenesis, largely as a result of changes in acyl-CoA/CoA distributions which inhibit pyruvate carboxylase activation by acetyl-CoA (Billington et al., 1978; Kean & Pogson, 1979).

Metabolism of hypoglycin, a cyclic analogue of leucine, is initiated by branched-chain aminotransferase and continued by branched-chain 2-oxo acid dehydrogenase (Tanaka, 1975). Due to the presence of appreciable amounts of the enzymes involved in BCAA metabolism in muscle, hypoglycin and its metabolites could owe part of their hypoglycaemic action to effects on the metabolism in muscle. Indeed, Osmundsen et al. (1978) have shown that hypoglycin almost completely abolishes glucose recycling via muscle lactate and alanine. Therefore the effect of hypoglycin on the BCAA-stimulated alanine release from muscle was studied. These experiments used incubations of intact soleus and EDL muscles. Both valine and leucine stimulated alanine production
from those tissues. The presence of hypoglycin (1 mM) abolished
the amino acid-stimulated release of alanine from muscles of
starved rats (Fig. 4.3).

Both valine and leucine-stimulated alanine release was
inhibited i.e. alanine production was blocked from both the
glucogenic and the ketogenic amino acid. This would imply that
hypoglycin inhibits the provision of amino nitrogen for alanine
formation rather than the pathway for conversion of valine carbon
into pyruvate for alanine formation. The structural similarity
of hypoglycin to BCAAs and the metabolism of hypoglycin by the
enzymes involved in BCAA oxidation (Fig. 4.4) suggested that
decreased alanine release was the result of inhibition of branched-
chain aminotransferase and a decrease in the formation of glutamate
for transamination to alanine. The activity of branched-chain
aminotransferase in homogenates of soleus and EDL muscles was
therefore studied. 1 mM-hypoglycin only caused a 13.5% and 5%
inhibition of enzyme activity in soleus and EDL homogenates
respectively.

An alternative proposal for the inhibition of alanine
release by hypoglycin could be sequestration of CoA through
metabolism of hypoglycin to methylenecyclopropylacetyl-CoA
(Bressler et al., 1969). In muscle, CoA sequestration would
inhibit branched-chain 2-oxo acid dehydrogenase causing an
accumulation of the branched-chain oxo acids and secondarily of
BCAAs. As it is suggested that inhibition of protein degradation
is mediated by an intermediate of leucine metabolism (Goldberg
& Tischler, 1981), and that in humans 4-methyl-2-oxopentanoate
FIG. 4.3 Effect of Hypoglycin on Alanine Release a) by Soleus Muscles In Vitro, and b) by EDL Muscles In Vitro, Prepared From 48 h-Starved Rats

Soleus and EDL preparations were incubated as described in Chapter 2. Results shown are increases in alanine release in the presence of branched-chain amino acids (3 mM-valine or 3 mM-leucine). The effect of the presence of 1 mM-hypoglycin on the valine- or leucine-stimulated alanine release is also shown. Results shown are means of at least 4 observations.

☐ 48 h-starved    ☒ 48 h-starved + hypoglycin

<table>
<thead>
<tr>
<th>Amino Acid Added:</th>
<th>VALINE</th>
<th>LEUCINE</th>
<th>VALINE</th>
<th>LEUCINE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in alanine release in presence of BCAA (μmol/2 h/g tissue)</td>
<td>+3.0</td>
<td>+2.0</td>
<td>+1.0</td>
<td>+1.0</td>
</tr>
<tr>
<td>a) Soleus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b) EDL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIG. 4.4  Catabolism of Leucine and Hypoglycin

Branched-chain aminotransferase

Branched-chain 2-oxo acid dehydrogenase

* possible sites of inhibition
clinically improves protein nitrogen balance (Mitch et al., 1981), it is tempting to consider that hypoglycin causes the decrease in alanine release observed in this experiment by two different means in addition to lowering BCAA transamination. With valine as substrate the proposed pathway of valine metabolism is blocked and hence pyruvate will not be formed for alanine synthesis. With leucine as substrate, the lowered levels of CoA due to hypoglycin metabolism may decrease leucine metabolism and cause a build-up of 4-methyl-2-oxopentanoate which is possibly the leucine metabolite responsible for decreasing protein breakdown. Thus less alanine will be derived via proteolysis when leucine is incubated in the presence of hypoglycin. Whatever the mechanism involved, an interrelationship between muscle BCAA metabolism and alanine release can be inferred from these experiments with hypoglycin.

4.2.2.3 Clodibric Acid

A further study of the relationship between BCAA metabolism, and alanine synthesis and release in skeletal muscle was conducted using the hypolipidaemic drug, clodibric acid (p-chlorophenoxyethylpropionic acid). Clodibric acid has been shown to interfere with BCAA metabolism in cultured skeletal muscle cells (Pardridge et al., 1980; 1981) and is associated with myopathy in man and experimental animals (Langer & Levy, 1968; Havel & Kane, 1973; Abowitz et al., 1979). The effect of clodibric acid on valine- and leucine-stimulated alanine release by diaphragm, soleus and EDL muscles is shown in Table 4.4. Tyrosine release
TABLE 4.4  Effect of Clofibric Acid on Muscle Alanine and Tyrosine Release Using Muscle Preparations from a) Fed Rats, and b) 48 h-Starved Rats

Hemi-diaphragm, soleus and EDL muscle preparations were incubated with the addition of 3 mM-valine or leucine, with and without 2 mM-clofibric acid. Values are given as means ± S.E.M. for 4 observations and statistical differences (by a paired t-test) between incubations with and without clofibric acid are denoted as: * p < 0.05, ** p < 0.01.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Additions to Incubation Medium</th>
<th>Alanine</th>
<th>Tyrosine</th>
<th></th>
<th>Alanine</th>
<th>Tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rate of Release (μmol/2 h/g tissue)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemi-Diaphragm</td>
<td>Valine</td>
<td>2.01 ± 0.29</td>
<td>0.42 ± 0.05</td>
<td>4.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Valine + Clofibric acid</td>
<td>2.00 ± 0.19</td>
<td>0.46 ± 0.08</td>
<td>4.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>2.67 ± 0.24</td>
<td>0.38 ± 0.03</td>
<td>7.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leucine + clofibric acid</td>
<td>1.51 ± 0.14*</td>
<td>0.50 ± 0.05*</td>
<td>3.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soleus</td>
<td>Valine</td>
<td>2.37 ± 0.10</td>
<td>0.38 ± 0.06</td>
<td>6.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Valine + clofibric acid</td>
<td>1.76 ± 0.24</td>
<td>0.48 ± 0.08</td>
<td>3.67</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>1.93 ± 0.19</td>
<td>0.25 ± 0.06</td>
<td>7.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leucine + clofibric acid</td>
<td>1.15 ± 0.14**</td>
<td>0.47 ± 0.11</td>
<td>2.45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
(TABLE 4.4 Continued)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Additions to Incubation Medium</th>
<th>Alanine</th>
<th>Tyrosine</th>
<th>Alanine Tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDL</td>
<td>Valine</td>
<td>1.92 ± 0.26</td>
<td>0.60 ± 0.12</td>
<td>3.20</td>
</tr>
<tr>
<td></td>
<td>Valine + clofibric acid</td>
<td>1.33 ± 0.25</td>
<td>0.62 ± 0.14</td>
<td>2.15</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>1.98 ± 0.16</td>
<td>0.25 ± 0.06</td>
<td>7.92</td>
</tr>
<tr>
<td></td>
<td>Leucine + clofibric acid</td>
<td>0.94 ± 0.14*</td>
<td>0.47 ± 0.14</td>
<td>2.00</td>
</tr>
</tbody>
</table>

b) Tissue From 48 h-Starved Rats

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Additions to Incubation Medium</th>
<th>Alanine</th>
<th>Tyrosine</th>
<th>Alanine Tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemi-diaphragm</td>
<td>Valine</td>
<td>1.17 ± 0.07</td>
<td>0.81 ± 0.05</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td>Valine + clofibric acid</td>
<td>1.12 ± 0.10</td>
<td>0.94 ± 0.07</td>
<td>1.19</td>
</tr>
<tr>
<td></td>
<td>Leucine</td>
<td>0.92 ± 0.03</td>
<td>0.55 ± 0.02</td>
<td>1.67</td>
</tr>
<tr>
<td></td>
<td>Leucine + clofibric acid</td>
<td>0.78 ± 0.12</td>
<td>0.75 ± 0.07*</td>
<td>1.04</td>
</tr>
</tbody>
</table>
into the incubation medium was also measured to give an indication of net proteolysis by the muscles.

With all three muscle types studied from fed rats, incubation with leucine produced a lower rate of tyrosine release than incubation with valine, thus confirming that leucine (or a metabolite) can inhibit muscle protein degradation.

Clofibrac acid greatly inhibited leucine-stimulated alanine release from diaphragm, soleus and EDL muscles in vitro. In contrast, valine-stimulated alanine release was not so dramatically affected by the drug. Tyrosine release, the measure of net protein breakdown, was stimulated by clofibrac acid (by 32, 88 and 88% from diaphragm, soleus and EDL respectively) in the presence of leucine. Again the effect of clofibrac acid on muscles incubated in valine was not so great. Further studies with diaphragm muscle using L-[1-14C]-amino acids, showed that clofibrac acid inhibited total leucine transamination (sum of 14CO2 and [1-14C]-2-oxo acid release) by 63%, but inhibited valine transamination by only 24% (Fig. 4.5). The observed inhibition of transamination is secondary to an inhibition of branched-chain 2-oxo acid dehydrogenase and accumulation of branched-chain 2-oxo acids as shown by the sizeable inhibition of 14CO2 production from [1-14C]-leucine and increase in 14C-2-oxo acid release. This inhibition of transamination accounts for the decrease in alanine release found in the presence of clofibrac acid since alanine formation is linked to the BCAAs by coupled transaminations. Clofibrac acid inhibition of BCAA oxidation and alanine release from cultured rat skeletal muscle cells has
FIG. 4.5  Effect of 2 mM-Clofibric Acid on the Rate of Oxidation and Transamination of 3 mM-[1-\textsuperscript{14}C]-Leucine and 3 mM-[1-\textsuperscript{14}C]-Valine by Hemi-Diaphragms Prepared From a) Fed Rats, and b) 48 h-Starved Rats

Results obtained from incubations with 2 mM-clofibric acid added are expressed as a percentage of paired values found in the absence of 2 mM-clofibric acid. Results are the means of four paired observations.

\[ \begin{array}{c}
\square 3 \text{ mM-Valine} \quad \square 3 \text{ mM-Leucine} \\
\end{array} \]

\begin{align*}
\text{TOTAL TRANSAMINATION} & \quad \text{RATE OF} \quad 14\text{CO}_2 \quad \text{RATE OF [1-14C]-2-OXO ACID PRODUCTION} \\
\end{align*}

<table>
<thead>
<tr>
<th>Percent of Control Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
</tr>
<tr>
<td>100</td>
</tr>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

a) Fed

b) 48 h-Starved
been reported by Pardridge et al., (1980; 1981). A similar
demonstration of the effect of clofibric acid on the metabolism
of the BCAAs has come from the work of Danner et al. (1982) who
demonstrated clofibric acid inhibition of the purified branched-
chain 2-oxo acid dehydrogenase from bovine liver.

An interesting observation from the experiments of
Pardridge et al. (1981) in which muscle cells were found to
export alanine and citrate during periods of active BCAA utilisation
and clofibric acid inhibited BCAA-stimulated alanine release, was
that clofibric acid also blocked citrate release and markedly
reduced the intracellular levels of citrate, malate, 2-oxo-
glutarate and pyruvate. These studies imply that BCAAs contribute
significantly to anaplerotic pathways in cultured skeletal muscle
cells and that these pathways lead to the net production of
alanine and citrate during periods of lowered carbohydrate
utilisation.

In addition to demonstrating further the dependence of
muscle alanine release on BCAA metabolism some other important
points arise from the experiments using clofibric acid in the
present study. Inhibition of protein degradation may be mediated
by an intermediate of leucine metabolism whereas stimulation of
protein synthesis apparently depends on leucine itself (Goldberg
& Tischler, 1981). In the present study inhibition of leucine
metabolism by clofibric acid led to an increase in net protein
breakdown (as measured by tyrosine release). Presumably this
effect is the result of preventing the formation of the leucine
metabolite which inhibits proteolysis. The inhibition of the
dehydrogenase by clofibric acid leads to a build-up of 4-methyl-2-oxopentanoate. This compound cannot then be the proteolysis-inhibiting leucine metabolite as has been suggested (Mitch et al., 1981). This interpretation would also invalidate the suggestion in the preceding section (4.2.2.2) that hypoglycin inhibition of leucine metabolism resulted in increased amounts of 4-methyl-2-oxopentanoate which decreased alanine release further by inhibiting proteolysis. However, it is possible that the inhibitory effect of hypoglycin on BCAA metabolism in muscle is at a stage beyond the oxidative decarboxylation of the branched-chain 2-oxo acids.

It has been reported that, when hypoglycin is injected into humans blood concentrations of branched-pentanoic acids (mostly isovaleric acid) increase to over 50 times the levels found in controls (Tanaka et al., 1971). These results correlate well with reports of several in vitro studies which suggest that a hypoglycin metabolite inhibits isovaleryl-CoA dehydrogenase activity (Tanaka, 1975). If this explanation of inhibition of BCAA metabolism by hypoglycin is accepted, together with the previous explanation for the increase in tyrosine release observed in the presence of leucine and hypoglycin, it is possible that the proteolysis-inhibiting metabolite of leucine is not 4-methyl-2-oxopentanoate but isovaleryl-CoA or isovalerate.

The primary effect of clofibric acid was to inhibit the oxidative decarboxylation of the 2-oxo acids derived from BCAAs. The inhibition of leucine metabolism led to an acceleration of protein catabolism and this effect of clofibric acid may, in part,
account for the myopathic side-effects of the drug. It is also of interest that valine metabolism was not inhibited to the same extent as the metabolism of leucine. This finding and the different responses of the metabolism of these two amino acids to starvation (Chapter 3) suggest either that regulatory changes occur during starvation and in response to clofibric acid which somehow affect the metabolism of leucine to a greater extent than that of valine, or that separate enzymes may exist for the transamination and/or oxidative decarboxylation of valine and leucine in muscle.

4.2.3 Muscle Alanine Formation From Tricarboxylic Acid Cycle Carbon

As mentioned previously (Chapter 1, 1.3.1), the accepted pathway for the catabolism of valine is via methylmalonyl-CoA to succinyl-CoA which feeds into the tricarboxylic acid cycle. For the carbon of valine to contribute to muscle pyruvate formation (and hence alanine formation) carbon must be withdrawn from the tricarboxylic acid cycle. In Chapter 1 (1.3.3), the possible enzymes involved in carbon withdrawal from the cycle were described, withdrawal being either via malate or oxaloacetate.

In order to examine whether tricarboxylic acid cycle carbon did contribute to the carbon of alanine released from muscle, experiments were conducted using sodium malonate, a highly specific inhibitor of succinate dehydrogenase at low concentrations (<0.01 M) (Mahler & Cordes, 1971). Addition of malonate to a respiring system leads to an abolition
of the catalytic effect of succinate dehydrogenase in promoting the conversion of succinate to fumarate and an accumulation of succinate results. Inhibition of succinate dehydrogenase was chosen as this enzyme is located in the cycle between the points where valine carbon feeds in and where carbon could be removed for pyruvate formation.

The effect of 8 mM-malonate on the release of alanine from muscle from fed and 48 h-starved rats is shown in Table 4.5. With muscle from fed rats no inhibition in alanine release was observed, whereas with muscle from 48 h-starved rats alanine release was significantly (p < 0.01) reduced. This decrease in alanine release was not due to decreased proteolysis. Tyrosine release was measured and on this basis no difference in net protein degradation was found in the presence or absence of 8 mM-malonate. It can therefore be concluded that the decreased alanine production was due to the inhibition of succinate dehydrogenase and that carbon derived from the tricarboxylic acid cycle is a source of carbon for alanine formation by muscle from 48 h-starved animals.

These results suggest that in the fed animal, pyruvate for muscle alanine formation is probably obtained from glycolysis or glycogenolysis in accordance with the proposal of the glucose-alanine cycle. However, they also show that muscle alanine in starved animals can be formed from pyruvate which is supplied via the tricarboxylic acid cycle from the amino acid valine. Alanine from such a source, released from muscle and arriving at the liver as a gluconeogenic precursor, would thus be contributing net
### TABLE 4.5 Effect of Sodium Malonate on the Release of Alanine From Hemi-Diaphragms Prepared From a) Fed Rats, and b) 48 h-Starved Rats

Hemi-diaphragms were incubated in the presence of 3 mM-valine with or without 8 mM-sodium malonate. Values represent the means of measurements from different rats and are given together with S.E.M. and the number of observations in parentheses. Statistical differences (by paired t-test) between incubations with and without sodium malonate are denoted as * $p < 0.05$, ** $p < 0.01$.

#### a) Fed

<table>
<thead>
<tr>
<th>Additions to medium</th>
<th>Alanine Release ($\mu$mol/2 h/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mM-valine</td>
<td>2.13 ± 0.14 (4)</td>
</tr>
<tr>
<td>3 mM-valine + 8 mM-malonate</td>
<td>2.26 ± 0.20 (4)</td>
</tr>
</tbody>
</table>

#### b)

<table>
<thead>
<tr>
<th>Additions to Medium</th>
<th>Alanine Release ($\mu$mol/2 h/g tissue)</th>
<th>Tyrosine Release ($\mu$mol/2 h/g tissue)</th>
<th>Ratio: Alanine Tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mM-valine</td>
<td>2.08 ± 0.13 (6)</td>
<td>0.91 ± 0.05 (4)</td>
<td>2.17 ± 0.10</td>
</tr>
<tr>
<td>3 mM-valine + 8 mM-malonate</td>
<td>1.68 ± 0.17 (6)**</td>
<td>0.87 ± 0.07 (4)</td>
<td>1.65 ± 0.21 (4)**</td>
</tr>
</tbody>
</table>
carbon to the total body glucose pool.

4.2.3 Enzymic Route of Pyruvate Formation From Tricarboxylic Acid Cycle Intermediates

The experiments described in this section were designed to establish more clearly the route by which four carbon units of the tricarboxylic acid cycle in skeletal muscle are withdrawn to form pyruvate for de novo alanine formation.

As discussed in the following chapter (Chapter 5) the most likely enzymic routes involved are via NADP-malic enzyme or via PEPCK and pyruvate kinase. Alanine release was therefore measured in the presence of specific inhibitors of both routes, in order to clarify which route is involved in the formation of alanine de novo by muscle.

The inhibitors used were hydroxymalonate (tartronate) for malic enzyme and 3-mercaptopicolinate for PEPCK. Hydroxymalonate has been shown to be a competitive inhibitor of cytosolic NADP-dependent malic enzyme (Schimerlik & Cleland, 1977) and a competitive inhibitor of both NAD- and NADP-dependent mitochondrial malic enzymes in heart muscle (Hiltunen & Davis, 1981). The effect of hydroxymalonate on the NADP-dependent malic enzyme activity of diaphragm muscle homogenate was investigated in the present study and is shown in Fig. 4.6 as a double reciprocal plot (1/V vs 1/[S]). From these graphs it was concluded that hydroxymalonate was a competitive inhibitor of the NADP-dependent malic enzyme of diaphragm muscle, with a calculated Ki of approximately 0.13 mM. At the concentration of hydroxymalonate
FIG. 4.6  Inhibition by Hydroxymalonate of Rat Diaphragm NADP-Malic Enzyme with Respect to Malate Concentration

NADP⁺-dependent malic enzyme activity in crude extracts of diaphragm from fed rats was measured in the presence of 50 mM-Tris/HCl, pH 7.4; 0.5 mM-NADP; 1 mM-MnCl₂, 2 μg/ml antimycin A and malate and hydroxymalonate as indicated.

a) no inhibitor: $1/V$ vs $1/[\text{malate}]$

\[
\frac{1}{V} \quad (\mu\text{mol}^{-1}.\text{min.} \cdot \text{g tissue})
\]

\[
\frac{1}{K_m} \quad 0 \quad 50 \quad 100
\]

$1/ [\text{malate}]$ (mM⁻¹)

b) with hydroxymalonate: $1/V$ vs $1/[\text{malate}]$

Hydroxymalonate concentration

\[
\frac{1}{V} \quad (\mu\text{mol}^{-1}.\text{min.} \cdot \text{g tissue})
\]

\[
-0.4 \quad 0 \quad 0.5 \quad 1.0
\]

$1/ [\text{malate}]$ (mM⁻¹)
used in the muscle incubations described below (2 mM), 70% inhibition of malic enzyme activity in the crude extract of diaphragm was observed when assayed with a malate concentration of 10 mM (1.72 and 0.55 μmol/min/g tissue in the absence and presence of 2 mM-hydroxymalonate respectively) and over 90% inhibition when assayed with a malate concentration of 1 mM (1.93 and 0.17 μmol/min/g tissue in the absence and presence of 2 mM-hydroxymalonate respectively). The malate content of rat skeletal muscle has been measured as approximately 100 nmol/g (Goodman et al., 1978). The malic enzyme activity of diaphragm preparations would therefore be expected to be substantially inhibited when incubated in the presence of 2 mM-hydroxymalonate.

3-mercaptopicolinate is a potent inhibitor of the cytosolic and mitochondrial forms of PEPCK (Robinson & Oei, 1975; Kostas et al., 1975). Most reports of the effect of 3-mercaptopicolinate on PEPCK activity have studied the reaction in the direction towards PEP formation (Robinson & Oei, 1975; Jomain-Baum et al., 1976; MacDonald & Lardy, 1978a). Owing to the low level of PEPCK activity in muscle, however, the more sensitive assay in the direction towards oxaloacetate formation was employed in the present study (PEPCK method A, Chapter 2). 3-mercaptopicolinate has been reported to be a competitive inhibitor of liver PEPCK activity with respect to phosphoenolpyruvate concentration when PEPCK is assayed in the direction of oxaloacetate synthesis (Reynolds, 1980a). That report is consistent with the inhibitory effect of 3-mercaptopicolinate on the activity of PEPCK in diaphragm muscle extracts shown in
Fig. 4.7. 1 mM-3-mercaptopicolinate produced 60% inhibition of PEPCK activity when assayed with a phosphoenolpyruvate concentration of 3 mM (23.8 and 10.4 nmoles/min/g tissue in the absence and presence of 1 mM-3-mercaptopicolinate respectively) and over 80% inhibition with a phosphoenolpyruvate concentration of 1 mM (25.4 and 5.0 nmoles/min/g tissue in the absence and presence of 1 mM-3-mercaptopicolinate respectively). 1 mM-3-mercaptopicolinate was used in diaphragm incubations described below. As the level of phosphoenolpyruvate in mammalian muscle has been reported as 10 - 20 μM (Newsholme & Start, 1973), additions of 1 mM-3-mercaptopicolinate should produce substantial inhibition of PEPCK activity in the diaphragm incubations.

The effects of hydroxymalonate and 3-mercaptopicolinate on the release of alanine from hemi-diaphragms incubated in the presence of the BCAAs, valine or leucine, are seen in Table 4.6. 2 mM-hydroxymalonate had no effect on alanine production in the presence of 3 mM-valine or 3 mM-leucine from hemi-diaphragms from fed or 48 h-starved rats. In contrast, 1 mM-mercaptopicolinate decreased alanine release. To make allowances for the contribution made to alanine release by possible effects of the inhibitors on protein degradation, tyrosine release was measured. Correction for the slight increases in net muscle proteolysis found in the presence of the inhibitors was made by expressing the data as the ratio of alanine release to tyrosine release (shown in Table 4.7). Again 3-mercaptopicolinate but not hydroxymalonate was found to inhibit muscle alanine production. These results provide support for a route of alanine synthesis from BCAA carbon in
FIG. 4.7 Inhibition by 3-Mercaptopicolinate (3MP) of Rat Diaphragm PEPCK with Respect to Phosphoenolpyruvate Concentration

PEPCK activity in crude extracts of diaphragm from fed rats by Method A, Chapter 2, except that phosphoenolpyruvate concentration was varied as indicated.

a) no inhibitor: \(\frac{1}{V}\) vs \(\frac{1}{[\text{Phosphoenolpyruvate}]}

\[
\begin{align*}
\frac{1}{V} &= 0.125 \\
\text{(nmoles}^{-1} \cdot \text{min.g tissue})
\end{align*}
\]

\[
\frac{1}{Km} = 0, 5, 10
\]

\[
\frac{1}{[\text{Phosphoenolpyruvate}]} \text{ (mM}^{-1})
\]

b) with 3-mercaptopicolinate: \(\frac{1}{V}\) vs \(\frac{1}{[\text{Phosphoenolpyruvate}]}

\[
\begin{align*}
\frac{1}{V} &= 0.15 \\
\text{(nmoles}^{-1} \cdot \text{min.g tissue})
\end{align*}
\]

\[
\frac{1}{[\text{Phosphoenolpyruvate}]} \text{ (mM}^{-1})
\]

3-mercaptopicolinate concentration
TABLE 4.6  Effect of 2 mM-Hydroxy malonate and 1 mM-3-Mercaptopicolinate on the Release of Alanine, Glutamine, Glutamate and Tyrosine by Hemi-Diaphragms Prepared From a) Fed and, b) 48 h- Starved Rats

Values shown are means ± S.E.M.; number of observations in parentheses. Statistical differences (by paired t-test) between incubations with and without hydroxymalonate or 3-mercaptopicolinate are denoted as: * p < 0.05, ** p < 0.01.

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>Alanine</th>
<th>Metabolite Release (µmol/2 h/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alanine</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>3 mM-valine</td>
<td>2.69 ± 0.18 (8)</td>
<td>0.65 ± 0.04 (8)</td>
</tr>
<tr>
<td>3 mM-valine + 2 mM-hydroxymalonate</td>
<td>2.56 ± 0.22 (4)</td>
<td>0.76 ± 0.03 (4)</td>
</tr>
<tr>
<td>3 mM-valine + 1 mM-mercaptopicolinate</td>
<td>1.98 ± 0.30 (4)*</td>
<td>0.85 ± 0.03 (4)**</td>
</tr>
<tr>
<td>3 mM-leucine</td>
<td>3.26 ± 0.26 (8)</td>
<td>0.70 ± 0.06 (8)</td>
</tr>
<tr>
<td>3 mM-leucine + 2 mM-hydroxymalonate</td>
<td>2.98 ± 0.43 (4)</td>
<td>0.71 ± 0.03 (4)</td>
</tr>
<tr>
<td>3 mM-leucine + 1 mM-mercaptopicolinate</td>
<td>2.40 ± 0.08 (4)**</td>
<td>0.96 ± 0.09 (4)**</td>
</tr>
<tr>
<td>Additions to Incubation Medium</td>
<td>Alanine</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>----------</td>
<td>-----------</td>
</tr>
<tr>
<td>3 mM-valine</td>
<td>1.73 ± 0.18 (8)</td>
<td>0.78 ± 0.03 (8)</td>
</tr>
<tr>
<td>3 mM-valine + 2 mM-hydroxymalonate</td>
<td>2.08 ± 0.38 (4)</td>
<td>0.94 ± 0.11 (4)</td>
</tr>
<tr>
<td>3 mM-valine + 1 mM-mercaptopicolinate</td>
<td>1.21 ± 0.11 (4)**</td>
<td>1.10 ± 0.08 (4)**</td>
</tr>
<tr>
<td>3 mM-leucine</td>
<td>1.42 ± 0.09 (8)</td>
<td>0.81 ± 0.03 (8)</td>
</tr>
<tr>
<td>3 mM-leucine + 2 mM-hydroxymalonate</td>
<td>1.43 ± 0.08 (4)</td>
<td>0.93 ± 0.04 (4)</td>
</tr>
<tr>
<td>3 mM-leucine + 1 mM-mercaptopicolinate</td>
<td>1.02 ± 0.09 (4)**</td>
<td>1.18 ± 0.07 (4)**</td>
</tr>
</tbody>
</table>
TABLE 4.7  Effect of 2 mM-Hydroxymalonate and 1 mM-Mercaptopicolinate on the Release of Alanine, Glutamine and Glutamate by Hemi-Diaphragms Prepared From a) Fed and, b) 48 h-Starved Rats

Amino acid release was calculated as a ratio to tyrosine release for each tissue incubation. For each pair of samples, ratios obtained in the presence of hydroxymalonate or mercaptopicolinate were expressed as percentages of ratios obtained in the absence of inhibitors.

a) Fed

<table>
<thead>
<tr>
<th>Amino Acid Release</th>
<th>Percentage of Control Value Caused by Presence of Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alanine</td>
</tr>
<tr>
<td>3 mM-valine + 2 mM-hydroxymalonate</td>
<td>88.8 ± 18.3 (4)</td>
</tr>
<tr>
<td>3 mM-valine + 1 mM-mercaptopicolinate</td>
<td>53.7 ± 4.9 (4)</td>
</tr>
<tr>
<td>3 mM-leucine + 2 mM-hydroxymalonate</td>
<td>88.9 ± 6.2 (4)</td>
</tr>
<tr>
<td>3 mM-leucine + 1 mM-mercaptopicolinate</td>
<td>54.8 ± 3.4 (4)</td>
</tr>
</tbody>
</table>
(TABLE 4.7 Continued)

b) 48 h-Starved

<table>
<thead>
<tr>
<th></th>
<th>Alanine</th>
<th>Glutamine</th>
<th>Glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mM-valine + 2 mM-hydroxymalonate</td>
<td>79.4 ± 3.0 (4)</td>
<td>88.2 ± 4.6 (4)</td>
<td>66.7 ± 6.7 (4)</td>
</tr>
<tr>
<td>3 mM-valine + 1 mM-mercaptopicolinate</td>
<td>63.8 ± 5.6 (4)</td>
<td>71.7 ± 4.8 (4)</td>
<td>77.1 ± 13.7 (4)</td>
</tr>
<tr>
<td>3 mM-leucine + 2 mM-hydroxymalonate</td>
<td>99.4 ± 12.4 (4)</td>
<td>88.1 ± 10.5 (4)</td>
<td>187.3 ± 45.6 (4)</td>
</tr>
<tr>
<td>3 mM-leucine + 1 mM-mercaptopicolinate</td>
<td>45.3 ± 3.8 (4)</td>
<td>77.7 ± 12.9 (4)</td>
<td>64.2 ± 8.5 (4)</td>
</tr>
</tbody>
</table>

Amino Acid Release

Percentage of Control Value Caused by Presence of Inhibitor
skeletal muscle involving PEPCK rather than malic enzyme.

Hydroxymalonate had no significant effect on glutamate or glutamine release but 3-mercaptopicolinate significantly (p < 0.05) reduced valine-stimulated glutamine release from hemi-diaphragms from fed rats and valine-stimulated glutamate release from hemi-diaphragms from 48 h-starved rats. The method by which inhibition of PEPCK by 3-mercaptopicolinate causes these effects is not clear. One possibility is that inhibition of the amount of carbon leaving the tricarboxylic acid cycle via PEPCK limits the amount of pyruvate available for acetyl-CoA formation. The lowered acetyl-CoA levels may then lead to lowered citrate and hence 2-oxoglutarate levels within the muscle. Consequently, the amount of glutamate and glutamine within the muscle may be decreased, limiting the amount released. In a separate experiment the effect of 3-mercaptopicolinate on the tissue content of alanine, glutamine and glutamate was measured using hemi-diaphragms from 48 h-starved rats. Incubation of hemi-diaphragms in 3 mM-valine significantly increased (p < 0.05, paired t-test) the tissue content of alanine and glutamate but not of glutamine (Table 4.8). However, the presence of 1 mM-3-mercaptopicolinate in the incubation media produced no change in the tissue levels of alanine and glutamate and the decrease in tissue content of glutamine observed in the presence of 3-mercaptopicolinate did not reach statistical significance.

The decreased alanine release into the incubation medium observed in the presence of 1 mM-3-mercaptopicolinate in the previous experiment (Tables 4.6 & 4.7) was not therefore merely
TABLE 4.8 Effect of 1 mM-3-Mercaptopicolinate on Diaphragm Tissue Content of Alanine, Glutamine and Glutamate

Tissue content of hemi-diaphragms prepared from 48 h-starved rats was determined following incubation as described below.

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>Tissue Content (µmol/g of muscle)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alanine</td>
</tr>
<tr>
<td>NONE</td>
<td>0.33 ± 0.03 (4)</td>
</tr>
<tr>
<td>3 mM-valine</td>
<td>0.59 ± 0.09 (4)</td>
</tr>
<tr>
<td>1 mM-3MP</td>
<td>0.34 ± 0.08 (4)</td>
</tr>
<tr>
<td>3 mM-valine + 1 mM-3MP</td>
<td>0.54 ± 0.07 (4)</td>
</tr>
</tbody>
</table>
due to 3-mercaptopicolinate inhibiting transport of alanine from the hemi-diaphragm but was in fact an inhibition of alanine formation in the muscle.

3-mercaptopicolinate was also found to inhibit PEPCK of EDL muscle (Table 4.9) and to substantially decrease the rate of valine-stimulated alanine production by that muscle (Table 4.10). No compensatory build-up of alanine in the muscle was observed, again indicating that 3-mercaptopicolinate inhibits alanine formation in muscle and not merely alanine release from muscle.

Other workers have exploited the inhibition of PEPCK activity in muscle by 3-mercaptopicolinate. Connett (1979) proposed a pathway for glyconeogenesis from lactate in frog striated muscle involving PEPCK based on experiments showing a decreased incorporation of lactate into glycogen in the presence of approximately 0.3 mM-mercaptopicolinate. A previous report from this laboratory (Snell & Duff, 1977b) and later work by Odedra & Palmer (1981) have indicated that 1 mM-mercaptopicolinate reduced alanine release from rat hemi-diaphragms incubated with glutamate, suggesting that the pathway involving PEPCK described in the present study was involved in withdrawing carbon derived from glutamate from the tricarboxylic acid cycle for alanine formation.

Lee & Davis (1979) however have proposed that malic enzyme is the more likely route for carbon withdrawal for alanine formation in skeletal muscle following their observation that 0.1 mM-3-mercaptopicolinate failed to affect alanine release in
TABLE 4.9  Effect of 3-Mercaptopicolinate on Muscle Phosphoenolpyruvate Carboxykinase (PEPCK) Activity

Values shown are means ± S.E.M. for measurements on crude muscle extracts from 4 fed rats. PEPCK activity was determined in each extract in the absence and presence of 1 mM-3-mercaptopicolinate (3MP) by method A as described in Chapter 2 i.e. [PEP] = 3 mM. Statistical differences (by paired t-test) between enzyme activity determinations in the presence and absence of 1 mM-3MP are denoted by: *** p < 0.001.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>PEPCK Activity (nmol/min/g of muscle)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-3MP</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>17.8 ± 2.8</td>
</tr>
<tr>
<td>Extensor</td>
<td>17.5 ± 2.4</td>
</tr>
<tr>
<td>digitorum longus</td>
<td></td>
</tr>
</tbody>
</table>
### TABLE 4.10 Effect of 1 mM-3-Mercaptopicolinate (3MP) on Valine-Stimulated Alanine Production by Extensor Digitorum Longus Muscle (EDL)

Values shown are means ± S.E.M. with the number of observations shown in parentheses. One of each pair of EDL muscle preparations from 48 h-starved rats was incubated in the absence and one in the presence of 3 mM-valine. Statistical differences (by paired t-test) between alanine determinations in the presence or absence of 3 mM-valine are denoted by: ** p < 0.01.

<table>
<thead>
<tr>
<th>Addition to Medium</th>
<th>Alanine Released (μmol/2 h/g of muscle)</th>
<th>Alanine Tissue Content (μmol/g of muscle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.67 ± 0.09 (6)</td>
<td>0.69 ± 0.26 (3)</td>
</tr>
<tr>
<td>3 mM-valine</td>
<td>1.18 ± 0.11 (6)**</td>
<td>0.87 ± 0.22 (3)</td>
</tr>
<tr>
<td>Mean difference</td>
<td>+0.51</td>
<td>+0.18</td>
</tr>
<tr>
<td>1 mM-3MP</td>
<td>0.73 ± 0.06 (6)</td>
<td>0.56 ± 0.18 (3)</td>
</tr>
<tr>
<td>3 mM-valine + 1 mM-3MP</td>
<td>1.00 ± 0.18 (6)</td>
<td>0.64 ± 0.15 (3)</td>
</tr>
<tr>
<td>Mean difference</td>
<td>+0.27</td>
<td>+0.08</td>
</tr>
</tbody>
</table>
hindlimb perfusions in the presence of 2 mM-propionate. The concentration of mercaptopicolinate used by these workers was relatively low in comparison with the present study. Furthermore it has been suggested that 3-mercaptopicolinate rapidly becomes inactivated in solutions not containing reducing agents and readily forms a disulphide with itself or other molecules (MacDonald & Grewe, 1981). It is possible that the high oxygen tension of the perfusion system of Lee & Davis (1979) which involved a fluorocarbon emulsion-based medium, could have oxidatively inactivated some of the 3-mercaptopicolinate. The further lowering of active mercaptopicolinate concentration could therefore have rendered it insufficient to cause appreciable inhibition of muscle PEPCK.

The results of the present experiments using 3-mercaptopicolinate and hydroxymalonate provide strong evidence that PEPCK and not malic enzyme is principally involved in the withdrawal of carbon from the tricarboxylic acid cycle for alanine synthesis de novo.

It is interesting that the inhibition of PEPCK by 3-mercaptopicolinate caused a significant decrease in alanine release from hemi-diaphragms incubated with 3 mM-leucine. Similar results have previously been reported from this laboratory (Snell & Duff, 1977b). In that study alanine release from hemi-diaphragms from rats starved for 2 days was $1.48 \pm 0.02$ and $2.07 \pm 0.12 \mu\text{mol}/2 \text{h/g tissue}$ when incubated with no addition to the medium and in the presence of 3 mM-leucine respectively, a mean increase of $0.59$ $\mu\text{mol}/2 \text{h/g tissue}$ in the presence of 3 mM-leucine. Alanine
release was 0.99 ± 0.14 and 1.55 ± 0.06 μmol/2 h/g tissue when incubated in the presence of 1 mM-3-mercaptopicolinate and 1 mM-mercaptoicolinate + 3 mM-leucine respectively, a mean increase of 0.56 μmol/2 h/g tissue in the presence of 3 mM-leucine. From those results it was concluded that 3-mercaptopicolinate had no effect on 3 mM-leucine-stimulated alanine release, although it was found to inhibit 3 mM-valine-stimulated alanine release by over 60% and 3 mM-glutamate-stimulated alanine release by over 50%. However, comparing the values for alanine release when leucine was present in the incubation medium, 2.07 ± 0.12 and 1.55 ± 0.06 μmol/2 h/g tissue in the absence and presence of 3-mercaptopicolinate respectively, it can be seen that they are similar to those of the present study (Table 4.6), the presence of 3-mercaptopicolinate producing a lower rate of alanine release. When the figures of the Snell & Duff (1977b) report are examined it can be seen that the presence of 3-mercapto- picolinate decreased alanine release even when no other additions were present in the incubation medium (1.48 ± 0.02 and 0.99 ± 0.14 μmol/2 h/g tissue in the absence and presence of 1 mM-3-mercaptoicolinate respectively for the unsupplemented incubations paired to those where 3 mM-leucine was present). Taken together with the results for alanine release from muscle incubated in 3 mM-leucine and 3 mM-leucine + 1 mM-3-mercaptoicolinate, these results suggest that the PEPCK inhibitor is blocking alanine formation from endogenous sources and not inhibiting alanine formation from the added leucine. However, this conclusion may not be valid in view of the observed effects of 3-mercaptoicolinate
and the reported effects of leucine (or one of its metabolites) on muscle protein breakdown (Goldberg & Tischler, 1981). It is possible that the inhibitory effect of leucine on net protein breakdown is affected by the presence of 3-mercaptopicolinate. The alanine release when leucine + 3-mercaptopicolinate are present may then be partially the result of decreased inhibition of proteolysis by leucine. Hence the increase in alanine release observed in the presence of leucine and 3-mercaptopicolinate may include a contribution from proteolysis which the increase in alanine release observed in the presence of leucine alone may not. Therefore before it can be definitely stated that 3-mercaptopicolinate does not affect leucine-stimulated alanine production some knowledge of the net protein breakdown in those different situations is required. If 3-mercaptopicolinate does affect leucine-stimulated alanine production it is possible that leucine may exert its effect of increasing muscle alanine release by stimulating the withdrawal of tricarboxylic acid cycle inter­mediates for pyruvate and hence alanine formation. The inter­mediates in question could not be supplied by the carbon skeleton of leucine itself, as leucine is considered to be a purely ketogenic amino acid (Meister, 1965). Perhaps leucine (or one of its metabolites) can channel the carbon of other amino acids, derived from muscle protein degradation, from the tricarboxylic acid cycle to provide the carbon skeleton of alanine.

Regardless of the mechanisms involved for leucine stimulation of muscle alanine release, 3-mercaptopicolinate was found to decrease alanine release from muscle without affecting
muscle alanine tissue content. As alanine release derived from proteolysis increased in the presence of 3-mercaptopicolinate this suggests that de novo alanine formation in muscle is decreased in the presence of the inhibitor and that alanine formation in muscle involves PEPCK.

Alternatively, 3-mercaptopicolinate may have additional effects to the inhibition of PEPCK which might interfere with a step common to both valine and leucine-stimulated alanine release, for example, inhibition of BCAA or alanine transamination or of alanine transport. However, the latter possibility seems unlikely in view of the observation that intracellular muscle alanine content was not increased in the presence of 3-mercaptopicolinate (Tables 4.8 & 4.10) and experiments described in Chapter 7 (Tables 7.1 & 7.4) have shown that 3-mercaptopicolinate does not inhibit BCAA transamination.

4.2.4 Proposed Pathway for Alanine Formation from Valine in Muscle

The proposed pathway for alanine formation from valine in muscle involves the coupling of branched-chain aminotransferase and alanine aminotransferase to bring about the transfer of nitrogen from the BCAAs to pyruvate. Reports of the sub-cellular locations of the enzymes involved, however, are conflicting (Ichihara et al., 1975; Odessey & Goldberg, 1979). Over 90% of muscle alanine aminotransferase activity has been reported to be in the cytosolic fraction (De Rosa & Swick, 1975). However, most of the branched-chain aminotransferase activity
(over 70%) of muscle has been reported to be located in the particulate fraction (Ichihara et al., 1975; but c.f. Odessey & Goldberg, 1979). In addition, oxidation of branched-chain 2-oxo acids is known to occur in the mitochondria (Dancis & Levitz, 1972; Van Hinsbergh et al., 1978; Odessey & Goldberg, 1979). These observations suggest that there must be a transfer of BCAA derived nitrogen from the mitochondria to the cytosol where it can be used for alanine formation by alanine aminotransferase. A possible carrier for this transfer is aspartate. Safer & Williamson (1973) have shown that in the perfused rat heart aspartate efflux from the mitochondria is associated with increased formation of alanine. Aspartate entering the cytosol from the mitochondria would serve as a carrier of BCAA nitrogen and of oxaloacetate, the carbon of which may be derived from the metabolism of the carbon skeleton of valine.

Transamination of aspartate in the cytosol by the enzyme aspartate aminotransferase would donate amino nitrogen for glutamate formation and hence, by further transamination, alanine formation. Cytosol aspartate transamination would also generate oxaloacetate. The oxaloacetate can then be converted to pyruvate for alanine formation via the enzymes PEPCK and pyruvate kinase. The presence of aspartate aminotransferase in both the cytosolic and the mitochondrial compartments is required for this scheme (Fig. 4.8a) which serves to channel both the nitrogen and carbon of valine into alanine. Evidence that aspartate transamination is involved in the formation of alanine was obtained from experiments using 2-amino-4-methoxy-
FIG. 4.8 Intracellular Compartmentalisation of the Proposed Pathway of Alanine Formation from Valine in Muscle of Starved Rats, Assuming a) Branched-Chain Aminotransferase is Located in Mitochondria, and b) Branched-Chain Aminotransferase is Located In the Cytosol.

a) Cytosol

- Phosphoenolpyruvate
- Pyruvate
- Glutamate
- Oxaloacetate
- Alanine
- 2-Oxoglutarate
- Aspartate

Mitochondria

- Valine
- 2-Oxoglutarate
- Aspartate
- 3-Methyl-2-oxobutyrate
- Glutamate
- Oxaloacetate
- Succinyl-CoA

b) Cytosol

- Valine
- 2-Oxoglutarate
- Alanine
- 3-Methyl-2-oxobutyrate
- Glutamate
- Pyruvate
- Phosphoenolpyruvate
- Oxaloacetate

Mitochondria

- 3-Methyl-2-oxobutyrate
- Aspartate
- 2-Oxoglutarate
- Glutamate
trans-but-3-enoic acid (AMB) which is a relatively specific inhibitor of aspartate aminotransferase (Rando et al., 1976; Smith et al., 1977; Snell, 1978). Table 4.11 shows that AMB decreased the valine-stimulated release of alanine by diaphragm muscle. This effect was particularly marked with muscle from 48 h-starved rats (Fig. 4.9). These results show that aspartate aminotransferase is involved in alanine formation and are consistent with the proposal that alanine can be derived from the carbon skeleton of valine, particularly in the fasted state. Alanine production by muscle from fed animals was not so affected by inhibition of aspartate transamination. The carbon for alanine formation in the fed state is therefore probably not supplied by valine to such an extent as during starvation. This is in agreement with the results of experiments involving sodium malonate inhibition of alanine formation. In the fed state a large proportion of the pyruvate required for alanine formation is most probably derived from glycogenolysis.

The increased lactate/pyruvate ratios obtained in the presence of 1 mM-AMB (Table 4.12) indicate its effectiveness as an inhibitor of aspartate aminotransferase. In aerobic muscle the mitochondrial electron-transport chain provides the obvious means of oxidation of glycolytically-derived NADH. A problem arises from the impermeability of the mitochondrial membrane to NADH. To overcome this problem in muscle, one means of transporting NADH from the cytoplasm into mitochondria is the malate-oxaloacetate shuttle (Newsholme & Start, 1973). In the cytosol oxaloacetate is reduced to malate (and NADH is oxidised
<table>
<thead>
<tr>
<th>Additions to Incubation</th>
<th>Alanine Control</th>
<th>+ 1 mM-AMB</th>
<th>Pancreatic</th>
<th>Pyruvate Control</th>
<th>+ 1 mM-AMB</th>
<th>Lactate Control</th>
<th>+ 1 mM-AMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.48 ± 0.14 (13)</td>
<td>1.21 ± 0.07 (9)</td>
<td>0.86 ± 0.10 (13)</td>
<td>0.24 ± 0.05** (9)</td>
<td>6.43 ± 0.70 (13)</td>
<td>8.38 ± 0.66* (8)</td>
<td></td>
</tr>
<tr>
<td>3 mM-valine</td>
<td>2.47 ± 0.15 (13)</td>
<td>1.81 ± 0.15* (3)</td>
<td>0.85 ± 0.13 (13)</td>
<td>0.45 ± 0.32 (3)</td>
<td>6.53 ± 0.48 (13)</td>
<td>8.91 ± 0.91* (3)</td>
<td></td>
</tr>
<tr>
<td>Difference</td>
<td>+0.99</td>
<td>+0.60</td>
<td>-0.01</td>
<td>+0.21</td>
<td>+0.10</td>
<td>+0.53</td>
<td></td>
</tr>
<tr>
<td>3 mM-leucine</td>
<td>2.57 ± 0.17 (5)</td>
<td>1.75 ± 0.18** (3)</td>
<td>0.38 ± 0.08 (5)</td>
<td>0.33 ± 0.10 (3)</td>
<td>7.44 ± 1.23 (5)</td>
<td>11.86 ± 0.18* (3)</td>
<td></td>
</tr>
<tr>
<td>Difference</td>
<td>+1.09</td>
<td>+0.54</td>
<td>-0.48</td>
<td>+0.09</td>
<td>+1.01</td>
<td>+3.48</td>
<td></td>
</tr>
</tbody>
</table>
(TABLE 4.11 Continued)

b) 48 h-Starved 

<table>
<thead>
<tr>
<th>Additions to Incubation</th>
<th>Alanine</th>
<th>Pyruvate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+1 mM-AMB</td>
<td>Control</td>
</tr>
<tr>
<td>Medium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1.06 ± 0.12</td>
<td>0.83 ± 0.06</td>
<td>1.43 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>(12)</td>
<td>(14)</td>
<td>(12)</td>
</tr>
<tr>
<td>3 mM-valine</td>
<td>1.93 ± 0.15</td>
<td>1.16 ± 0.09**</td>
<td>1.19 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>(?)</td>
<td>(5)</td>
<td>(?)</td>
</tr>
<tr>
<td>Difference</td>
<td>+0.87</td>
<td>+0.33</td>
<td>-0.24</td>
</tr>
<tr>
<td>3 mM-leucine</td>
<td>2.07 ± 0.11</td>
<td>1.85 ± 0.12</td>
<td>0.35 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(3)</td>
<td>(3)</td>
</tr>
<tr>
<td>Difference</td>
<td>+1.01</td>
<td>+1.02</td>
<td>-1.08</td>
</tr>
<tr>
<td>3 mM-glutamate</td>
<td>3.25 ± 0.33</td>
<td>1.50 ± 0.29**</td>
<td>0.79 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(3)</td>
<td>(4)</td>
</tr>
<tr>
<td>Difference</td>
<td>+2.19</td>
<td>+0.67</td>
<td>-0.64</td>
</tr>
</tbody>
</table>

Metabolite Release (μmol/2 h/g tissue)
FIG. 4.9 Effect of 1 mM-AMB on Valine-Stimulated Alanine Formation by Hemi-Diaphragms Prepared From Fed and 48 h-Starved Rats

Valine concentration was 3 mM.
Controls were incubated in the absence of 3 mM-valine.
TABLE 4.12 Effect of 1 mM-2-Amino-4-methoxy-trans-but-3-enoate (AMB) on the Ratio of Lactate to Pyruvate Released by Hemi-Diaphragms From a) Fed and, b) 48 h-Starved Rats

Values are ratios of the mean values of lactate release to the mean values of pyruvate release shown in Table 4.10.

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>Control</th>
<th>+ 1 mM-AMB</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.5</td>
<td>34.9</td>
</tr>
<tr>
<td>3 mM-valine</td>
<td>7.7</td>
<td>19.8</td>
</tr>
<tr>
<td>3 mM-leucine</td>
<td>19.6</td>
<td>35.9</td>
</tr>
<tr>
<td>b) 48 h-Starved</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additions to Incubation Medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>3.6</td>
<td>45.4</td>
</tr>
<tr>
<td>3 mM-valine</td>
<td>3.9</td>
<td>79.0</td>
</tr>
<tr>
<td>3 mM-leucine</td>
<td>12.8</td>
<td>53.5</td>
</tr>
<tr>
<td>3 mM-glutamate</td>
<td>9.2</td>
<td>120</td>
</tr>
</tbody>
</table>
to NAD) by malate dehydrogenase. The malate crosses the mito-
chondrial membrane and is then oxidised to oxaloacetate by
mitochondrial malate dehydrogenase, hence transferring.
reducing equivalents from the cytosol into mitochondria.
Oxaloacetate, however, does not cross the mitochondrial membrane
very readily and so leaves as aspartate which requires trans-
amination reactions in both the cytosolic and mitochondrial
compartments. Thus aspartate aminotransferase is involved in
the malate-oxaloacetate shuttle in muscle. Inhibition of
aspartate aminotransferase by AMB would therefore prevent the
transfer of NADH from the cytosol by the shuttle, thereby making
the cytosol more reduced and so increasing the ratio of lactate/
pyruvate release.

As noted above, in contrast to Ichihara et al. (1975),
Odessey & Goldberg (1979) have reported that branched-chain
aminotransferase activity is largely (~70%) cytosolic in
muscle. If branched-chain aminotransferase activity is indeed
located in the cytosol a different scheme for the reactions
involved in alanine formation from valine is required (Fig. 4.8). In this scheme, aspartate aminotransferase is still required to
provide cytosolic oxaloacetate in order to produce pyruvate for alanine formation by the pathway involving PEPCK. However, in
this scheme no net transfer of amino nitrogen occurs from the
mitochondria to the cytosol; the amino groups of aspartate entering
the cytoplasm return to the mitochondria as glutamate.

The experiments reported in this chapter indicate that
muscle has the metabolic capacity to utilise the carbon and
nitrogen of valine (and possibly other amino acids whose
catabolism results in a net formation of tricarboxylic acid
cycle intermediates) for the formation of alanine. The metabolic
route involves the enzymes PEPCK and aspartate aminotransferase
and it is more active during starvation. The extent to which
this metabolic route operates during starvation in vivo is
unknown but it does provide an explanation for the net conversion
of amino acids arising from proteolysis into alanine which,
following its release from muscle, can be used by the liver as
a gluconeogenic precursor.

4.2.5 **Appendix: Apparent Stimulation of PEPCK Activity by
3-Aminopicolinate**

The effect of an inhibitor of muscle PEPCK, 3-mercaptopicocolinate, on valine-stimulated alanine release provided evidence
for the proposed pathway for alanine formation from valine
carbon detailed above. Experiments were also carried out,
however, on the effect of 3-aminopicolinate, (3-AP), (an activator
of PEPCK activity, MacDonald & Lardy, 1978b) on valine-stimulated
alanine release. 3-AP increased the valine-stimulated release
of alanine by EDL muscle preparations without increasing net
protein degradation (as measured by tyrosine release) (Table 4.13).
However, 3-AP did not increase valine-stimulated alanine release
from soleus muscle (Table 4.13). A compound which stimulates
muscle PEPCK activity would be expected, from the evidence of
preceding experiments, to increase valine-stimulated alanine
release. The lack of increase in alanine release could not be
**TABLE 4.13**  Effect of 1 mM-3-Aminopicolinate (3AP) on the Valine-Stimulated Alanine Release From a) EDL and b) Soleus Muscles From 48 h-Starved Rats

Values shown are means ± S.E.M., number of observations are shown in parentheses. The alanine and tyrosine contents of the muscles were also determined at the end of the incubation period.

a) **EDL**

<table>
<thead>
<tr>
<th>Addition to Incubation Medium</th>
<th>Metabolite Release (μmol/2 h/g tissue)</th>
<th>Tissue Content (μmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alanine</td>
<td>Glutamine</td>
</tr>
<tr>
<td>NONE</td>
<td>0.68 ± 0.06 (11)</td>
<td>2.75 ± 0.16 (8)</td>
</tr>
<tr>
<td>3 mM-valine</td>
<td>0.92 ± 0.10 (11)</td>
<td>3.58 ± 0.29 (8)</td>
</tr>
<tr>
<td>Mean Difference</td>
<td>+0.24</td>
<td>+0.83</td>
</tr>
<tr>
<td>1 mM-3AP</td>
<td>0.67 ± 0.07 (11)</td>
<td>3.80 ± 0.29 (8)</td>
</tr>
<tr>
<td>1 mM-3AP + 3 mM-valine</td>
<td>1.02 ± 0.07 (11)</td>
<td>5.44 ± 0.28 (8)</td>
</tr>
<tr>
<td>Mean Difference</td>
<td>+0.35</td>
<td>+1.64</td>
</tr>
</tbody>
</table>
(TABLE 4.13 Continued)

b) Soleus

<table>
<thead>
<tr>
<th>Addition to Incubation Medium</th>
<th>Alanine</th>
<th>Glutamine</th>
<th>Tyrosine</th>
<th>Alanine</th>
<th>Tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>NONE</td>
<td>0.79 ± 0.07 (10)</td>
<td>3.95 ± 0.51 (7)</td>
<td>0.36 ± 0.03 (12)</td>
<td>0.32 ± 0.09 (4)</td>
<td>0.24 ± 0.01 (4)</td>
</tr>
<tr>
<td>3 mM-valine</td>
<td>1.08 ± 0.08 (11)</td>
<td>4.53 ± 0.36 (8)</td>
<td>0.35 ± 0.02 (12)</td>
<td>0.42 ± 0.08 (4)</td>
<td>0.22 ± 0.04 (4)</td>
</tr>
<tr>
<td>Mean Difference</td>
<td>+0.29</td>
<td>+0.58</td>
<td>-0.01</td>
<td>+0.10</td>
<td>-0.02</td>
</tr>
<tr>
<td>1 mM-3AP</td>
<td>0.76 ± 0.09 (11)</td>
<td>4.56 ± 0.34 (7)</td>
<td>0.32 ± 0.02 (12)</td>
<td>0.24 ± 0.05 (4)</td>
<td>0.21 ± 0.05 (4)</td>
</tr>
<tr>
<td>1 mM-3AP + 3 mM-valine</td>
<td>0.97 ± 0.12 (11)</td>
<td>6.13 ± 0.52 (8)</td>
<td>0.32 ± 0.02 (12)</td>
<td>0.31 ± 0.07 (4)</td>
<td>0.19 ± 0.02 (4)</td>
</tr>
<tr>
<td>Mean Difference</td>
<td>+0.21</td>
<td>+1.57</td>
<td>0</td>
<td>+0.07</td>
<td>-0.02</td>
</tr>
</tbody>
</table>
accounted for by decreased protein breakdown in the presence of 3-AP because tyrosine release was similar in the presence or absence of 3-AP. It was observed however, that 3-AP increased the valine-stimulated release of glutamine from both soleus and EDL muscles. This is a further demonstration that the BCAA-stimulated release of alanine and glutamine by muscle do not necessarily respond in the same manner to certain circumstances.

The effects of 3-AP on alanine release were puzzling. In order to clarify the situation, experiments were carried out to measure the effect of 3-aminopicolinate on muscle and liver enzyme activities. Liver was used as previous work which had demonstrated 3-AP to be an activator of PEPCK activity employed liver PEPCK (MacDonald & Lardy, 1978b). PEPCK activity was assayed by method A as described in Chapter 2. No activation of liver cytosol PEPCK activity, or the PEPCK activities in crude extracts of soleus and EDL muscles were observed (results not shown). The effect of 3-AP on the activity of PEPCK was also determined by assaying in the direction of PEP formation (i.e. by method D, Chapter 2). As is mentioned in the following chapter (Chapter 5), no muscle PEPCK activity was measurable at the limits of detection of this assay and so no effect of 3-AP could be observed. However, liver PEPCK activity was slightly inhibited by 1 mM-3-aminopicolinate (by 15%).

It has previously been reported that when liver PEPCK, 3-AP, dithiothreitol and a divalent metal ion (Fe$^{2+}$ or Mn$^{2+}$) are pre-incubated before assay, 3-AP can activate the enzyme (MacDonald
In the present study pre-incubation with Fe\textsuperscript{2+} and dithiothreitol was found to slightly increase muscle and liver PEPCK activities (\sim 10\%) but the presence of 1 mM-3-aminopicolinate in the pre-incubation caused no further stimulation of PEPCK activities. Reynolds (1980b) has also failed to observe activation of rat liver PEPCK activity by 3-aminopicolinate in the presence of Fe\textsuperscript{2+} and dithiothreitol. The same author has also reported that the PEPCK ferroactivator protein of rat liver acts by protecting PEPCK from inactivation when the enzyme is in the presence of Fe\textsuperscript{2+} (Reynolds, 1980c). He suggests that PEPCK may be in dynamic equilibrium between inactivation by ferrous ions and reactivation by thiol compounds and that 3-AP may act in the same manner as the ferroactivator protein by chelating the potentially inhibitory metal ions (Reynolds, 1980b). However, it is not known whether muscle PEPCK activity can be regulated in this manner by Fe\textsuperscript{2+}. No PEPCK ferroactivator was detected in rat skeletal muscle (MacDonald et al., 1978). Nevertheless, incubations with 1 mM-3-aminopicolinate stimulated alanine (and glutamine) release from EDL muscle. Investigations on the effect of 3-AP on muscle pyruvate kinase activity were also carried out (Fig. 4.10). 3-AP acted as an inhibitor of muscle pyruvate kinase activity in a non-competitive manner with respect to phosphoenolpyruvate concentration. In addition experiments on LDH activity were conducted with crude extracts of muscle and liver cytosol pre-incubated in the presence of 0.1 mM-FeCl\textsubscript{2} and 0.8 mM-dithiothreitol, with and without 3-AP. Pre-incubating either liver or muscle samples in the presence of
FIG. 4.10  Effect of 1 mM-3-Aminopicolinate on Muscle Pyruvate Kinase Activity

- 3-aminopicolinate
- + 3-aminopicolinate

\[ \text{"V"} \]

(\(\mu\text{mol/min/g tissue}\))

\[ \frac{1}{V} \]

(\(\mu\text{mol}^{-1}\text{min.g tissue} \times 10^{-3}\))
3-AP greatly inhibited LDH activity (Table 4.14). The inhibition apparently depended more on the presence of thiols in the pre-incubation mixture than Fe\(^{2+}\), although substituting Mn\(^{2+}\) for Fe\(^{2+}\) in the pre-incubation mixture relieved 3-AP inhibition of LDH activity.

The assay for pyruvate kinase makes use of LDH as a coupling enzyme. It is therefore possible that the observed inhibitory effects of 3-AP on pyruvate kinase were in fact secondary to the inhibition of the added LDH. The inhibition of LDH by 3-AP may also have been responsible for the slight inhibition found in liver PEPCK activity when assayed in the direction of phosphoenolpyruvate synthesis. The PEPCK assay method in this direction involves measuring phosphoenolpyruvate produced in a given time, by making use of a coupled enzyme system involving commercial preparations of pyruvate kinase and LDH.

With regard to the effect of 3-AP on alanine release observed in the present study, a possible explanation may be that 3-AP inhibited LDH to such an extent that more pyruvate was available for alanine formation. White muscle (EDL) has a higher rate of glycolysis than red muscle (soleus). If this explanation is correct then more pyruvate would be expected to build up in white muscle in the presence of 3-AP and this could account for the increase in alanine release found with EDL in the presence of 3-AP but not with soleus muscle. The increase in glutamine release observed in the presence of 3-AP may be a result of the increased amount of pyruvate permitting more acetyl-
TABLE 4.14  Effect of 1 mM-3-Aminopicolinate on Liver and Muscle (EDL) Lactate Dehydrogenase Activity

Tissue samples were pre-incubated for 10 min at 0°C prior to assay in the presence of 1 mM-FeCl₂, 0.8 mM-dithiothreitol (DTT) and with or without 2.5 mM-3AP. The amount of pre-incubation mixture taken for assaying for lactate dehydrogenase activity was such that [3AP] in the assay mixture = 1 mM.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Pre-incubation Conditions</th>
<th>Lactate Dehydrogenase Activity (μmol/min/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle</td>
<td>-3AP + Fe²⁺ + DTT</td>
<td>230</td>
</tr>
<tr>
<td>Muscle</td>
<td>+3AP + Fe²⁺ + DTT</td>
<td>47.3</td>
</tr>
<tr>
<td>Liver</td>
<td>-3AP + Fe²⁺ + DTT</td>
<td>425</td>
</tr>
<tr>
<td>Liver</td>
<td>+3AP + Fe²⁺ + DTT</td>
<td>22.4</td>
</tr>
<tr>
<td>Liver</td>
<td>+3AP - Fe²⁺ + DTT</td>
<td>15.2</td>
</tr>
<tr>
<td>Liver</td>
<td>+3AP + Fe²⁺ - DTT</td>
<td>52.1</td>
</tr>
<tr>
<td>Liver</td>
<td>-3AP - Fe²⁺ + 1 mM-Mn²⁺ + DTT</td>
<td>402</td>
</tr>
<tr>
<td>Liver</td>
<td>+3AP - Fe²⁺ + 1 mM-Mn²⁺ + DTT</td>
<td>322</td>
</tr>
</tbody>
</table>
CoA to be formed via pyruvate dehydrogenase. This could stimulate citrate formation and hence increase levels of 2-oxoglutarate which could be removed from the tricarboxylic acid cycle to form glutamine. It should be noted that in the present study, experiments were not conducted on purified enzyme preparations. 3-AP may well activate purified PEPCK from muscle (and liver, as shown by MacDonald & Lardy, 1978b) but the inhibitory effect on LDH activity may confound this observation in tissue incubation studies or with crude tissue extracts.

In view of the doubt as to the action of 3-aminopicolinate on muscle enzyme activities, the results obtained from these experiments did not provide any useful information for the elucidation of the proposed pathway from valine to alanine.

3-mercaptopicolinate was found to have no effect on muscle LDH and pyruvate kinase when assayed with or without the pre-incubation procedure detailed above.
CHAPTER FIVE

STUDIES ON MUSCLE ENZYMES IMPLICATED IN THE REMOVAL OF CARBON FROM THE TRICARBOXYLIC ACID CYCLE TO FORM PYRUVATE
5.1 INTRODUCTION

As discussed previously, leucine is regarded as a solely ketogenic amino acid (Meister, 1965) in that the accepted route of catabolism of leucine results in the formation of acetyl-CoA and acetoacetate. Leucine would only be able to contribute carbon for pyruvate formation if another, as yet unknown, pathway for leucine catabolism existed in muscle.

The accepted route of catabolism of valine, on the other hand, involves the formation of propionyl-CoA which can feed into the tricarboxylic acid cycle. Hence carbon derived from valine metabolism can contribute to the carbon skeleton of alanine if a metabolic route exists in muscle for the removal of carbon from the cycle to yield pyruvate.

The existence of muscle enzymes which are capable of removing carbon from the cycle for the formation of pyruvate has been reported. Substantial amounts of NADP-dependent (Nolte et al., 1972) and NAD-dependent (Lee & Davis, 1979) malic enzymes have been reported in rat skeletal muscles, and Opie & Newsholme (1967) have reported the existence of PEPCK activity in muscle.

The main aim of the work described in this chapter was to further investigate the enzymes implicated in carbon withdrawal from the tricarboxylic acid cycle, with a view to discovering by which of the possible alternative routes (see Chapter 1,
5.2 RESULTS AND DISCUSSION

Alternative Routes for Alanine Synthesis from Valine in Muscle

5.2.1 Muscle Oxaloacetate Decarboxylase Activity

A similar situation to the present one involving muscle was encountered by Watford et al., (1979) who were studying the route of alanine formation from the carbon of glutamine by enterocytes. The possible enzymes involved were as indicated above. Of the three enzymes, malic enzyme, oxaloacetate decarboxylase and PEPCK, oxaloacetate decarboxylase had the highest activity in enterocytes and could have been responsible for the formation of pyruvate. For this reason the oxaloacetate decarboxylase activity of muscle was measured.

Oxaloacetate decarboxylase activity can theoretically be measured by following oxaloacetate consumption and/or pyruvate production. Oxaloacetate is a rather unstable compound and undergoes spontaneous decarboxylation to pyruvate. Therefore in order to study the enzymatic decarboxylation both the assay methods used (Dean & Bartley, 1973; Wojtczak & Walajtys, 1974) employ conditions which are optimal for the enzymatic process and which decrease the non-enzymatic reaction. Unfortunately the decrease in oxaloacetate observed during the reactions proved to be highly variable and so oxaloacetate decarboxylase...
activity was measured only by the increase in pyruvate production.

The production of pyruvate by crude extracts (600 g supernatants) of soleus muscle are shown in Fig. 5.1. An increase in pyruvate production was found. However, the rate of pyruvate production by soleus extracts was no greater than the rate when boiled soleus muscle extract replaced the test extract in the assay mixture. This finding was not restricted to red muscles as is shown in Fig. 5.2. Fresh extracts of the white muscle, EDL, formed pyruvate at no greater rate than heat-inactivated extracts.

In order to show that the enzyme assay itself was not defective, the activity of oxaloacetate decarboxylase in 100,000 g supernatant of rat liver homogenate was followed. Fig. 5.3 shows that liver has in fact substantial enzymatic activity (1.30 ± 0.28 (4) μmol/min/g) being the difference between the total pyruvate production rate observed with test tissue extract and the pyruvate production rate observed with "boiled liver supernatant". This suggests that the enzyme assay itself is not defective and that muscle contains no appreciable oxaloacetate decarboxylase activity. Similarly, Wojtczak & Walajtys (1974) detected no skeletal muscle mitochondrial oxaloacetate decarboxylase activity.

5.2.2 Muscle "Malic Enzyme" Activity

There is little doubt as to the existence of NADP-malic enzyme activity in skeletal muscle (Nolte et al., 1972; Newsholme & Williams, 1978; Zahlten & Nejtek, 1979; Nagel et al., 1980;
FIG. 5.1  "Oxaloacetate Decarboxylase" in Soleus Muscle

- test
- "boiled tissue" blank

![Graph showing Pyruvate formation vs. Incubation Time](image-url)
FIG. 5.2 "Oxaloacetate Decarboxylase" in EDL Muscle

- test
- "boiled tissue" blank

Pyruvate formation (E490) vs. Incubation Time (min)
FIG. 5.3 "Supernatant Oxaloacetate Decarboxylase" in Liver

- test
- "boiled tissue" blank

Pyruvate formation (E440 x 1000)

Incubation time (min)
Swierczynski et al., 1980; Swierczynski, 1980; Swierczynski & Davis, 1981). Two types of malic enzyme have been reported to be present in several mammalian tissues (Sauer; 1973a; 1973b; Lin & Davis, 1974; Nagel et al., 1980): one of the enzyme forms uses only NADP$^+$ as coenzyme; the other reduces either NAD$^+$ or NADP$^+$, with NAD being the preferred substrate (Swierczynski & Davis, 1981). Lee & Davis, (1979) recently stated that rat skeletal muscle contained NAD$^+$-dependent malic enzyme activity.

The activities of NADP-malic enzyme, and NAD(P)-malic enzyme (using NAD and NADP as cofactors) were measured in the present study. The activity of NADP-malic enzyme was measured on crude extracts of diaphragm muscles from fed rats and found to be $1.90 \pm 0.15 \ (3) \ \mu$mol/min/g tissue at $30^\circ$C by the method of Swierczynski et al. (1980) and $1.96 \pm 0.18 \ (3) \ \mu$mol/min/g of tissue by the method of Nagel et al. (1980).

The activity of the NAD(P)-malic enzyme from various tissues has been reported to be increased when assayed in the presence of fumarate and inhibited by ATP (Sauer, 1973b; Mandella & Sauer, 1975; Sauer & Dauchy, 1978; Sauer et al., 1979). For this reason 5 mM-sodium fumarate was included in the mixture used for the assay of NAD(P) activity as described by Nagel et al. (1980). With NADP$^+$ as cofactor an activity of $1.10 \pm 0.21 \ (3) \ \mu$mol/min/g tissue was obtained whereas no activity was detectable using NAD$^+$ as the cofactor. These findings are in agreement with those of Nagel et al., (1980) who were also unable to confirm the findings of Lee & Davis (1979).

A recent paper (Swierczynski & Davis, 1981), however,
has again reported a cytoplasmic malic enzyme from rat skeletal muscle which can use NAD\(^+\) as a cofactor. The enzyme converts malate to pyruvate with either co-enzyme present at approximately the same maximal rates but the Km's for malate and nucleotide are many-fold higher with NAD\(^+\) as coenzyme than with NADP\(^+\).

Regardless of which enzyme form is predominant in skeletal muscle it was observed that malic enzyme (with NADP\(^+\) as cofactor) is present in sufficient activities to convert valine-derived carbon to pyruvate for alanine formation.

5.2.3 **Muscle Phosphoenolpyruvate Carboxykinase and Pyruvate Kinase Activity**

This route for the provision of pyruvate from tricarboxylic acid cycle carbon differs from those above in that two enzymatic steps are involved: a decarboxylation of oxaloacetate to phosphoenolpyruvate by PEPCK, followed by conversion to pyruvate by pyruvate kinase.

PEPCK activity was measured in crude extracts of rat diaphragm using PEPCK assay method A (Chapter 2). The mean value (0.0246 \(\mu\)mol/min/g tissue) was low in comparison with muscle malic enzyme activity but was more than sufficient to account for the valine-stimulated increase in alanine formation observed in diaphragm incubations in vitro being 2.95 \(\mu\)mol/2 h/g tissue compared with the mean valine-stimulated increase in alanine production of 0.99 \(\mu\)mol/2 h/g tissue from diaphragm preparations from fed rats and 0.87 \(\mu\)mol/2 h/g tissue from diaphragm preparations from 48 h-starved rats (Table 3.1).
Two methods for assaying PEPCK activity in crude extracts of muscle have been employed by various workers, both of which supposedly follow the formation of oxaloacetate from phosphoenolpyruvate and are coupled to malate formation from oxaloacetate by added malate dehydrogenase. One method is a continuous spectrophotometric assay based on the decrease in absorbance at 340 nm due to the oxidation of NADH by the conversion of oxaloacetate, formed in the assay, to malate by malate dehydrogenase (Opie & Newsholme, 1967; Crabtree et al., 1972). The other method (method A, Chapter 2) measures incorporation of $^{14}$CO$_2$ into oxaloacetate which is trapped for radioactive counting as acid-stable malate (Nolte et al., 1972) and is based on the assay developed for liver PEPCK (Chang & Lane, 1966; Ballard & Hanson, 1967).

Although the reaction mixtures for the two methods are very similar, two important differences have been observed between PEPCK activities in crude extracts of muscle determined by the two techniques (Table 5.1). The PEPCK values obtained using assay method A showed that, although in agreement with values reported by Nolte et al., (1972), the $^{14}$CO$_2$-incorporation assay method gave muscle PEPCK activities approximately one order of magnitude lower than the spectrophotometric assay (Opie & Newsholme, 1967; Crabtree et al., 1972; Newsholme & Williams, 1978; Newsholme et al., 1979; McLane & Holloszy, 1979; Odedra & Palmer, 1981). In addition, the radioactive assay method indicated that red muscle had PEPCK activity similar to or slightly higher than white muscle (Nolte et al., 1972), whereas the spectro-


**TABLE 5.1** Activities of PEPCK Determined by Four Different Methods, Pyruvate Kinase, and Lactate Dehydrogenase in Crude Extracts of Diaphragm, Soleus and Extensor Digitorum Longus Muscles

Activities were measured as described in Chapter 2. Values represent the means of measurements from different rats and are given ± S.E.M. with numbers of observations in parentheses. N.D. indicates activity was not detectable (see text)

<table>
<thead>
<tr>
<th>Muscle Type</th>
<th>PEPCK A (μmol/min/g of muscle at 30°C)</th>
<th>PEPCK B (μmol/min/g of muscle at 30°C)</th>
<th>PEPCK C (μmol/min/g of muscle at 30°C)</th>
<th>PEPCK D (μmol/min/g of muscle at 30°C)</th>
<th>Pyruvate Kinase</th>
<th>Lactate Dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diaphragm</td>
<td>0.0246 ± 0.0021 (5)</td>
<td>0.36 ± 0.06 (4)</td>
<td>1.03 ± 0.15 (4)</td>
<td>N.D.</td>
<td>104.6 ± 0.7 (3)</td>
<td>219.4 ± 19.3 (3)</td>
</tr>
<tr>
<td>Soleus</td>
<td>0.0253 ± 0.0015 (5)</td>
<td>0.30 ± 0.06 (7)</td>
<td>0.64 ± 0.03 (4)</td>
<td>N.D.</td>
<td>68.1 ± 9.5 (3)</td>
<td>159.4 ± 13.2 (3)</td>
</tr>
<tr>
<td>Extensor digitorum longus</td>
<td>0.0211 ± 0.0013 (5)</td>
<td>0.62 ± 0.08 (6)</td>
<td>2.96 ± 0.18 (5)</td>
<td>N.D.</td>
<td>215.6 ± 13.6 (4)</td>
<td>321.5 ± 22.2 (3)</td>
</tr>
</tbody>
</table>
photometric assay showed substantially higher activity in white muscle than in red muscle (McLane & Holloszy, 1979). In this study the basis for the differences between the two assay methods was investigated.

Table 5.1 shows the PEPCK activity of crude extracts of diaphragm, soleus and EDL muscles determined by the four PEPCK assay methods described in Chapter 2. The continuous spectrophotometric assays (methods B and C) gave apparent PEPCK activities which were in agreement with other workers (Opie & Newsholme, 1967; Crabtree et al., 1972; Newsholme & Williams, 1978; McLane & Holloszy, 1979; Odedra & Palmer, 1981) but which were at least ten times greater than the activities obtained using the $^{14}$CO$_2$-incorporation method (method A). Assay of muscle PEPCK in the direction of phosphoenolpyruvate formation (method D) showed negligible activity for all three muscle studies. However, it should be noted that the PEPCK activity reported using the $^{14}$CO$_2$-incorporation assay (method A) would be below the level of detection of activity using the phosphoenolpyruvate formation method (D).

The specificity of the $^{14}$CO$_2$-incorporation assay for PEPCK activity was tested using 3 mM-Na pyruvate in place of Na phosphoenolpyruvate. Less than 5% of the $^{14}$CO$_2$ incorporated with phosphoenolpyruvate as substrate was found with pyruvate under method A assay conditions.

Spectrophotometric PEPCK assays by methods B and C were performed using NaH$^{14}$CO$_3$ in place of "cold" NaHCO$_3$ and the incorporation of $^{14}$CO$_2$ into acid-stable products under these
assay conditions compared with the formation of malate as judged by NADH oxidation (Table 5.2). Product formation measured by radioactivity incorporated did not correspond with that measured by the decrease in [NADH] during the assay.

The two continuous spectrophotometric assays for PEPCK activity (methods B and C) were compared. Method B (where NaHCO₃ was omitted from controls) showed that the presence of NaHCO₃ increased NADH oxidation. The higher [NaHCO₃] in method C (50 mM) produced a greater rate of NADH oxidation than method B in which [NaHCO₃] was 17 mM (Table 5.1). NADH oxidation in the absence of phosphoenolpyruvate (i.e. control of method C) was negligible (0.1 μmol/min/g of muscle), whereas in the presence of phosphoenolpyruvate but in the absence of NaHCO₃ (i.e. control in method B), NADH oxidation was appreciable (0.51 ± 0.08 (4) μmol/NADH oxidised/min/g of muscle (mean ± S.E.M.) for EDL extracts). Substantial NADH oxidation was also found when NaHCO₃ was omitted (but phosphoenolpyruvate was present) in method C (0.76 ± 0.09 (4) μmol NADH oxidised/min/g of muscle for EDL extracts). As method C included antimycin A, an inhibitor of NADH oxidase (Wainio, 1970), some factor other than mitochondrial NADH oxidation was responsible for the phosphoenolpyruvate-dependent NADH oxidation which was stimulated by NaHCO₃. Both assay methods B and C had control and test assay mixtures where the measured pH was equal (pH 7.5 for method B, and pH 7.1 for method C). Varying [NaHCO₃] in assay B would have altered the pH of the test assay mixture compared with controls and so confused any effect of altering [NaHCO₃] per se. However, using method C, where NADH oxidation
TABLE 5.2  Muscle PEPCK Activities Determined by NADH Oxidation and $^{14}\text{CO}_2$-Incorporation in Individual Assays

PEPCK was assayed by methods B and C as described in Chapter 2, except that NaHCO$_3$ was replaced with NaH$^{14}$CO$_3$. PEPCK activity is expressed for each assay as $\mu$mol NADH oxidised/min per g of muscle and as $\mu$mol $^{14}$CO$_2$ incorporated/min per g of muscle (both at 30°C). Spectrophotometric assays were followed for 15 min after addition of muscle extract and terminated by mixing with 1 ml of 2M-HCl. $^{14}$CO$_2$ not incorporated into acid-stable products was removed by bubbling with carbon dioxide for 10 min. 0.5 ml of sample was removed for counting as described in Chapter 2.

<table>
<thead>
<tr>
<th>Assay Method</th>
<th>Muscle type</th>
<th>PEPCK activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\mu$mol NADH oxidised/min per g of muscle</td>
</tr>
<tr>
<td>B Soleus</td>
<td>0.18</td>
<td>0.0188</td>
</tr>
<tr>
<td></td>
<td>Extensor digitorum longus</td>
<td>0.52</td>
</tr>
<tr>
<td>C Soleus</td>
<td>0.62</td>
<td>0.0109</td>
</tr>
<tr>
<td></td>
<td>Extensor digitorum longus</td>
<td>3.38</td>
</tr>
</tbody>
</table>
was measured with phosphoenolpyruvate omitted from controls, the test and control samples had a similar pH at each \([\text{NaHCO}_3]\) in assays of PEPCK activity in crude extracts of soleus and EDL muscles. Increasing \([\text{NaHCO}_3]\) stimulated NADH oxidation (Fig. 5.4) but this did not account for the phosphoenolpyruvate-dependent NADH oxidation observed in the absence of \(\text{NaHCO}_3\). 

NaCl was substituted for \(\text{NaHCO}_3\), in this case using assay method B as the pH would not be so greatly affected by this substitution as would assay C. The results with extracts of EDL muscle are shown in Fig. 5.5. Assaying soleus muscle extracts in the presence of 17 mM-\(\text{NaHCO}_3\) or 17 mM-\(\text{NaCl}\) gave NADH oxidation rates of 0.37 ± 0.09 (3) and 0.41 ± 0.09 (3) \(\mu\text{mol/min/g of muscle}\) respectively (having subtracted the NADH-oxidation attributable to the controls to which neither \(\text{NaHCO}_3\) nor \(\text{NaCl}\) was added). Together these findings indicated that \([\text{Na}^+]\) and not \([\text{HCO}_3^-]\) was responsible for the stimulation of NADH oxidation found with increasing \([\text{NaHCO}_3]\). This would account for the NADH oxidation found in controls where \(\text{NaHCO}_3\) was omitted. Sodium salts of phosphoenolpyruvate, IDP and NADH were used in method B, the sodium concentration in the assay mixture in the absence of \(\text{NaHCO}_3\) being 4.5 mM. Similarly, method C used sodium salts of phosphoenolpyruvate, IDP and NADH but also included a contribution from the Hepes-NaOH buffer employed. Approximately 18 mM-NaOH was required to adjust stock 250 mM-Hepes to pH 6.5. The resulting sodium ion concentration in the assay due to the buffer was therefore 3.6 mM. Thus method C contains a sodium ion concentration of approximately 8.1 mM before the addition of \(\text{NaHCO}_3\).
NADH-oxidation attributable to apparent PEPCK activity was measured by method C, as described in Chapter 2.

Δ soleus  ● extensor digitorum longus
FIG. 5.5 Effect of Substituting NaCl for NaHCO₃ on NADH-Oxidation in Assays for Apparent PEPCK Activity in Crude Extracts of EDL Muscle

NADH oxidation was measured by PEPCK assay method B, as described in Chapter 2.

▲ NaCl  ○ NaHCO₃
The question arose as to what in the crude muscle extracts was causing the Na\textsuperscript{+}-stimulated NADH oxidation, which was not dependent on HCO\textsubscript{3}\textsuperscript{-}. Examination of the components of the assay mixtures of methods B and C suggested that lactate formation from phosphoenolpyruvate, by the joint action of pyruvate kinase and lactate dehydrogenase could be responsible. Lactate and malate produced during assays of apparent PEPCK activity by methods B and C were measured. Negligible malate was produced whereas the lactate formed is shown in Table 5.3. It was found that lactate production could indeed account for the oxidation of NADH which had previously been attributed to malate formation by apparent PEPCK activity. As for apparent PEPCK activity, lactate production was dependent on Na\textsuperscript{+} and was greater in EDL muscle extracts than in soleus muscle extracts (Fig. 5.6).

Assays for apparent PEPCK activity were performed by method C substituting commercial purified preparations of pyruvate kinase and lactate dehydrogenase (both from rabbit muscle) for crude muscle extracts. The approximate activities of the two enzymes found in diaphragm extracts (see Table 5.1), equivalent to 1 mg of tissue, were added (i.e. at 30\textdegree C pyruvate kinase 0.15 \(\mu\)mol/min; lactate dehydrogenase 0.25 \(\mu\)mol/min). The NADH oxidation observed was found to be dependent on the amount of pyruvate kinase added to the assay mixture (Table 5.4).

Pyruvate kinase activities in crude extracts of muscle were measured under various conditions by omitting and/or substituting various components of the assay system of Zammit et al., (1978), in order to obtain an indication of the pyruvate
Lactate production under the conditions of PEPCK assay was measured as described in the Materials and Methods chapter. Apparent PEPCK activity as measured by NADH oxidation is compared with lactate production in each assay.

<table>
<thead>
<tr>
<th>Muscle type</th>
<th>Assay method</th>
<th>PEPCK activity (μmol/min per g of muscle at 30°C)</th>
<th>Lactate production (μmol/min per g of muscle at 30°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diaphragm</td>
<td>C</td>
<td>0.92</td>
<td>0.81</td>
</tr>
<tr>
<td>Soleus</td>
<td>C</td>
<td>0.66</td>
<td>0.66</td>
</tr>
<tr>
<td>Soleus</td>
<td>C</td>
<td>0.61</td>
<td>0.87</td>
</tr>
<tr>
<td>Extensor digitorum longus</td>
<td>C</td>
<td>3.38</td>
<td>3.54</td>
</tr>
<tr>
<td>Extensor digitorum longus</td>
<td>C</td>
<td>3.20</td>
<td>3.40</td>
</tr>
<tr>
<td>Soleus</td>
<td>B</td>
<td>0.15</td>
<td>0.08</td>
</tr>
<tr>
<td>Extensor digitorum longus</td>
<td>B</td>
<td>0.47</td>
<td>0.57</td>
</tr>
<tr>
<td>Soleus control (−NaHCO₃)</td>
<td>B</td>
<td>-</td>
<td>0.12</td>
</tr>
<tr>
<td>Extensor digitorum longus control (−NaHCO₃)</td>
<td>B</td>
<td>-</td>
<td>0.44</td>
</tr>
</tbody>
</table>
FIG. 5.6 Lactate Production During the Continuous Spectrophotometric PEPCK Assay a) Time Course of Lactate Production by Crude Extracts of Soleus and EDL; b) Effect of Na⁺ on Lactate Production by Extract of EDL Muscle

Lactate production, in assays of PEPCK activity by method C, was measured as described in Chapter 2.

\[ \text{soleus} \quad \text{extensor digitorum longus} \]

![Graph](image-url)
NADH oxidation, previously attributable to PEPCK activity, was determined by PEPCK assay method C as described in the Materials and Methods chapter. 0.15 µmol/min at 30°C and 0.25 µmol/min at 30°C are approximate maximal activities of pyruvate kinase and lactate dehydrogenase respectively, determined in crude extracts of diaphragm, equivalent to 1 mg of muscle (see Table 5.1). Results are expressed as a percentage of the highest rate of NADH oxidation observed. Phosphoenolpyruvate was omitted from controls.

<table>
<thead>
<tr>
<th>Commercial enzyme activity added (µmol/min at 30°C)</th>
<th>% of maximum NADH oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate kinase</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>0.15</td>
<td>0.25</td>
</tr>
<tr>
<td>0.15</td>
<td>0.75</td>
</tr>
<tr>
<td>0.45</td>
<td>0.25</td>
</tr>
<tr>
<td>0.45</td>
<td>0.75</td>
</tr>
<tr>
<td>Control</td>
<td>0.15</td>
</tr>
<tr>
<td>Control</td>
<td>0.45</td>
</tr>
</tbody>
</table>
kinase activity of crude muscle extracts under the conditions
used in the assays of apparent PEPCK activity by methods B and
c (Table 5.5). K⁺, Mg²⁺ and NH₄⁺ are all known to stimulate
muscle pyruvate kinase (Bucher & Pfleiderer, 1955; Zammit et al.,
1978), but none of these ions was present in the spectrophotometric
assays for PEPCK by methods B and C. NH₄⁺ is present in the
commercial lactate dehydrogenase preparation normally used
in the pyruvate kinase assay. An aliquot of the commercial
preparation was therefore extensively dialysed against distilled
water to remove NH₄⁺. Cofactor and metal ion conditions
similar to those used in the assay of PEPCK by methods B and
C gave low pyruvate kinase activity in crude muscle extracts
compared to the maximum value obtained. Although Na⁺ was slightly
inhibitory to pyruvate kinase activity when K⁺ was present, in
the absence of K⁺, Mg²⁺ and NH₄⁺ (as in the assay of PEPCK by
methods B and C) Na⁺ stimulated muscle pyruvate kinase activity
(Table 5.5).

To summarise these studies, it can be stated that the
continuous spectrophotometric assay methods for PEPCK activity
in crude extracts of muscle principally measure NADH oxidation
due to lactate formation, and not malate formation. The crude
extracts of muscle include the enzymes pyruvate kinase and
lactate dehydrogenase. Under the conditions of PEPCK assay
by methods B and C, NADH-oxidation, previously attributed to
PEPCK activity is due to lactate production by lactate dehydrogenase
from pyruvate formed from phosphoenolpyruvate by a low
activity of pyruvate kinase. Compared with the activity of
TABLE 5.5  Pyruvate Kinase Activities of Crude Extracts of (a) Extensor Digitorum Longus and (b) Soleus Muscles Assayed Under Various Conditions

"Normal" activity denotes that the assay mixture contained metal ion, cofactor and substrate concentrations as described by Zammit et al., (1978): 160 mM-triethanolamine/HCl buffer, 10 mM-\(\text{MgCl}_2\), 80 mM-KCl, 0.17 mM-NADH (sodium salt), 5 mM-ADP (sodium salt), 2 mM-phosphoenolpyruvate (sodium salt) and 25\(\mu\)g of lactate dehydrogenase (5\(\mu\)l containing 16 \(\mu\)mol of \(\text{(NH}_4\text{)}_2\text{SO}_4\)). "Dialysed LDH" indicates that lactate dehydrogenase, which had been extensively dialysed against distilled water to remove \(\text{NH}_4^+\), replaced the normal commercial preparation of lactate dehydrogenase in \((\text{NH}_4\text{)}_2\text{SO}_4\) in the assay mixture. "PEPCK conditions" indicates that 1.54 mM-IDP replaced 5 mM-ADP, 1 mM-phosphoenolpyruvate replaced 2 mM-phosphoenolpyruvate and dialysed lactate dehydrogenase replaced lactate dehydrogenase in \((\text{NH}_4\text{)}_2\text{SO}_4\) solution in the assay mixture, and that 1 mM-MnCl\(_2\) was added but that \(\text{MgCl}_2\) and KCl were omitted. "PEPCK conditions-LDH" indicates that the assay conditions were as described for "PEPCK conditions" except that lactate dehydrogenase in \((\text{NH}_4\text{)}_2\text{SO}_4\) solution replaced the dialysed lactate dehydrogenase preparation.
(TABLE 5.5 continued)

<table>
<thead>
<tr>
<th>Assay Conditions and Additions</th>
<th>Enzyme Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>((\mu \text{mol/min per g of muscle at } 30^\circ \text{C}))</td>
</tr>
</tbody>
</table>

(a) Extensor digitorum longus

| Normal | 209.6 | 100 |
| + 17 mM NaHCO\(_3\) | 199.2 | 95.0 |
| PEPCK conditions - LDH | 17.9 | 8.5 |
| Dialysed LDH | 342.0 | 163 |
| PEPCK conditions | 0.31 | 0.15 |
| PEPCK conditions + 17 mM NaCl | 0.78 | 0.37 |
| PEPCK conditions + 50 mM NaCl | 1.35 | 0.64 |
| PEPCK conditions + 100 mM NaCl | 1.90 | 0.90 |
| PEPCK conditions + 150 mM NaCl | 2.37 | 1.13 |
| PEPCK conditions + 100 mM NaHCO\(_3\) | 2.08 | 0.99 |

(b) Soleus

| Normal | 56.3 | 100 |
| Dialysed LDH | 63.8 | 113.4 |
| PEPCK conditions | 0.05 | 0.09 |
| PEPCK conditions + 17 mM NaCl | 0.22 | 0.40 |
| PEPCK conditions + 50 mM NaCl | 0.35 | 0.62 |
| PEPCK conditions + 100 mM NaCl | 0.59 | 1.06 |
| PEPCK conditions + 150 mM NaCl | 0.48 | 0.86 |
| PEPCK conditions + 100 mM NaHCO\(_3\) | 0.51 | 0.91 |
muscle pyruvate kinase assayed under optimal conditions, Na\(^+\)-stimulated pyruvate kinase activity in the absence of K\(^+\), Mg\(^{2+}\) and NH\(_4\)\(^+\) ions reached a maximum value of 1.2 % of optimal. This is in close agreement with the findings of Kachmar & Boyer (1953), who found that although Na\(^+\) inhibited rabbit muscle pyruvate kinase in the presence of K\(^+\), Na\(^+\) stimulated pyruvate kinase activity in the absence of K\(^+\) although never to greater than 1.5 % of the maximum activity obtained.

Although the Na\(^+\) stimulation of pyruvate kinase activity is relatively small, it is nevertheless sufficient to account for the NADH oxidation previously attributed to PEPCK activity determined by methods B and C. Table 5.1 shows that in crude extracts of diaphragm, soleus and EDL muscles, apparent PEPCK activity as determined by method B was substantially less than 1 % of the corresponding pyruvate kinase maximum activity.

Na\(^+\)-stimulation of muscle pyruvate kinase also probably accounts for the previous findings that crude extracts of red muscle have lower apparent PEPCK activity than extracts of white muscle (Opie & Newsholme, 1967; McLane & Holloszy, 1979), because red muscle contains less pyruvate kinase activity than white muscle. Table 5.1 confirms that extracts of muscle display spectrophotometrically-determined apparent PEPCK activity and pyruvate kinase activity in the order: soleus < diaphragm < EDL.

The continuous spectrophotometric method for measuring PEPCK activity in crude extracts of muscle has been widely used (Opie & Newsholme, 1967; Crabtree et al., 1972; Zammit & Newsholme, 1976; 1978; Newsholme & Williams, 1978; Newsholme et al., 1979;
McLane & Holloszy, 1979; Odedra & Palmer, 1981). However, the present study indicates that the assay conditions employed largely measure Na\(^+\)-stimulated pyruvate kinase activity and demonstrates that this method is unsuitable for PEPCK activity determination in muscle preparations which have not been extensively purified to remove pyruvate kinase and lactate dehydrogenase activities, although it may still prove suitable for extracts of muscles which lack lactate dehydrogenase (Zammit & Newsholme, 1976).

An alternative continuous spectrophotometric PEPCK assay method has recently been described (Petrescu et al., 1979) in which pyruvate kinase activity is limited by employing deoxyguanosine 5'-diphosphate (dGDP) in place of GDP. This method was used to measure PEPCK activity spectrophotometrically in guinea pig liver preparations (where pyruvate kinase activity exceeds that of PEPCK by 5 to 15 fold) (Petrescu et al., 1979). Rat muscle preparations however contain much lower maximal PEPCK activities than liver and have maximal pyruvate kinase activities which exceed those of PEPCK (measured by method A) by approximately 2,500 to 10,000-fold. As dGDP still permits slight activity of rabbit muscle pyruvate kinase (Petrescu et al., 1979) a large error in muscle PEPCK activity estimation could still result using this modified spectrophotometric method.

Experiments were run to test this possibility. Pyruvate kinase activity was measured in crude extracts of diaphragm muscle. Activity measured as described by Zammit et al., (1978) was taken as 100%. Replacing 5 mM-ADP from the assay mixture
with 5 mM-IDP decreased pyruvate kinase activity to 70% whereas replacing with dGDP decreased activity to 8% of maximal. Thus the use of dGDP still permits an appreciable activity of muscle pyruvate kinase. Table 5.6 shows the effect of using dGDP in place of IDP when measuring apparent PEPCK activity in crude extracts of muscle by assay method C (Chapter 2). Although the use of dGDP substantially lowers the apparent PEPCK activity measured by the spectrophotometric assay the activities obtained were still much higher than those obtained using the $^{14}$CO$_2$-incorporation assay of PEPCK activity. The use of dGDP brought about the same percentage decrease in apparent PEPCK activity in all three muscles studied. The white muscle (EDL) still had substantially higher activity than the red muscle (soleus) suggesting that what was being measured in the crude extracts of muscle, under the conditions of PEPCK assay method C, with dGDP in place of IDP, was still pyruvate kinase activity.

From the above studies it was decided that PEPCK activity in crude extracts of muscle is best determined by the $^{14}$CO$_2$-incorporation method. As has already been mentioned, despite the relatively low activity of PEPCK in muscle, the PEPCK activity observed in rat diaphragm is more than sufficient to account for the valine stimulated increase in alanine formation observed in diaphragm incubations in vitro (Chapter 3). The proposed pathway of pyruvate formation for muscle alanine synthesis involving PEPCK also requires the participation of pyruvate kinase. Table 5.1 shows that this is present greatly in excess of the requirements for alanine synthesis.
TABLE 5.6  Effect of Substituting Deoxyguanosine 5'-diphosphate (dGDP) for Inosine 5'-diphosphate (IDP) in the Continuous Spectrophotometric Assay of Apparent PEPCK Activity of Crude Extracts of Muscle

Apparent PEPCK activity was measured by assay method C, as described in Chapter 2.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Apparent PEPCK Activity (μmol/min/g of tissue)</th>
<th>Activity in presence of dGDP expressed as a percentage of +IDP activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+IDP</td>
<td>+dGDP</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>0.66</td>
<td>0.13</td>
</tr>
<tr>
<td>Soleus</td>
<td>0.60</td>
<td>0.11</td>
</tr>
<tr>
<td>EDL</td>
<td>1.57</td>
<td>0.31</td>
</tr>
</tbody>
</table>
5.2.4 Effect of Conditions Which Cause Increased Muscle Alanine Release on the Activities of Muscle PEPCK and Malic Enzyme

It is possible that valine-derived carbon may leave the tricarboxylic acid cycle to form pyruvate for alanine synthesis either via malic enzyme or via PEPCK and pyruvate kinase. In situations where muscle alanine release and/or BCAA metabolism are increased the activity of the enzymes involved in such a pathway may undergo adaptive changes.

5.2.4.1 Effect of Starvation on the Activities of Muscle PEPCK and Malic Enzyme

During starvation muscle valine metabolism, and the requirement for the carbon of alanine synthesis de novo to be derived from valine, increase. Branched-chain aminotransferase (BCAT) activity was determined in homogenates of skeletal muscle from fed and 48 h-starved rats using both valine and leucine as substrates (Table 5.7). Starvation caused an increase in valine aminotransferase activity in both soleus \( p < 0.05 \) and EDL \( p < 0.01 \) homogenates whereas leucine aminotransferase activity was not so greatly affected. Karl et al., (1976) have also reported increased BCAT activity in preparations of muscle from 48 h-starved rats compared with muscle from fed rats. These workers, however, found that the increases in BCAT activity were similar with either leucine or valine as substrate.

The results of the present study are in close agreement with the response to starvation of transamination values obtained in a different manner with incubated hemi-diaphragm
TABLE 5.7  Effect of 48 h-Starvation on Branched-Chain
Aminotransferase Activity in Rat Skeletal Muscles

Branched-chain aminotransferase activity was measured
on crude extracts of skeletal muscle as described in Chapter 2
with leucine or valine as substrate.

Results shown are means ± S.E.M. of activity
measured in muscle homogenates from 3 fed and 3 48 h-starved rats
on 3 separate days.

Statistical differences between activities in
extracts of muscles prepared from fed and 48 h-starved rats were
calculated by Student's t-test using paired analysis for assays
performed on the same day. Differences are shown by * p < 0.05,
** p < 0.01.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Substrate</th>
<th>Nutritional State</th>
<th>Branched-chain Aminotransferase activity (μmol/min/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soleus</td>
<td>Valine</td>
<td>Fed</td>
<td>0.63 ± 0.06</td>
</tr>
<tr>
<td>Soleus</td>
<td>Valine</td>
<td>48 h-starved</td>
<td>0.88 ± 0.12 *</td>
</tr>
<tr>
<td>Soleus</td>
<td>Leucine</td>
<td>Fed</td>
<td>0.61 ± 0.09</td>
</tr>
<tr>
<td>Soleus</td>
<td>Leucine</td>
<td>48 h-starved</td>
<td>0.76 ± 0.11</td>
</tr>
<tr>
<td>EDL</td>
<td>Valine</td>
<td>Fed</td>
<td>0.53 ± 0.07</td>
</tr>
<tr>
<td>EDL</td>
<td>Valine</td>
<td>48 h-starved</td>
<td>0.78 ± 0.10 **</td>
</tr>
<tr>
<td>EDL</td>
<td>Leucine</td>
<td>Fed</td>
<td>0.56 ± 0.06</td>
</tr>
<tr>
<td>EDL</td>
<td>Leucine</td>
<td>48 h-starved</td>
<td>0.61 ± 0.06</td>
</tr>
</tbody>
</table>
preparations (Figs. 3.2 and 3.6). Valine transamination by tissue from 48 h-starved rats was higher than that from fed rats, whereas leucine transamination was the same or slightly lower in "48 h-starved" hemi-diaphragms than "fed" tissue. It has already been shown that as starvation proceeds a greater amount of valine carbon escapes losses either through complete oxidation or through release as the branched-chain 2-oxo acid (Table 3.6). Also the potential for valine-stimulated alanine release is higher in muscle from starved rats than from fed rats (Fig. 3.1). Therefore as the withdrawal of valine carbon from the tricarboxylic acid cycle was expected to increase as starvation proceeded, the activities of malic enzyme and PEPCK were studied to see whether changes occurred during starvation (Fig. 5.7). Starvation caused an increase in the activity of PEPCK whereas malic enzyme activity was decreased (diaphragm homogenates) or unchanged (EDL homogenates) by starvation.

In agreement with these findings, Karl et al., (1976) reported no difference between the malic enzyme activities of epitrochlearis muscle from fed and 48 h-starved rats, and Nolte et al., (1972) found decreases in the malic enzyme activities of rat rectus femoris and soleus muscles after 3 days of starvation in comparison with the activities found in muscles from fed controls. Newsholme & Williams (1978) also reported that rat muscle malic enzyme activity was not altered by 48 h starvation and in addition stated that muscle PEPCK activity increased substantially. However, these workers measured PEPCK activity in crude extracts of muscle by the spectrophotometric
Crude extracts of the muscles were prepared, and enzyme activities measured, as described in Chapter 2. PEPCK activity was determined by method A.

Each point represents the mean of measurements from 4 different rats. Vertical bars indicate S.E.M.

- Diaphragm
- EDL

**FIG. 5.7 Effect of Starvation on Muscle Enzyme Activities**

- PEPCK
- Malic enzyme

**Graphs:**
- PEPCK activity (units/g tissue) vs Starvation period (h)
- Malic enzyme activity (units/g) vs Starvation period (h)
method discussed previously (5.2.3) and so their PEPCK activity measurements are open to criticism.

5.2.4.2 Effect of Dietary Supplementation With Valine on Muscle PEPCK and Malic Enzyme Activities

Rats were fed for 14 days on powdered diet, (Spratts Laboratory Diet 2), supplemented with an extra 5 % by weight of L-valine. Feeding rats a diet rich in BCAAs has previously been reported to increase muscle BCAA metabolism as measured by branched-chain 2-oxo acid dehydrogenase activity (Shinnick & Harper, 1977). The valine-supplemented group showed increased alanine release by hemi-diaphragms incubated with 3 mM-valine (Fig. 5.8) when compared with those fed a normal unsupplemented diet (p < 0.01, Student's t-test). It was found in this situation of increased muscle BCAA metabolism and resulting increased alanine release, that as is the case during starvation, the activity of muscle PEPCK was increased (p < 0.05, Student's t-test), whereas that of malic enzyme remained the same or decreased.

Some rats were fed for 14 days on powdered-diet supplemented with an extra 5 % by weight of L-leucine. Hemi-diaphragms of the leucine-supplemented rats did not show increased alanine release when incubated with 3 mM-leucine when compared with those from rats fed an unsupplemented diet (2.87 ± 0.30 (4) and 3.02 ± 0.19 (4) μmole/min/g tissue respectively). However, hemi-diaphragms of the leucine-supplemented rats had significantly higher PEPCK activities (p < 0.001) 28.2 ± 0.6 (4) vs 19.7 ± 1.5 (4)
FIG. 5.8  Effect of Dietary Valine Supplementation on Diaphragm Metabolism

Rats were fed for 14 days on a normal diet supplemented with an extra 5% by weight of L-valine. NADP-dependent malic enzyme and PEPCK activities were assayed in diaphragm muscle at the end of the period, and alanine release by hemi-diaphragms incubated in a glucose-free bicarbonate medium containing 3 mM-valine was measured. Open columns are normally fed controls and hatched columns are valine-supplemented animals.

Results shown are means of at least 3 determinations ± S.E.M.
nmol/min/g tissue; and lower malic enzyme activities ($p < 0.05$): 0.84 ± 0.04 (4) vs 0.98 ± 0.03 (4) μmol/min/g tissue; than those fed a normal unsupplemented diet.

5.2.4.3 Effect of L-Triiodothyronine (L-T$_3$) Treatment on Muscle PEPCK and Malic Enzyme Activities

Carter et al., (1981) observed that isolated epitrochlaris muscle preparations from rats which had received daily injections of triiodothyronine (L-T$_3$) for 6 days released more alanine in vitro than tissue from rats injected only with vehicle. Rats were therefore treated with L-T$_3$ and the activities of muscle malic enzyme and PEPCK measured. Rats injected with L-T$_3$ showed an approximately 30% lower weight gain over the 6 day treatment period than vehicle only-injected rats (33.2 ± 1.5 g vs 45.0 ± 3.3 g, n=6 in each group). No differences were observed in malic enzyme and PEPCK activities between the groups (Table 5.8) although Carter et al., (1981) had found that the muscles of the thyroid hormone-treated group released alanine to a greater extent than the other. Nolte et al., (1972) also reported that triiodothyronine treatment was without effect on muscle malic enzyme activity but stated that the response of muscle PEPCK activity to triiodothyronine was varied, being slightly increased in red muscle but decreased in white muscle.

An explanation for the increased alanine release from muscle of L-T$_3$ treated rats may be provided by the later findings of Carter et al., (1982) that L-T$_3$ increases the rate of skeletal muscle protein breakdown. Hence the increase in alanine release
TABLE 5.8 Effect of L-Triiodothyronine (L-T<sub>3</sub>) Treatment on Muscle PEPCK and Malic Enzyme Activities

Rats were injected i.p. with 250 µg L-T<sub>3</sub>/kg body weight dissolved in 0.9 % NaCl-5 mM-NaOH, daily for 6 days. Control animals were injected over the same time period with equivalent volumes of vehicle. Results given are means ± S.E.M., number of observations in parentheses.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Malic Enzyme Activity (units/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Control</strong></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Diaphragm</td>
<td>0.97 ± 0.12 (4)</td>
</tr>
<tr>
<td>Soleus</td>
<td>0.50 ± 0.04 (4)</td>
</tr>
<tr>
<td>EDL</td>
<td>0.61 ± 0.06 (4)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Muscle</th>
<th>PEPCK Activity (munits/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Control</strong></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Diaphragm</td>
<td>19.3 ± 4.0 (3)</td>
</tr>
<tr>
<td>Soleus</td>
<td>22.1 ± 4.8 (3)</td>
</tr>
<tr>
<td>EDL</td>
<td>18.2 ± 0.6 (3)</td>
</tr>
</tbody>
</table>
was not due to an increase in alanine formation de novo and an adaptive change in PEPCK (or malic enzyme) activity might not be expected.

Karl et al., (1976) have previously found that thyroxine (T₄) pretreatment substantially stimulated alanine release by rat epitrochlaris muscle without greatly affecting glutamine release. If the effect of the thyroid-hormone was only to increase proteolysis it would have been expected that both alanine and glutamine release would have been increased to the same extent. Taken together with the finding of Karl et al., (1976) of no change in malic enzyme activity in T₄ treated rat muscle, these results suggested that PEPCK might have been involved in the formation of the increased amount of alanine released by T₄ treated muscle. However, as no change was observed in PEPCK activity in the present study a more likely explanation for the alanine release could be as follows. The increased alanine release without concomitant increased glutamine release was observed from epitrochlaris muscle preparations incubated in medium containing 5 mM-glucose and insulin (100 munits /ml). Such conditions would ensure a plentiful supply of pyruvate within the muscle preparations. The increased proteolysis induced by thyroid-hormone treatment would make available a substantial amount of amino acids stimulating transamination reactions and thus increasing the formation of alanine. Thus the substantial amounts of alanine released from the muscles of T₄ pretreated rats would be derived not only from proteolysis but also from transamination of pyruvate with amino groups.
derived from other amino acids made available by proteolysis.

5.2.5 Conclusion

The results reported in this chapter show that the activities of either PEPCK + pyruvate kinase or NADP-malic enzyme measured under optimal conditions in muscle homogenates are sufficient to account for the observed rates of valine-stimulated de novo synthesis of alanine in vitro. It is recognised that the assay of PEPCK activity was in the opposite direction to that proposed in its role for removing oxaloacetate from the tricarboxylic acid cycle (measurements in that direction were below the sensitivity of the assay method). Nevertheless, the work of Pogson & Smith, (1975) on the liver and kidney PEPCK activities suggests that the values in either direction, i.e. measurements of phosphoenolpyruvate formation or $^{14}$C-incorporation into oxaloacetate, are not greatly dissimilar.

Although the activity of NADP-malic enzyme is much greater (100-fold) than that of PEPCK, the latter enzyme showed adaptive changes that were more appropriate to a physiological role in diverting valine-derived tricarboxylic acid cycle carbon into pyruvate and alanine formation. Thus, both starvation and dietary valine supplementation, which increase BCAA metabolism and alanine formation, evoked adaptive increases in PEPCK activity but not malic enzyme activity. This circumstantial evidence favours the route of alanine formation from valine proceeding via PEPCK and pyruvate kinase rather than that via malic enzyme (Fig. 1.3) in agreement with the conclusions reached from
experiments using inhibitors of those routes described in the
previous chapter (Chapter 4).
CHAPTER SIX

SUBCELLULAR DISTRIBUTION OF ENZYMES INVOLVED IN BRANCHED-CHAIN AMINO ACID METABOLISM AND ALANINE FORMATION IN MUSCLE
6.1 INTRODUCTION

As stated in the discussion of Chapter 4, in order to further elucidate the suggested pathway of alanine formation from BCAAs by rat muscle preparations, some information was required concerning the subcellular distribution of the enzymes proposed to be involved in the pathway. The enzymes studied included branched-chain aminotransferase, alanine aminotransferase, aspartate aminotransferase and PEPCK. It was of particular importance to establish the location of branched-chain aminotransferase within the muscle cell as published reports on its subcellular location provide conflicting conclusions. Odessey & Goldberg (1979) reported the enzyme to be mainly cytosolic (approximately 70%), Knox's group (Cappuccino et al, 1978; Kadowaki & Knox, 1982), found it was 45% cytosolic, whereas Ichihara et al (1975) stated that only 29% of muscle branched-chain aminotransferase activity was located in the cytosol.

6.2 RESULTS AND DISCUSSION

Several methods were employed for the subcellular fractionation of muscle tissue from fed rats, as described in Chapter 2. Initially, method 1 was used and the distribution of the activities of branched-chain aminotransferase and succinate dehydrogenase in fractions obtained from a mixture
of soleus and EDL muscles is shown in Fig. 6.1. Succinate dehydrogenase was employed as a mitochondrial "marker" enzyme as its location within the cell is assumed to be solely within the mitochondrion (Greville, 1969). Fig. 6.1 shows that the majority (63%) of the branched-chain aminotransferase activity (determined with leucine as substrate) was located in the soluble (cytosol) fraction and that the activity of the mitochondrial marker, succinate dehydrogenase, was negligible in this fraction. A certain amount of leucine aminotransferase activity (28%) was detected in fractions with substantial succinate dehydrogenase activity. From the distributions of the two enzyme activities among the particulate fractions it was assumed that the non-cytosolic leucine aminotransferase activity was mitochondrial. In case the apparent dual location of branched-chain aminotransferase was due to different intracellular locations of the enzyme in the two different muscle types, the subcellular distribution of branched-chain aminotransferase was determined separately, in soleus alone and EDL alone (Fig. 6.2). Differences were detected between the muscles in that whole homogenates of soleus muscle exhibited higher aminotransferase activity than EDL homogenates with both valine and leucine as substrates. However, the subcellular distributions of the enzyme activities were found to be very similar in both muscles and agree with the results of Odessey & Goldberg (1979), who found that 70 - 80% of the branched-chain aminotransferase activity of rat hindlimb muscles was recovered in the cytosol fraction of the cell, and the remaining
Subcellular fractionation was carried out by method 1 as described in Chapter 2. Values shown are representative of 3 fractionations.

N = nuclear 800 g x 10 min pellet suspension
MT = mitochondrial 20,000 g x 10 min pellet suspension
MC = microsomal 100,000 g x 60 min pellet suspension
C = cytosol 100,000 g x 60 min supernatant
Subcellular fractions prepared according to fractionation method 1. Figures in columns represent % of whole homogenate activity. Columns and bars show the mean and range of 2 determinations.

BCAT = branched-chain aminotransferase; EDL = extensor digitorum longus.

H = homogenate; C = cytosol; M = mitochondrial
amount in the mitochondrial fraction.

Using the same fractionation method as that used to determine the location of branched-chain aminotransferase activity within the muscle cell (method 1), the subcellular distributions of muscle PEPCK and "malic enzyme" (NADP-dependent malate dehydrogenase) were measured in rat diaphragm muscle and in the previously-used mixture of red and white leg muscles. Table 6.1 shows the results obtained. 75 - 80% of the recovered PEPCK activity was found in the 100,000 g cytosol fraction with a small percentage (9 - 12%) recovered in the mitochondrial fraction. A similar distribution was seen for malic enzyme activity with, in diaphragm, 86% being found in the cytosolic fraction and 9% recovered in the mitochondrial fraction. It had already been reported by Nolte et al (1972) that in red muscle about 70% of the total activity of both enzymes was located in the mitochondria, whereas in white muscle up to 70% of the total activity of both enzymes was found in the cytosol. It therefore appeared surprising that in our muscle preparations, containing both red and white muscles, such a large proportion of the total activity was located in the cytosolic fraction.

A possible reason for the observed difference between the results of this study and those of Nolte et al (1972) could be deduced from the use of succinate dehydrogenase as the mitochondrial marker enzyme in my study (Table 6.1). Succinate dehydrogenase is located within the inner mitochondrial membrane and is found in the membrane fractions obtained on
TABLE 6.1 Subcellular Distribution of PEPCK, NADP-Dependent Malic Enzyme, Glutamate Dehydrogenase, and Succinate Dehydrogenase Activities in Muscle

Subcellular fractions prepared according to fractionation method 1. Values shown are means of 2 determinations. N.M. = not measured.

A) In Diaphragm

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme Activity in Subcellular Fraction (μmol/min/g tissue)</th>
<th>% of whole homogenate activity in parentheses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole homogenate</td>
<td>800 g pellet (nuclear)</td>
</tr>
<tr>
<td>PEPCK</td>
<td>0.0163 (100)</td>
<td>N.M.</td>
</tr>
<tr>
<td>Malic enzyme</td>
<td>1.57 (100)</td>
<td>N.M.</td>
</tr>
<tr>
<td>Glutamate dehydrogenase</td>
<td>1.96 (100)</td>
<td>0.11 (6)</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>5.13 (100)</td>
<td>0.92 (18)</td>
</tr>
</tbody>
</table>
### TABLE 6.1 (continued)

#### B) Mixture Of Soleus and EDL - PEPCK Activity

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity (µmol/min/g tissue)</th>
<th>% of Whole Homogenate activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole homogenate</td>
<td>0.0184</td>
<td>100</td>
</tr>
<tr>
<td>20,000 g pellet (mitochondrial)</td>
<td>0.0022</td>
<td>12</td>
</tr>
<tr>
<td>100,000 g pellet (microsomal)</td>
<td>0.0005</td>
<td>3</td>
</tr>
<tr>
<td>100,000 g supernatant (cytosol)</td>
<td>0.0138</td>
<td>75</td>
</tr>
</tbody>
</table>
further fractionation of disrupted mitochondria (De Haan et al., 1973). Damage to the mitochondria during homogenisation or centrifugation would therefore still result in succinate dehydrogenase activity being centrifuged down in the "mitochondrial pellet" whereas mitochondrial enzymes which are not membrane-bound would be freed into the soluble fractions. To test the possibility of mitochondrial damage during the fractionation procedure, a more suitable mitochondrial marker enzyme was employed, which is believed to be located solely within the mitochondrial matrix, glutamate dehydrogenase (Pette, 1966).

Table 6.1 shows the subcellular distribution of succinate dehydrogenase and glutamate dehydrogenase activities obtained from fractions prepared by method 1. Although the membrane-bound mitochondrial enzyme activity (succinate dehydrogenase) was principally recovered in the 20,000 g pellet (i.e. the designated mitochondrial fraction), the majority of the glutamate dehydrogenase activity was found in the 100,000 g supernatant. As these results indicated the presence of damaged mitochondria, other published schemes for the subcellular fractionation of muscle were applied. These schemes (methods 1 - 4) were described in Chapter 2. Methods 1 - 4 differed with respect to the centrifugation speeds employed and the composition of the homogenising medium. The initial low speed centrifugation employed to sediment unbroken cells, nuclei and cell debris, differed in all 4 methods [Crabtree et al., (1972) 800 g x 10 min; Odessey & Goldberg,
(1979) 800 g x 5 min; Ernster & Nordenbrand, (1967) 600 g x 7 min; Surholt & Newsholme, (1981) 600 g x 5 min]. The supernatants resulting from this initial spin were therefore contaminated with cell debris and nuclei to varying degrees. In addition the amount of mitochondria sedimented in the initial pellet varied between methods.

Similarly, differing amounts of mitochondria were sedimented by the second stage of centrifugation using the four different methods [Crabtree et al, (1972) 20,000 g x 10 min; Odessey & Goldberg, (1979) 10,000 g x 10 min; Ernster & Nordenbrand, (1967) 14,000 g x 10 min; Surholt & Newsholme, (1981) 5,000 g x 20 min].

The composition of the homogenising media differed in methods 1 - 4 (see Chapter 2). All, however, contained EDTA or EGTA. Mitochondria of poor quality (poor phosphorylating efficiency) would otherwise be obtained due to the relatively high content of Ca$^{2+}$ in muscle tissue which absorbs to the mitochondria during homogenisation (Ernster & Nordenbrand, 1967).

It has been reported that skeletal muscle mitochondrial preparations made in sucrose medium may have very low yields of mitochondria (Ernster & Nordenbrand, 1967). The problem of yield arises from the fact that skeletal muscle, when homogenised in a non-electrolyte medium, often assumes a gelatinous consistency which makes it difficult to obtain a sufficient disintegration of the myofibrils. Thus the method of Odessey and Goldberg (1979) may suffer from this problem. The non-sucrose containing medium used in method 3 (Ernster & Nordenbrand, 1967) was
designed by Chappell & Perry (1954) so that the medium maintained the myofibrils in such a physical state that homogenisation and subsequent differential centrifugation could easily be performed with the resulting mitochondrial preparation theoretically being satisfactory both quantitatively and qualitatively.

The homogenising medium used in method 4 (Surholt & Newsholme, 1981) differed from that employed in the other methods as it included 0.1% (w/v) bovine serum albumin (BSA) in an attempt to produce mitochondria of good quality. BSA is an inert protein which acts to stabilise the mitochondria (Davis, 1967) possibly by binding "toxic" substances released when the cell is disrupted ("toxic" to oxidative phosphorylation), such as long-chain fatty acids, which are potent uncoupling agents (Pressman & Lardy, 1956).

Application of fractionation method 2, as described by Odessey & Goldberg (1979), revealed only a small amount of glutamate dehydrogenase activity associated with the "mitochondrial pellet" with by far the majority of the activity being obtained in the cytosolic fraction (Table 6.2).

Odessey & Goldberg (1979) used succinate dehydrogenase as their only mitochondrial marker enzyme. In view of the present findings of damaged mitochondria, and subsequent leakage of mitochondrial enzyme activities into the soluble fraction obtained using their muscle fractionation scheme, some doubt must be placed on their interpretation of the subcellular distribution of branched-chain aminotransferase.
### TABLE 6.2 Subcellular Distribution of Glutamate Dehydrogenase Activity in Diaphragm Muscle

Subcellular fractions prepared according to fractionation method 2 (Odessey & Goldberg, 1979).

Values are the mean of 2 determinations.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity (µmol/min/g tissue)</th>
<th>% of Whole homogenate activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole homogenate</td>
<td>3.18</td>
<td></td>
</tr>
<tr>
<td>800 g pellet (nuclear)</td>
<td>0.90</td>
<td>28</td>
</tr>
<tr>
<td>10,000 g pellet (mitochondrial)</td>
<td>0.40</td>
<td>13</td>
</tr>
<tr>
<td>100,000 g pellet (microsomal)</td>
<td>0.27</td>
<td>8</td>
</tr>
<tr>
<td>100,000 g supernatant (cytosol)</td>
<td>2.36</td>
<td>74</td>
</tr>
</tbody>
</table>
Method 3, as described by Ernster & Nordenbrand (1977), was followed. Table 6.3 shows that the activity of muscle glutamate dehydrogenase was recovered mainly in the "cytosolic" fraction, again indicating damage to the mitochondria to such an extent as to make estimates of the subcellular distribution of enzyme activities practically impossible by this method.

Method 4, as described by Surholt & Newsholme (1981), was also applied. Although the damage to the mitochondria was not as great using this method for subcellular fractionation (Table 6.4) as in the previous methods, substantial glutamate dehydrogenase activity was still recovered in the "cytosolic" fraction. A similar pattern was obtained for citrate synthase, which is also located in the mitochondrial matrix (Pette, 1966).

The main cause of the damage to mitochondria seemed to occur on homogenising muscle. Being a tough tissue, large shear forces were required to produce "good" muscle homogenates but this also caused damage to the mitochondria. In view of the amount of damage caused to muscle mitochondria when following the various subcellular fractionation methods, it became apparent that trying to identify the subcellular location of enzyme activities by using these methods which depended on homogenisation followed by differential centrifugation, was impractical. Consequently another approach was made to the problem and an attempt at locating enzyme activities within the muscle cell was made using the fractional extraction procedure described for "difficult" tissues by Taylor et al., (1978), and pioneered by Pette, (1966). The principle of this
### TABLE 6.3 Subcellular Distribution of Glutamate Dehydrogenase and Succinate Dehydrogenase Activities in Diaphragm Muscle

Subcellular fractions prepared according to fractionation method 3 (Ernster & Nordenbrand, 1967).

Values are means of 2 determinations. N.D. = no detectable activity.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Glutamate Dehydrogenase</th>
<th>Succinate Dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity (μmol/min/g tissue)</td>
<td>% of whole homogenate activity</td>
</tr>
<tr>
<td>Whole homogenate</td>
<td>2.19</td>
<td></td>
</tr>
<tr>
<td>600 g pellet (nuclear)</td>
<td>0.47</td>
<td>21</td>
</tr>
<tr>
<td>14,000 g pellet (mitochondrial)</td>
<td>0.48</td>
<td>22</td>
</tr>
<tr>
<td>100,000 g pellet (microsomal)</td>
<td>0.13</td>
<td>6</td>
</tr>
<tr>
<td>100,000 g supernatant (cytosol)</td>
<td>1.23</td>
<td>56</td>
</tr>
</tbody>
</table>
### TABLE 6.4 Subcellular Distribution of Glutamate Dehydrogenase and Citrate Synthase Activities in Diaphragm Muscle

Subcellular fractions prepared according to fractionation method 4 (Surholt & Newsholme, 1981).

Values are the means of 2 determinations.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Glutamate Dehydrogenase</th>
<th>Citrate Synthase</th>
<th>Citrate Synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity (µmol/min/g tissue)</td>
<td>% of whole homogenate activity</td>
<td>Activity (µmol/min/g tissue)</td>
</tr>
<tr>
<td>Whole homogenate</td>
<td>2.22</td>
<td></td>
<td>37.8</td>
</tr>
<tr>
<td>600 g pellet (nuclear)</td>
<td>1.06</td>
<td>48</td>
<td>19.6</td>
</tr>
<tr>
<td>5,000 g pellet (mitochondrial)</td>
<td>0.69</td>
<td>31</td>
<td>10.6</td>
</tr>
<tr>
<td>100,000 g pellet (microsomal)</td>
<td>0.13</td>
<td>6</td>
<td>1.1</td>
</tr>
<tr>
<td>100,000 g supernatant (cytosol)</td>
<td>0.54</td>
<td>24</td>
<td>13.9</td>
</tr>
</tbody>
</table>
procedure consists of submitting a tissue sample to repeated extractions with buffer solutions which, by means of increasing osmotic and mechanical aggressiveness, disclose successively different cellular compartments (Pette, 1966). The modification of this method used here primarily achieves the separation of extra- and intra-mitochondrial enzymes.

Pyruvate kinase and LDH activities were assayed as cytosolic markers, whereas glutamate dehydrogenase and citrate synthase activities were measured as mitochondrial enzyme markers. The percentage of total activity of citrate synthase and glutamate dehydrogenase found after the first extraction, was a measure of the degree of damage to mitochondria during the relatively mild homogenisation and first extraction. As this percentage varied from experiment to experiment no attempt was made to pool results from individual experiments for statistical analysis. Instead, the results of individual experiments using the fractional extraction technique are shown in Fig. 6.3, and the total tissue activities of the various enzymes studied (assayed on homogenates prepared by ground-glass homogenisation of tissue in deoxycholate-containing buffer) are shown in Table 6.5. Enzyme activities shown in the fractional extraction profiles were expressed as the percentage of the sum of the enzyme activities found in the five fractions i.e. three extractions in KCl-based medium, one extraction in phosphate buffer and the homogenate of the sediment from the phosphate extraction. Recoveries of enzyme activities in the five fractions obtained by this technique were always 80 - 110%
FIG. 6.3 Fractional Extraction of Extra- and Intra-Mitochondrial Enzyme Activities in Rat Diaphragm Muscle. The Percentage of the Total Cellular Activity Extracted at Different Steps is Plotted Against the Time Course of the Extraction

Enzyme activities shown in the fractional extraction profiles are expressed as percentages of the sum of the enzyme activities found in the five fractions i.e. three extraction in KCl-based medium, one extraction in phosphate buffer and the homogenate of the sediment from the phosphate extraction.

The results of 3 extractions are shown overleaf.

AlaAT = Alanine aminotransferase
AspAT = Aspartate aminotransferase
BCAT = Branched-chain aminotransferase
CS = Citrate synthase
GDH = Glutamate dehydrogenase
LDH = Lactate dehydrogenase
PEPCK = Phosphoenolpyruvate carboxykinase
PK = Pyruvate kinase
FIG. 6.3

% of Total Activity Extracted

Extraction time (min)

Extraction time (min)
**TABLE 6.5 Whole Tissue Activities of Enzymes Studied During Fractional Extraction of Diaphragm Muscle**

Whole tissue activities were assayed on homogenates of diaphragm prepared by ground-glass homogenisation of tissue in deoxycholate-containing buffer. Values represent the means of measurements from different rats and are given ± S.E.M. with the number of observations in parentheses.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity (μmol/min/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate dehydrogenase</td>
<td>319 ± 29 (3)</td>
</tr>
<tr>
<td>Pyruvate Kinase</td>
<td>217 ± 2 (3)</td>
</tr>
<tr>
<td>Citrate Synthase</td>
<td>36.3 ± 2.2 (3)</td>
</tr>
<tr>
<td>Glutamate Dehydrogenase</td>
<td>3.54 ± 0.41 (3)</td>
</tr>
<tr>
<td>Alanine Aminotransferase</td>
<td>5.86 ± 0.41 (3)</td>
</tr>
<tr>
<td>Aspartate Aminotransferase</td>
<td>178 ± 10 (3)</td>
</tr>
<tr>
<td>Branched-chain Aminotransferase</td>
<td>1.15 ± 0.12 (3)</td>
</tr>
<tr>
<td>Phosphoenolpyruvate Carboxykinase</td>
<td>0.033 ± 0.005 (3)</td>
</tr>
</tbody>
</table>
of the enzyme activities of the minced tissue homogenised in deoxycholate-containing buffer.

From the fractional profiles obtained (Fig. 6.3), branched-chain aminotransferase was found to be extracted with the mitochondrial matrix markers, citrate synthase and glutamate dehydrogenase. This suggested a mitochondrial location for the bulk of the branched-chain aminotransferase activity in contrast to what had been inferred from the differential centrifugation studies (Table 6.1) and by Odessey and Goldberg, (1979).

On this basis, PEPCK, aspartate aminotransferase and alanine aminotransferase activities were all located in both the cytosol and mitochondrial fractions. However, some idea of the relative distribution of these activities between the two compartments can be obtained. In extracts of diaphragm, higher percentages of aspartate aminotransferase and PEPCK activities were located in the mitochondrial fraction than alanine aminotransferase, and aspartate aminotransferase and PEPCK activities were slightly more mitochondrial than cytosolic. Alanine aminotransferase activity on the other hand, was located to a greater extent in the cytosol fraction of the muscle cell than in the mitochondrial fraction.

Unfortunately, malic enzyme activity could not be assayed in the fractions obtained by this technique. It appeared that a component of the fractionation medium interfered with the malic enzyme assay and so no information concerning the subcellular location of malic enzyme could be obtained. However,
studies by other workers have shown that NADP-specific malic enzyme is located in both the mitochondrial (Swierczynski, 1980) and cytosolic (Swierczynski et al., 1980) compartments.

The finding that branched-chain aminotransferase activity is predominantly located in the mitochondrial fraction is in contrast to most other reports of its intracellular location. These reports can be criticised, as subcellular fractionation methods have been employed which, in my experience, cause varying amounts of damage to mitochondria, allowing enzyme activities to leak into the soluble fractions. Odessey & Goldberg (1979), who reported a predominantly cytosolic location for branched-chain aminotransferase, used the membrane-bound succinate dehydrogenase as their mitochondrial marker, and so would not have detected the damage caused to mitochondria by their fractionation procedure. Kadowaki & Knox (1982), although finding a partial mitochondrial location, prepared skeletal muscle mitochondria by the method of Ernster & Nordenbrand (1967) which in the present study was found to result in mitochondrial damage. No mention was made in that study of marker enzymes for subcellular fractions of skeletal muscle.

Cappuccino et al. (1978), who reported a similar partially mitochondrial location, obtained their values for soluble and particulate activities by measuring activity in whole homogenates, centrifuging the homogenate at 30,000 g for 30 min, assaying the supernatant for enzyme activity and denoting this the cytosolic activity. The particulate activity was obtained
from the difference between the cytosolic and whole homogenate activities and therefore assumed a total of 100% recovery of branched-chain aminotransferase activity between the particulate and cytosol fractions. It was also assumed that no damage to the subcellular particles occurred during the fractionation procedure although no mitochondrial marker enzymes were employed to establish this.

The findings of Ichihara et al (1975) that muscle branched-chain aminotransferase activity was 70% mitochondrial come closest to the findings of the present study. The present finding of a predominantly mitochondrial location for branched-chain aminotransferase would suggest that the pathway of alanine formation from valine in skeletal muscle was as proposed in a previous chapter (Fig. 4.8a).

Using the present fractional extraction of muscle experiments, alanine aminotransferase was shown to be mainly cytosolic, in agreement with the previous findings of De Rosa & Swick, (1975). This finding and the observation that aspartate aminotransferase activity was associated with both the cytosolic and the mitochondrial fractions provide further support for the proposed pathway (Fig. 4.8a).

However, the intracellular location of PEPCK activity observed did not fit so neatly into the scheme as suggested. PEPCK activity was found associated with the cytosol and the mitochondria. The scheme of alanine formation from valine assumed a solely cytosolic location for PEPCK, thus enabling valine-derived oxaloacetate, exported from mitochondria in the
form of aspartate, to be converted to PEP and from there to pyruvate and then alanine. The present finding of substantial mitochondrial PEPCK activity in skeletal muscle was however, not altogether unexpected. As mentioned earlier, Nolte et al (1972) have previously reported that PEPCK activity of red muscle was approximately 70% mitochondrial. Although PEPCK activity was found to be associated with both the cytosolic and the mitochondrial fractions, this does not necessarily mean that the pathway for conversion of valine carbon to alanine cannot proceed as previously proposed. It is difficult to propose a pathway for the formation of alanine from valine which involves mitochondrial PEPCK and adheres to the properties already discovered for the pathway. For example, it was found using the aspartate aminotransferase inhibitor, AMB, that in muscle from 48 h-fasted rat, blocking aspartate aminotransferase activity inhibited the formation of alanine (Chapter 4). A pathway involving mitochondrial PEPCK would require the export of PEP from the mitochondria following oxaloacetate decarboxylation and would not therefore require aspartate aminotransferase. Therefore it is probable that a substantial proportion of the decarboxylation of valine-derived oxaloacetate occurs in the cytosol. The function of mitochondrial PEPCK activity with regard to valine metabolism could possibly be explained by the following suggestion which concerns the cofactor requirement of the PEPCK reaction. When oxaloacetate is decarboxylated to PEP by PEPCK, GTP is converted to GDP. The enzyme succinate thiokinase, which converts succinyl-CoA to succinate and is
the point of entry of valine carbon into the tricarboxylic acid cycle, also uses those factors, converting GDP to GTP with succinate formation. As high GTP/GDP ratios would inhibit the thiokinase reaction, the conversion of oxaloacetate to PEP, and hence GTP to GDP by mitochondrial PEPCK, would lower the GTP/GDP ratio and potentially increase the rate of entry of valine-derived carbon into the tricarboxylic acid cycle intermediates pool.

There are, however, other ways in which mitochondrial PEPCK activity may be involved with muscle alanine production and these will be discussed in Chapter 8.
CHAPTER SEVEN

INCORPORATION OF $^{14}$C-LABEL FROM [U-$^{14}$C] BRANCHED-CHAIN AMINO ACIDS INTO OTHER AMINO ACIDS IN MUSCLE TISSUE IN VITRO
7.1 INTRODUCTION

Branched-chain amino acids have been observed to stimulate the de novo synthesis of alanine by muscle tissue preparations. Evidence has been presented that supports a pathway for branched-chain amino acid-stimulated alanine formation that involves the withdrawal of carbon from the tricarboxylic acid cycle and decarboxylation via the enzyme PEPCK to yield 3-carbon precursors (Chapters 4 & 5). Such a pathway is consistent with the proposal that valine itself is a source of the carbon skeleton of the alanine synthesised de novo. The experiments described in this chapter were performed to test the above proposal by measuring the incorporation of $^{14}$C from the $[U-{^{14}}C]$ BCAAs into the carbon skeletons of amino acids which were subsequently released by muscle tissue.

Hemi-diaphragm preparations were incubated in the presence of 3 mM-L-$[U-{^{14}}C]$-valine or L-$[U-{^{14}}C]$-leucine. The tissues were incubated for 2 h after which time the amino acids present in the incubation medium were isolated by ion-exchange chromatography and separated and identified by thin-layer electrophoresis (TLE) and thin layer chromatography (TLC). Radioactivity that was found to be associated with particular amino acids was measured. Of particular interest was the extent to which $^{14}$C from $[U-{^{14}}C]$-valine was incorporated into alanine which was released by the hemi-diaphragm preparations.
The following experiments were conducted to investigate the route of valine-stimulated alanine formation. Using paired tissue incubations, the ratio of $^{14}\text{C}\text{O}_2$ produced from [U-$^{14}\text{C}$]-labelled amino acids to $^{14}\text{C}\text{O}_2$ produced from [1-$^{14}\text{C}$]-labelled amino acids (when both [U-$^{14}\text{C}$] and [1-$^{14}\text{C}$] are initially present at the same specific radioactivity) yields information on the extent to which the amino acid skeleton is oxidised (see Chapter 3). If the metabolism of valine were consistent with it being a precursor for the carbon skeleton of alanine, valine would be oxidised, according to the proposed pathway, via PEPCK. In this case, the addition of the PEPCK inhibitor, 3-mercaptopicolinate (3MP) to incubations containing either [1-$^{14}\text{C}$] or [U-$^{14}\text{C}$]-valine would be expected to lower the ratio of $^{14}\text{C}\text{O}_2$ produced from [U-$^{14}\text{C}$]-valine relative to [1-$^{14}\text{C}$]-valine. The PEPCK-catalysed decarboxylation and hence some of the $^{14}\text{C}\text{O}_2$ produced from [U-$^{14}\text{C}$]-valine would be inhibited.

Experiments were carried out with paired hemi-diaphragm preparations from 48 h-starved rats in which valine-stimulated de novo alanine synthesis has been shown to involve PEPCK (Chapter 4). The ratios of $^{14}\text{C}\text{O}_2$ produced from [U-$^{14}\text{C}$]-valine to that produced from [1-$^{14}\text{C}$]-valine in paired incubations were compared with ratios obtained in similar incubations containing 1 mM-3MP. Similar experiments were also carried out using the malic enzyme inhibitor, hydroxymalonate, with the intention of comparing these with the 3MP experiments to evaluate further the proposal that valine metabolism, and conversion to alanine, does not occur via malic enzyme, but proceeds via PEPCK.
7.2 RESULTS AND DISCUSSION

7.2.1 Effect of Metabolic Inhibitors on Valine Metabolism

In Vitro

The effect of 1 mM-3MP on the ratio of $^{14}$CO$_2$ released from L-[U-$^{14}$C]-valine to L-[1-$^{14}$C]-valine is shown in Table 7.1. No decrease in the ratio of $^{14}$CO$_2$ released from incubations in the presence of L-[U-$^{14}$C]-valine to those incubated with L-[1-$^{14}$C]-valine was found when tissue preparations were incubated in the presence of the PEPCK inhibitor. This result suggests that PEPCK is not involved in the metabolism and oxidation of valine by muscle. In addition, no change in the [U-$^{14}$C]-valine-derived to [1-$^{14}$C]-valine-derived $^{14}$CO$_2$ production ratio was observed when hemi-diaphragms were incubated in the presence of the malic enzyme inhibitor, hydroxymalonate. Thus neither PEPCK nor malic enzyme appears to be involved, to any great extent, in the further metabolism of the carbon skeleton derived from valine. However, it is possible that, when the oxidative pathway via PEPCK is inhibited, the valine-derived carbon is removed from the tricarboxylic acid cycle via malic enzyme. Similarly, when malic enzyme is inhibited, metabolism might proceed via PEPCK. Assuming that valine is catabolised as is conventionally accepted, (Meister, 1965; Rodwell, 1969), the predicted lowering of the $^{14}$CO$_2$ release ratio from [U-$^{14}$C] to [1-$^{14}$C]-valine can not have been countered by any other diversion e.g. of the "blocked" carbon into the tricarboxylic acid cycle with subsequent loss of $^{14}$CO$_2$. The "blocked" carbon skeleton could not be oxidised
**TABLE 7.1**  Effect of 1 mM-3-Mercaptopicolinate and 2 mM-Hydroxymalonate on the Metabolism of 3 mM-L-[U-\(^{14}\)C]-Valine and 3 mM-L-[\(^{1-14}\)C]-Valine by Hemi-Diaphragm Preparations from 48 h-Starved Rats

<table>
<thead>
<tr>
<th>Addition to Incubation Medium</th>
<th>Oxidation to (^{14})CO(_2) ((\mu)mol/2 h/g tissue)</th>
<th>Ratio of (^{14})CO(_2) Release</th>
<th>3-Methyl-2-oxobutanoate released ((\mu)mol/2 h/g tissue)</th>
<th>Transamination of 3 mM-Valine ((\mu)mol/2 h/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mM-[U-(^{14})C]-Valine</td>
<td>2.37 ± 0.20</td>
<td>0.53 ± 0.03</td>
<td>0.51 ± 0.15</td>
<td>5.04 ± 0.43</td>
</tr>
<tr>
<td>3 mM-[1-(^{14})C]-Valine</td>
<td>4.52 ± 0.50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2.46 ± 0.21</td>
<td>0.58 ± 0.03</td>
<td>0.65 ± 0.02</td>
<td>4.83 ± 0.14</td>
</tr>
<tr>
<td>1 mM-3-Mercaptopicolinate</td>
<td>2.46 ± 0.21</td>
<td>0.58 ± 0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 mM-hydroxymalonate</td>
<td>3.10 ± 0.63</td>
<td>0.55 ± 0.05</td>
<td>0.42 ± 0.03</td>
<td>5.91 ± 0.59</td>
</tr>
</tbody>
</table>
directly by the cycle. As the cycle oxidises only the carbon skeleton of acetyl-CoA, the "blocked" carbon would first require to be converted into pyruvate and then into acetyl-CoA (Goldstein & Newsholme, 1976). Thus it might appear that, when PEPCK is inhibited, pyruvate is produced from the carbon skeleton via malic enzyme and so no change in the $^{14}$CO$_2$ production ratio could be observed. This explanation for the lack of change in the $^{14}$CO$_2$ production ratio is considered unlikely as it has previously been shown that the net valine-stimulated alanine production is inhibited to a much greater extent by the PEPCK inhibitor than by the malic enzyme inhibitor. If the suggested routes operated as alternatives for removing carbon from the tricarboxylic acid cycle then the presence of either $^{3}$MP or hydroxymalonate would have had no effect on valine-stimulated alanine production.

Thus this experiment casts some doubt on the proposed pathway of valine carbon metabolism to alanine via PEPCK in muscle.

7.2.2 Incorporation of $^{14}$C-Label from [U-$^{14}$C]-Branched-Chain Amino Acids Into Other Amino Acids Released by Muscle In Vitro

It has previously been shown that the carbon skeletons of branched-chain amino acids are only partially oxidised by skeletal muscle (Chapter 3) and thus make available to the muscle carbon which can possibly be utilised for the formation de novo of compounds, such as alanine, which are subsequently
released by muscle. In this set of experiments the incorporation of $^{14}C$-label from [U-$^{14}C$]-valine and [U-$^{14}C$]-leucine into other amino acids released by hemi-diaphragm muscle in vitro was studied. Muscle may be able to form alanine from valine carbon by a pathway involving PEPCK and so one of each pair of hemi-diaphragms was incubated in the presence of 1 mM-3MP (an inhibitor of PEPCK) to determine whether this would decrease the incorporation of $^{14}C$ into alanine. Hemi-diaphragm preparations were incubated in the presence of valine or leucine at an initial specific radioactivity of 167 mCi. mole$^{-1}$. As each incubation was performed with an initial [BCAA] of 3 mM in a 2 ml volume, each hemi-diaphragm was therefore incubated in the presence of 6 μmol BCAA and thus the radioactivity in each flask was 1 μCi. (2.2 x $10^6$ d.p.m.) at the start of incubation. The relatively high specific activity of BCAA employed was required because it was calculated, assuming a 100% recovery of radioactivity at each stage involved in the processing of incubation medium for amino acid separation i.e. after acidification, neutralisation, ion-exchange chromatography, freeze-drying and taking the residue up in solution again, that only about 1/15 of the original amount of radioactivity could be applied to the thin-layer plates for TLE and TLC. The main reason for this was that although the minimum volume of H$_2$O which could be used to reproducibly take up the residue obtained after freeze-drying was 200 μl, no more than 20 μl of sample could be applied to the TLC plate for separation of the amino acids.

The actual recoveries of radioactivity at different
stages of sample preparation were calculated. For example, recovery up to the point of applying sample onto the TLC plate was found to be approximately 68% for valine (48 h-starved rat hemi-diaphragm). This, of course, was partly because some radioactivity associated with compounds other than amino acids would be removed by the ion-exchange chromatography. \([U-^{14}C]3\)-methyl-2-oxobutyrate, for example, was removed from the sample at this stage. The subsequent recovery of radioactivity from the plates in the form of the amino acids being investigated was between 73 and 82% (3 observations) of that applied.

Thus, a figure of only about 1/30 of the original amount of radioactivity was expected to be recovered from the plate i.e. approximately 75,000 d.p.m., and from this the incorporation of \(^{14}\)C-label into other amino acids was calculated. No more sample could be applied to the plate because problems in separation arose when the plate was loaded with sample volumes > 20μl. As it was not practical to measure the actual amounts of each amino acid present in the incubation medium at the end of the incubation period (as explained above, the medium was required to ensure that radioactivity applied to the plate was as high as possible), actual measurements of radiolabelling of amino acids could not be expressed on a d.p.m./μmol basis. Therefore the amounts of radioactivity associated with individual amino acids were expressed as percentages of the sum of the radioactivities associated with the amino acids studied.

The rates of oxidation of 3 mM-[\(^{14}\)C]-leucine and 3 mM-[\(^{14}\)C]-valine observed in these experiments were similar to
those described previously (Chapter 3). For example, 3 mM-[U-\(^{14}\)C]-leucine was oxidised at a rate of 2.40 ± 0.03 (3) and 2.55 ± 0.15 (3) µmol/2 h/g tissue, in the absence and presence of 1 mM-3MP respectively, by hemi-diaphragms from 48 h-starved rats. 3 mM-[U-\(^{14}\)C]-valine was oxidised at a rate of 1.68 ± 0.13 (3) and 1.70 ± 0.12 (3) µmol/2 h/g tissue in the absence and presence of 1 mM-3MP respectively, by hemi-diaphragms from 48 h-starved rats.

The incorporation of \(^{14}\)C-label from [U-\(^{14}\)C]-leucine into other amino acids which were released by hemi-diaphragm preparations is shown in Table 7.2. Incorporation was mainly into glutamine and glutamate in both the fed and 48 h-starved states. This is in agreement with the findings of Hutson & Zapalowski (1981) who studied the incorporation of \(^{14}\)C-label from [U-\(^{14}\)C]-leucine into the perfused hindquarter free dispensable amino acid pool of fed and 3-day starved rats. Similarly Spydevold (1976) found substantially more \(^{14}\)C-label from [U-\(^{14}\)C]-leucine was incorporated into glutamate than into alanine and aspartate released by perfused hind-limbs of fasted rats. In the present study, much more label appeared in glutamine than in glutamate in the fasted state, whereas in the fed state more label was found in glutamate than glutamine. The presence of 1 mM-3MP appeared to cause an increase in the percentage of \(^{14}\)C appearing in glutamine, glutamate and alanine but decreased the amount of \(^{14}\)C appearing in aspartate. However, these differences were not statistically significant (p > 0.05, Student's t-test).

As leucine is reported to be a purely ketogenic amino
TABLE 7.2 Incorporation of $^{14}$C-Label from 3 mM-$[U-^{14}C]$-Leucine into Amino Acids Released by Hemi-Diaphragm In Vitro

<table>
<thead>
<tr>
<th>Condition</th>
<th>1 mM-3-mercaptopicolinate</th>
<th>% of Total Radioactivity in Amino Acids Studied</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Leucine</td>
</tr>
<tr>
<td>Fed</td>
<td>-</td>
<td>97.3 ± 0.6</td>
</tr>
<tr>
<td>Fed</td>
<td>+</td>
<td>96.5 ± 0.7</td>
</tr>
<tr>
<td>48 h-fasted</td>
<td>-</td>
<td>96.5 ± 0.2</td>
</tr>
<tr>
<td>48 h-fasted</td>
<td>+</td>
<td>96.0 ± 0.5</td>
</tr>
</tbody>
</table>

L-$[U-^{14}C]$-leucine was added at an initial specific activity of 167 mCi./mol.

Results shown are means ± S.E.M. of 3 observations.
acid, its catabolism leading to acetyl-CoA and acetoacetate production, (Rodwell, 1969), a problem arose as to how $^{14}$C from $[{U-^{14}}C]$-leucine could have appeared in alanine and aspartate released by the muscle.

The $^{14}$C-labelling of glutamate and glutamine can be simply explained by the formation of citrate from endogenous oxaloacetate and $^{14}$C-labelled acetyl-CoA derived from the metabolism of $[U-^{14}C]$-leucine. On the formation of 2-oxoglutarate from isocitrate derived from this $^{14}$C-citrate, $^{14}$CO$_2$ would be evolved. However, this would still leave one of the carbon atoms of 2-oxoglutarate radiolabelled. Glutamate and glutamine formed from such 2-oxoglutarate would therefore contain $^{14}$C on one carbon atom/molecule.

The $^{14}$C-labelling of aspartate observed with $[U-^{14}C]$-leucine as the sole source of $^{14}$C was very much less than that found of glutamate and glutamine. An explanation for the finding of $^{14}$C-aspartate could be as follows; A net increase in the level of tricarboxylic acid cycle intermediates in skeletal muscle has been reported under conditions when the acetyl-CoA concentration is elevated (Lee & Davis, 1979) and the mechanism responsible for this net increase is thought to be the fixation of carbon dioxide via carboxylation of pyruvate or phosphoenolpyruvate (Lee & Davis, 1979). If the carbon dioxide fixed was $^{14}$CO$_2$ derived from $[U-^{14}C]$-leucine oxidation, then the oxaloacetate formed would be radiolabelled. Transamination of this oxaloacetate would therefore, yield $^{14}$C-containing aspartate.
enolpyruvate could also account for the finding of $^{14}$C-labelled alanine albeit in very small amounts in the present study in which [$U-{^{14}}C$]-leucine was the sole source of $^{14}$C.

It is possible that the $^{14}$C-oxaloacetate formed by the fixation can undergo rapid equilibration with malate, fumarate and succinate. Radioactivity in citrate subsequently formed, would then be distributed evenly between the 1- and 3-carboxyl-GC-positions. Some $^{14}$C-labelling of pyruvate could then occur via decarboxylation of a four carbon unit derived from the labelled citrate mixture and would depend on randomisation of label between the two carboxyl groups via the symmetrical 4-carbon carboxylic acids. Transamination of the $^{14}$C-pyruvate so formed would thus yield the $^{14}$C-alanine observed. A similar explanation has been reported by Lee & Davis (1979) for finding $^{14}$C-lactate when $^{14}$C-labelled bicarbonate was added to perfusions of rat hindquarter.

The incorporation of $^{14}$C-label from 3 mM-$[U-{^{14}}C]$-valine into other amino acids released by rat hemi-diaphragm preparations is shown in Table 7.3. Very little incorporation of $^{14}$C into other amino acids took place, (again in agreement with Hutson & Zapolowski, 1981), although more label was found associated with glutamate, glutamine and particularly alanine in the fed state. In the experiments using hemi-diaphragms from 48 h-starved rats only a very small amount of label was found in the alanine released into the medium, the majority being found in glutamine and glutamate. These findings are similar to those reported by Chang & Goldberg (1978a) using incubated quarter-diaphragms.
<table>
<thead>
<tr>
<th>Condition</th>
<th>1 mM-3-mercapto-</th>
<th>% of Total Radioactivity in Amino Acids Studied</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>picollinate</td>
<td>Valine</td>
</tr>
<tr>
<td>Fed</td>
<td>-</td>
<td>99.2 ± 0.3</td>
</tr>
<tr>
<td>Fed</td>
<td>+</td>
<td>99.2 ± 0.1</td>
</tr>
<tr>
<td>48 h-fasted</td>
<td>-</td>
<td>99.8 ± 0.2</td>
</tr>
<tr>
<td>48 h-fasted</td>
<td>+</td>
<td>99.7 ± 0.2</td>
</tr>
</tbody>
</table>

L-[U-\(^{14}C\)]-valine was added at an initial specific activity of 167 mCi./mol.

Results shown are means ± S.E.M. of 3 observations.
In the experiments reported here, amino acid spots were visualised prior to radioactivity counting by spraying the thin-layer chromatography plates with ninhydrin reagent which would have caused decarboxylation of the amino acids. Thus assuming a uniform distribution of label in amino acids derived from $^{[U-14]C}$-valine, the ensuing percentage loss of label by a three carbon amino acid, such as alanine, is proportionately greater than by a larger amino acid, such as glutamate. However; even allowing for alanine decarboxylation by the ninhydrin reaction, the amount of $^{14}C$-label recovered in alanine seemed very small. The increase in alanine release by hemi-diaphragms from 48 h-starved rats, brought about by incubating in the presence of 3 mM-valine, has previously been shown to have a mean value of 0.87 µmol/2 h/g tissue (Table 3.1). If this alanine was formed de novo in muscle from the carbon of valine by the pathway proposed (Fig. 4.8 a), the radioactivity expected to be associated with alanine in the present experiment could be calculated.

Assuming a hemi-diaphragm weight of about 60 mg, approximately 50 nmol of alanine would be formed de novo from valine carbon in 2 h. If carbon from valine was not utilised for any purpose other than the formation of alanine the ratio of alanine/valine in the incubation medium would be 50/5950. This figure is an under-estimate as no account is taken for the amount of valine oxidised, released as 3-methyl-2-oxobutyrate or metabolised to other products. As alanine contains 3 carbon atoms and valine contains 5 carbon atoms, the radioactivity expected to be
associated with alanine would be $3/5 \times 50/5950$ of that associated with valine. However, as the detection method employed in these experiments caused decarboxylation of the amino acids, a minimum of $2/4 \times 50/5950$ of the recovered radioactivity associated with valine would be expected to be recovered with alanine. Thus $0.42\%$ of the radioactivity associated with valine would be expected with alanine and as stated previously, this is an under-estimate. The observed values of $99.8\%$ of the total radioactivity associated with valine, alanine, glutamine, glutamate and aspartate being found associated with valine and only $0.02\%$ associated with alanine suggest that the quantitative contribution of valine carbon to de novo alanine synthesis by muscle is not very great. Taken together with the previous finding that valine oxidation was not affected by inhibiting PEPCK or malic enzyme, these results may implicate an alternative route of valine metabolism in muscle which would not include the formation of a four-carbon compound and subsequent decarboxylation to yield a three-carbon precursor for alanine formation.

It has been proposed that leucine can be formed from valine via $\beta$-leucine (Poston, 1980a), although the physiological role of such a pathway is not clear.

The proposed steps involved are: transamination and decarboxylation to yield isobutyryl-CoA; incorporation of acetate to give 4-methyl-3-oxopentanoate ($\beta$-oxoisocaproate); transamination to $\beta$-leucine; conversion of $\beta$-leucine to leucine catalysed by the enzyme leucine 2,3-aminomutase (Poston, 1980b).
Valine carbon, after the formation of leucine by this path- 
way, could then be oxidised as for leucine i.e. catabolism to acetyl-CoA and subsequent oxidation in the tricarboxylic acid cycle. The reaction sequence involved in forming leucine from valine carbon involves two irreversible steps. Thus this proposed pathway can bring about the formation of leucine from valine, but not the reverse transformation.

It is possible that the above pathway could form part of an alternative route of valine oxidation in skeletal muscle. In support of this suggestion is the report that although leucine 2,3-aminomutase is widely distributed in rat tissues the activity of the enzyme is highest in skeletal muscle (Poston, 1980b). If valine was indeed metabolised via leucine by muscle it would explain why inhibition of PEPCK and malic enzyme had no significant effect on the oxidation of valine by muscle from 48 h-fasted rats.

The question arises as to whether the "alternative" route of valine oxidation is merely a consequence of the relatively high valine concentration used in the tissue incubations of this study. If valine metabolism were limited via the "conventional" route e.g. at the stage of isobutyryl-CoA, then the build up of isobutyryl-CoA could conceivably drive metabolism towards the
putative alternative oxidative pathway via leucine.

To examine this point the effect of the PEPCK inhibitor, 3-MP, on valine oxidation was studied using a more physiological concentration of valine, 0.2 mM (Table 7.1). Again it was shown that the $^{14}$CO$_2$ ratio was not affected by the presence of the inhibitor (c.f. Table 7.1). In comparison with the experiment using 3 mM-valine, it was observed with 0.2 mM-valine that a much higher proportion of the 3-methyl-2-oxobutyrate formed was released and hence that a lower proportion was oxidised. The transamination rate observed with 0.2 mM-valine was only about 10% of that found with 3 mM-valine. Similar results have been reported by Spydevold (1979) who showed that the oxidation rate of valine by perfused rat hindquarters increased approximately 30 times when the concentration of valine was increased from 0.1 mM to 5 mM. No difference in the $[^{14}C]$-valine / $[^{14}C]$-valine ratio was observed in the presence or absence of 1 mM-3MP, suggesting that even at physiological concentrations of valine, muscle preparations oxidise valine carbon by a route which does not involve PEPCK.

7.3 CONCLUSION

Although valine stimulates de novo alanine synthesis by muscle from fasted rats and although valine-stimulated de novo alanine synthesis proceeds via PEPCK, exogenous valine is not appreciably metabolised via PEPCK and is not itself a major source of carbon for the formation of alanine by muscle. The source of carbon for the PEPCK-dependent formation of alanine remains obscure.
TABLE 7.4  Effect of 1 mM-3-Mercaptopicolinate on the Metabolism of 0.2 mM-L-[\textsuperscript{14}C]-Valine and 0.2 mM-L-[\textsuperscript{1}C]-Valine by Hemi-Diaphragm Preparations Prepared from 48 h-Starved Rats

<table>
<thead>
<tr>
<th>Additions to Incubation Medium</th>
<th>None</th>
<th>1 mM-3-mercapto-picolinate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oxidation of [\textsuperscript{1}C]-valine</strong> ((\mu\text{mol}/2\ h/g\ tissue)</td>
<td>0.40 ± 0.04</td>
<td>0.55 ± 0.11</td>
</tr>
<tr>
<td><strong>Oxidation of [\textsuperscript{14}C]-valine</strong> ((\mu\text{mol}/2\ h/g\ tissue)</td>
<td>0.27 ± 0.04</td>
<td>0.36 ± 0.13</td>
</tr>
<tr>
<td>3-methyl-2-oxobutyrate released ((\mu\text{mol}/2\ h/g\ tissue)</td>
<td>0.15 ± 0.02</td>
<td>0.10 ± 0.04</td>
</tr>
<tr>
<td><strong>Transamination of [\textsuperscript{1}C]-valine</strong> ((\mu\text{mol}/2\ h/g\ tissue)</td>
<td>0.55 ± 0.03</td>
<td>0.65 ± 0.06</td>
</tr>
<tr>
<td><strong>Oxidation ratio [\textsuperscript{14}C]-valine</strong></td>
<td>0.69 ± 0.10</td>
<td>0.64 ± 0.12</td>
</tr>
</tbody>
</table>
CHAPTER EIGHT

GENERAL DISCUSSION: BRANCHED-CHAIN AMINO ACID METABOLISM AND ALANINE SYNTHESIS BY MUSCLE
GENERAL DISCUSSION: BRANCHED-CHAIN AMINO ACID METABOLISM AND ALANINE SYNTHESIS BY MUSCLE

In the present study a possible relationship between BCAA catabolism and alanine synthesis by muscle has been investigated.

Alanine released by muscle is a very important precursor for hepatic gluconeogenesis during starvation (see Introduction). It has been proposed that alanine is formed by the transamination of glycolytically-derived pyruvate i.e. glucose enters the muscle and is metabolised to pyruvate and converted to alanine by transamination. The alanine then is released from muscle into the bloodstream where it is taken up by the liver and converted back to glucose. This concept, the glucose-alanine cycle, (Mallette et al., 1969a; Felig et al., 1970) thus makes available the 2-oxo acid which is obtained from amino acid transamination for oxidation in the muscle. However, on a whole body basis, this cycle does not account for any net synthesis of glucose during starvation. The present study was therefore in part concerned with determining whether muscle was capable of releasing alanine which had been formed de novo in muscle via transamination with non-glycolytically derived pyruvate, as such a situation would enable a net contribution to the whole body glucose pool to be made as would be required during starvation.

The alanine released by muscle preparations incubated in unsupplemented medium is comprised in part of alanine derived from net protein degradation and from de novo synthesis. When
muscle is incubated in the presence of BCAAs, alanine release is stimulated. As the presence of BCAAs does not lower the intracellular content of alanine and does not increase net protein breakdown (as measured by tyrosine release) (see Chapter 4) it can be concluded that BCAA-stimulated alanine release is the result of de novo alanine formation. Experiments using inhibitors of transamination reactions (cycloserine, amino-oxyacetate) verified that the increased alanine release observed when muscle preparations are incubated in the presence of BCAAs is largely due to increased de novo alanine formation via transamination of pyruvate.

The source of the pyruvate was then investigated. Muscle preparations from fed animals mobilised substantial amounts of glycogen during incubations carried out in the absence of glucose whereas, under similar conditions, very little net glycogenolysis was observed in muscle from 48 h-starved rats. Thus although muscle glycogen was a possible source of the pyruvate required for de novo alanine synthesis by muscle from fed animals, the rate of net glycogen breakdown by muscle preparations from 48 h-starved rats was insufficient to provide the pyruvate for de novo alanine synthesis. Similar results have been reported by Goldstein & Newsholme (1976). A lack of relationship between the rate of muscle alanine release and glycolysis has been demonstrated using metabolic inhibitors of glycolysis (Goldstein & Newsholme, 1976; Garber et al., 1976a; but c.f. Chang & Goldberg, 1978b) and using insulin which in the present study was observed to increase the rate of glycolysis in muscle without producing
a corresponding increase in alanine release. These findings could appear to contradict the glucose-alanine cycle as proposed (Mallette et al., 1969a; Felig et al., 1970).

Incubated muscle tissue from 48 h-starved rats was observed to release appreciable amounts of lactate, pyruvate and alanine even when no substrate was added to the incubation medium which suggested that pyruvate (and hence lactate and alanine) can be formed from endogenous precursors present in muscle. As mentioned above, when valine was added to muscle incubations alanine release from muscle from 48 h-starved rats increased and occurred without lowering the tissue content of alanine, implying that the increased release of alanine was not a result of increased alanine loss from an intracellular pool. Goldstein & Newsholme (1976) have obtained similar results, observing that when alanine release from rat hemi-diaphragm preparations is stimulated by 3 mM-isoleucine or 3 mM-glutamate, the tissue content of pyruvate is increased.

Studies on the transamination and decarboxylation of valine and to what extent the carbon skeleton of valine was metabolised in the incubations of the present study showed that, as starvation proceeded, increased amounts of valine were transaminated and more valine-derived carbon appeared to be made available to the muscle. Together with the observations from experiments using clofibrate acid and hypoglycin, which showed that when BCAA metabolism beyond transamination was inhibited by these agents muscle alanine release decreased, these results suggested that the carbon skeleton of valine can provide the carbon
for pyruvate formation and hence for alanine synthesis in muscle.

It should be noted however that only $^{14}$CO$_2$ and BC0A release from muscle were measured. It is possible that further metabolites of the BCAA beyond the BC0A stage were released from the muscle. If this did in fact occur the calculated BCAA-derived carbon made available to the muscle would be an overestimate.

Valine metabolism is reported to proceed via propionyl-CoA and, after carboxylation, to form an intermediate of the tricarboxylic acid cycle, succinyl-CoA. For pyruvate to be formed from valine-derived carbon would therefore require a step whereby carbon could be withdrawn from the cycle to yield a 3-carbon compound. Enzymes which might be involved in this step are oxaloacetate decarboxylase, NAD/NADP-dependent malic enzyme(s) and PEPCK. No oxaloacetate decarboxylase activity was detected in skeletal muscle. Although both malic enzyme and PEPCK activities were found present in muscle, only PEPCK activity exhibited metabolic adaptations in situations of increased BCAA catabolism and increased muscle alanine release which were consistent with it being involved in the proposed pathway: during starvation and on feeding rats dietary supplements of BCAA, the PEPCK activity of muscle increased while malic enzyme activity did not. Further support for the proposal that, in muscle from 48 h-starved rats, valine contributed carbon for de novo pyruvate formation came from experiments using the specific PEPCK inhibitor, 3-mercaptopicolinate. Blocking PEPCK activity with this agent caused a substantial inhibition of valine-stimulated alanine release from muscle. However, against all
expectations, experiments using [U-14C]-valine failed to verify that the alanine carbon was to any great extent derived from the carbon of the added valine.

Leucine was also found to stimulate alanine release from muscle. Again the enzyme PEPCK was implicated in the formation of alanine; inhibition of the enzyme with 3-mercaptopicolinate caused a decrease in leucine-stimulated alanine release and supplemental feeding with leucine stimulated muscle PEPCK activity. However, leucine is a ketogenic amino acid which is oxidised to acetoacetate and acetyl-CoA and so cannot itself be a precursor for de novo pyruvate and hence alanine synthesis. This was verified in experiments using [U-14C]-leucine. Very little 14C was found incorporated into the alanine released from the muscle preparations. Therefore, although BCAAs stimulated muscle alanine release and a close relationship between BCAA catabolism and muscle alanine release has been observed, the BCAAs themselves did not appear to serve as the source of carbon for alanine synthesis to any great extent.

BCAA stimulation of alanine release has been observed with muscle preparations from 48 h-starved rats where negligible glycogenolysis occurred and the BCAAs were the only substrates added to the incubation medium. As the BCAAs were not the principal precursors for pyruvate formation, it would appear that other amino acids derived from muscle protein breakdown played an important role in this respect. This source of the alanine released from muscle would be consistent with the suggestion that during fasting there is net conversion of amino
acids to glucose (Cahill et al., 1966) and that muscle, being a major store for protein, is capable of being an important metabolic regulatory organ helping to maintain acceptable levels of glucose in the circulation (Daniel et al., 1977).

The pool of tricarboxylic acid cycle intermediates in muscle is increased in fasting, in diabetes (Davis et al., 1980) and by fatty acids and ketone bodies (Lee & Davis, 1979). Thus the concentration of cycle intermediates is increased when muscle is metabolising a non-carbohydrate fuel which results in the formation of acetyl-CoA. The metabolism of glucose is suppressed under these conditions (Williamson & Krebs, 1961; Shipp et al., 1961; Newsholme et al., 1962), the inhibition being most marked at the level of pyruvate dehydrogenase which is inactivated at high acetyl-CoA/CoA ratios (Davis & Quastel, 1964; Randle et al., 1970). The addition of exogenous leucine to starved muscle preparations (leucine is metabolised to acetyl-CoA) may therefore cause an increase in the levels of tricarboxylic acid cycle intermediates present in muscle mitochondria. Leucine has also been reported to inhibit glucose and particularly pyruvate oxidation by muscle from fasted animals (Chang & Goldberg, 1978c).

Differential compartmentation of leucine for oxidation and for protein synthesis has recently been reported in cultured skeletal muscle (Schneible et al., 1981). In cells cultured in buffered saline with 0.05 mM-leucine, 70% of the oxidised leucine originated extracellularly while 60% of the leucine for protein synthesis was derived from degraded protein. In fact these workers found that the preferential use of extracellular leucine
for oxidation was greatly enhanced at higher external leucine concentrations, the relative contribution of extracellular leucine to oxidation was 99% at 5 mM-leucine and the proportion coming from protein degradation was greatly decreased.

Comparing that situation with the present study it can be concluded that exogenous leucine (or exogenous valine via its possible transformation to leucine; Poston, 1980a, b) would be an important source of acetyl-CoA which could subsequently be oxidised in the tricarboxylic acid cycle.

It is possible that some of the added valine carbon is catabolised to form metabolites capable of feeding into the tricarboxylic acid cycle. However the negligible amount of $^{14}$C-labelling of alanine found when [U-$^{14}$C]-valine was added to the incubation medium shows that the amount of alanine which is derived from metabolites of valine which are withdrawn from the tricarboxylic acid cycle is very small. A possibility for negligible $^{14}$C-alanine formation from [U-$^{14}$C]-valine is that the rate at which carbon derived from endogenous amino acids feeds into the tricarboxylic acid cycle intermediates pool is very much greater than the rate at which exogenous valine-derived carbon can enter.

A further possibility is that at the relatively high valine concentration employed in these incubations, (3 mM), valine in excess of the amount that can be metabolised via succinyl-CoA may be metabolised via the "leucine formation" route described by Poston, 1980a, b).

It has been suggested that an increase in the levels of intermediates of the tricarboxylic acid cycle allows for a more
sensitive control of metabolic processes and that there is a continuous flux of intermediates in and out of the cycle pool (Lee & Davis, 1979). It has been reported by Świerczynski (1980) that the extramitochondrial malic enzyme of skeletal muscle is readily reversible whereas the mitochondrial malic enzyme preferentially catalyses the decarboxylation reaction. Therefore in some situations malic enzyme may carboxylate pyruvate extramitochondrially to malate which is transferred into the mitochondria as a tricarboxylic acid cycle intermediate which could help fulfil the requirement for increased pool levels in certain situations e.g. when muscle is metabolising fatty acids or ketone bodies. In other circumstances malic enzyme may catalyse the formation of pyruvate from malate should the need arise.

It has been shown (Chapters 4 & 5) that PEPCK activity is apparently linked with muscle alanine formation de novo and release, particularly during starvation. Proposals of how this enzyme could be involved were put forward in Chapters 4 & 6. It is possible that the PEPCK catalysed reaction proceeds towards oxaloacetate formation. However in the situation where amino acids are being used as oxidative fuels by muscle in times of carbohydrate sparing, it is doubtful whether phosphoenolpyruvate would be generated in sufficient amounts to permit this to any great extent. Possibly PEPCK functions as an overflow system whereby, when the level of tricarboxylic acid cycle intermediates becomes elevated the excess can be removed. Phosphoenolpyruvate so formed would then cross the mitochondrial membrane to the cytosol. This may occur via the tricarboxylate carrier in exchange
for malate as reported to occur in kidney (Bryla & Dzik, 1981) and liver (Robinson, 1971). Once in the cytosol phosphoenolpyruvate may be converted to pyruvate via pyruvate kinase and subsequently via transamination to alanine.

Thus in fasting it is proposed that muscle has the capability to metabolise amino acids as a source of fuel for the muscle itself, spare carbohydrate and, when the muscle has adequate energy provision, to mobilise more amino acids than are required to merely fuel the muscle to provide alanine as a precursor for hepatic gluconeogenesis which will subsequently increase the total body glucose pool.

The involvement of aspartate aminotransferase in the pathway for muscle alanine formation was suggested by the experiments in which 1 mM-AMB greatly inhibited alanine formation by hemidiaphragms from fasted rats (Chapter 4, Fig. 4.8a). However such an involvement depended on a cytosolic location for PEPCK whereas the majority of muscle PEPCK activity was subsequently shown to be mitochondrial (Chapter 6).

It is possible, though, that aspartate aminotransferase is not principally concerned with the transfer of carbon into the cytosolic compartment but of nitrogen. The nitrogen of BCAAs is made available in the mitochondria and as alanine aminotransferase is mainly cytosolic, aspartate efflux from mitochondria would serve to transport the nitrogen (Fig. 8.1).

These studies have provided an insight into the properties and capabilities of muscle with regard to supplying a major precursor for hepatic gluconeogenesis, alanine, and how BCAA.
FIG. 8.1 Proposed Pathway of Alanine Formation in Muscle from Starved Rats
metabolism is involved in this process.

It should be borne in mind that the findings of this study show the metabolic capabilities of rat muscle but are not necessarily indicative of muscle metabolism in vivo.

Further work is required to assess the possible pathway for valine catabolism via leucine in muscle (Poston, 1980a, b), in view of the results shown in Chapter 7, and to determine its role in physiological circumstances. Such a pathway would obviously preclude the contribution of valine to the net formation of tricarboxylic acid cycle intermediates and would require a re-evaluation of some of the concepts put forward in this thesis.

Experiments similar to those of the present study in which muscle preparations were incubated with $[^14C]$-valine or $[^14C]$-leucine should be repeated and by measuring the specific radioactivities of intracellular metabolites and intermediates on the pathways of amino acid metabolism the fate of the $^{14}C$ should be traced. It would be of interest to investigate the fate of $^{14}C$ when muscle from starved rat is incubated in the presence of physiological concentrations of $[^14C]$-BCAAs and other oxidisable substrates such as fatty acids and ketone bodies. Future work could also include in vivo investigations of BCAA turnover and its role in alanine formation in various dietary states.

The observations of the present study have been made on the basis of exogenously provided amino acids and require extension to explore their validity to amino acids generated endogenously in order to investigate whether the carbon of the alanine released by muscle can be derived from amino acids made available by
protein degradation. Further understanding of the mobilisation of muscle amino acids and its control by the BCAAs could prove useful in developing treatments for a variety of muscle wasting conditions, including diabetes, muscular dystrophy and tumour-induced cachexia.
REFERENCES

Adibi, S.A., Krzysik, B.A., Morse, E.L., Amin, P.M. & Allen, E.R.
J. Physiol. 228, 432 - 435.
Aikawa, T., Matsutaka, H., Takezawa, K. & Ishikawa, E. (1972)
Biochim. Biophys. Acta 272, 234 - 244.
Aikawa, T., Matsutaka, H., Yamamoto, H., Okuda, T., Ishikawa, E.,
74, 1003 - 1017.
154, 689 - 700.
Diabetologia 12, 59 - 69.
33, 671 - 679.

Barbieri, P., di Marco, A., Fuoco, L., Julita, P., Migliacci, A.  


Analysis" (Bergmeyer, H.U., ed.), pp 1704 - 1708, Verlag  

Biochem. Pharmacol. 27, 2892 - 2900.


5, 101 - 108.


21, 105 - 130.


(Colowick, S.P. & Kaplan, N.O., eds.), vol. 1, pp 435 - 440,  

Bucher, T., Czok, R., Lamprecht, W. & Latzko, E. (1963) in  
"Methods of Enzymatic Analysis" (Bergmeyer, H.U., ed.),  
J. Biol. Chem. 247, 8085 - 8096.


Fehlmann, M., Le Cam, A., Kitabgi, P., Rey, J-F & Freychet, P.


Felig, P. (1979) in "Endocrinology" (DeGroot, L.J., Cahill, G.F.,
Martini, L., Nelson, D.H., Odell, W.D., Potts, J.T.,
Steinberger, E. & Winegrad, A.I., eds.) vol. 3, pp 1927 -
1940, Grune & Stratton, New York & London.

Felig, P. & Koivisto, V. (1979) in "Contemporary Metabolism"
(Freinkel, N., ed.) vol. 1, pp 359 - 384, Plenum Medical


Science 167, 1003 - 1004.

Invest. 51, 1195 - 1202.

Felig, P., Wahren, J., Karl, I., Cerasi, E., Luft, R. & Kipnis, D.M.

70, 1775 - 1779.


Ross, B.D., Hems, R. & Krebs, H.A. (1967) Biochem. J. 102,
942 - 951.

Diabetes 26, 1120 - 1124.


Biophys. 200, 336 - 345.

12, 1 - 16.


184, 185 - 188.


Schimmel, R.J. & Knobil, E. (1970) Amer. J. Physiol. 218,
1540 - 1547.

Schmidt, E. (1963) in "Methods of Enzymatic Analysis" (Bergmeyer,


Waterlow, J.C., Garlick, P.J. & Millward, D.J. (1978a) "Protein Turnover in Mammalian Tissues and in the Whole Body", pp 481 - 528, North Holland Publishing Company, Amsterdam.


