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Protein And Amino Acid Metabolism In Skeletal Muscle

by

Guoyao Wu

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Protein And Amino Acid Metabolism In Skeletal Muscle submitted by Guoyao Wu in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Animal Biochemistry.

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Supervisor

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Date July 28, 1989

This thesis is dedicated to

my country,

my parents,

and all of my teachers in China and Canada

Abstract

Isolated chick extensor digitorum communis (EDC) muscles and, in some experiments, rat skeletal muscles were used to study a number of aspects of protein and amino acid metabolism. (1) Chick EDC muscles synthesise and release large amounts of alanine and glutamine, which indirectly obtain their amino groups from branched-chain amino acids (BCAA). (2) Acetoacetate or DL-ß-hydroxybutyrate (4 mM) decrease ($P<0.01$) alanine synthesis and BCAA transamination in EDC muscles from 24-h fasted chicks by decreasing ($P<0.01$) intracellular concentrations of pyruvate due to inhibition of glycolysis. (3) Glutamine is extensively degraded in skeletal muscles from both chicks and rats, thus challenging the traditional view that glutamine oxidation is negligible in skeletal muscle. The cytosolic glutamine aminotransferases L and K in the rat and the mitochondrial phosphate-activated glutaminase in the chick play important roles in the conversion of glutamine to α-ketoglutarate for further oxidation. (4) Although methionine has been reported to be extensively transaminated in rat skeletal muscle preparations in the absence of other amino acids, transamination of methionine is absent or negligible in chick and rat skeletal muscles in the presence of physiological concentrations of amino acids. (5) Glutamine at 1.0-15 mM increases ($P<0.01$) protein synthesis (^{3}H-phenylalanine incorporation), and at 10.0-15.0 mM decreases ($P<0.05$) protein degradation (^{3}H-phenylalanine release from prelabelled protein in vivo) in EDC muscles from fed chicks as compared to muscles incubated in the absence of glutamine. (6) Acetoacetate or DL-ß-hydroxybutyrate (4 mM) has a small but significant inhibitory effect ($P<0.05$) on the

rate of protein synthesis, but has no effect ($P>0.05$) on the rate of protein degradation in EDC muscles from fed chicks. A large decrease in intramuscular glutamine concentration due to an increase in its oxidation in the presence of 4 mM ketone bodies may account for the inhibitory effect of ketone bodies on protein synthesis in EDC muscles from fed chicks. (7) Ketone bodies (4 mM) decrease ($P<0.05$) both protein synthesis and protein degradation in EDC muscles from 24-h fasted chicks. It is unlikely that α-ketoisocaproic acid, a transamination product of leucine, is involved in the action of

bodies on muscle protein degradation. It remains to be determined how ketone bodies inhibit protein turnover in skeletal muscle from the fasted chick.

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I. Introduction

This thesis addresses a number of aspects of amino acid and protein metabolism in skeletal muscle. The skeletal musculature is the largest tissue in animals and humans, accounting for 40-45% of their body weight [1]. A thorough understanding of skeletal muscle metabolism is of importance in both animal agriculture to increase the efficiency of meat production and in medicine to decrease muscle wasting associated with various stressors. In order to understand the mechanisms of muscle growth or atrophy, research has been directed towards elucidating the metabolism of muscle proteins as they are the major components in this tissue on a dry matter basis. It is well established that proteins in all animal tissues including skeletal muscle undergo continuous synthesis and degradation [2], which is referred to as protein turnover (Fig. 1.1). The balance between the rate of protein synthesis and the rate of protein degradation regulates cellular protein levels and determines whether a tissue grows or atrophies [3].

Skeletal muscle is rich in free amino acids, particularly glutamine [1]. The intramuscular free amino acids are in dynamic equilibrium with protein turnover [3] (Fig. 1.1). Free amino acids in skeletal muscle are important precursors for protein synthesis and some, particularly alanine and glutamine, play an important role in interorgan metabolism of nitrogen and carbon [4]. Within the skeletal musculature, some amino acids are extensively degraded, others are extensively synthesised while others are neither synthesised nor

degraded [5]. Therefore, characterization of the metabolism of amino acids and their metabolites in skeletal muscle is of particular importance to the development of our understanding of the contribution of amino acids to metabolism, nutrition and homeostasis under a variety of physiological and pathological conditions in the animal.

The objectives of this chapter are three fold: (1) to outline protein and amino acid metabolism in skeletal muscle, (2) to review related methodologies, and (3) to introduce the research projects presented in this thesis.

A. Protein turnover in skeletal muscle

1. Protein synthesis

The mechanism of protein synthesis has been well characterized in both prokaryotic and eukaryotic organisms, and has been reviewed in detail elsewhere [6,7]. First, amino acids are esterified to tRNA by their specific aminoacyl-tRNA synthetases. This process requires energy from the conversion of ATP to AMP. Methionine and N-formylmethionine are the initiating amino acids for protein synthesis on the ribosomes and within the mitochondria of eukaryotes, respectively.

The activation of amino acids is followed by formation of ribosomal 80S subunits as the initiation complex for polypeptide chain synthesis. First, a free 40S ribosomal subunit binds to eukaryotic elongation factor 3 (eIF-3) to form complex 1, which is then bound to a mRNA to form complex 2. Complex 2 combines with GTP-eIF-2 and Met-tRNA (or N-formyl-Met-tRNA) to produce complex 3. A free 60S ribosomal subunit then binds to complex 3 with the hydrolysis of GTP

to GDP to yield the functional 80S subunit or initiation complex.

Following the formation of the initiation complex, polypeptide chain elongation begins. A new aminoacyl-tRNA is bound to the acceptor site (A site) on the ribosome and the former aminoacyl group is transferred from its tRNA to the new in-coming amino acid in a reaction catalysed by a peptidyl transferase. The ribosome moves along the mRNA towards its 3' end by one genetic codon, resulting in a shift of the peptide from the A site to the peptidyl site (P site) and the release of the tRNA. This process requires elongation factor G and GTP. The ribosome is now ready for another cycle.

The completion of polypeptide chain elongation is recognized by the terminating signal on mRNA. The newly synthesised peptide is hydrolysed from its terminal tRNA, and the 80S ribosome is dissociated into its 60S and 40S subunits. The polypeptide may undergo cotranslational and posttranslational modifications, yielding the native protein with biological activity.

2. Protein degradation in skeletal muscle

A number of proteolytic systems in skeletal muscle have been identified to play a role in the degradation of cellular proteins. These systems can be classified as lysosomal or nonlysosomal according to the location of their proteases [8].

2.1. Lysosomal system of protein degradation in skeletal muscle

Lysosomes are membranous vesicles which contain a large number of acid hydrolases and acid proteases (cathepsins) capable of degrading cellular proteins [8,9]. The mechanism by which intracellular proteins enter the lysosome in skeletal muscle is largely unknown [10]. Once a

protein has entered the lysosome, it is denatured due to the low pH and the peptide bonds are cleaved by proteases such as cathepsins B, C, and D. Lysosomes have been shown to be involved in the degradation of ^{3}H-ethylmaleimine-modified proteins or ^{3}H-leucine labeled normal myofibrillar proteins [11]. The lysosomal system is also responsible, in part, for the degradation of nonmyofibrillar proteins in skeletal muscle [11]. It has been demonstrated that the lysosomal proteolytic system in skeletal muscle is sensitive to regulation by hormones and nutritional deprivation [10]. However, the relative contribution of the lysosomal system to muscle protein degradation under physiological and pathological conditions remains to be determined [8,10].

2.2. Nonlysosomal system of protein degradation in skeletal muscle

The discovery of nonlysosomal proteases was an important step in the development of our understanding of intracellular protein degradation. Calcium-dependent proteases have been identified and are thought to play an important role in extralysosomal protein degradation in skeletal muscle [8,9]. In addition, there are two recently identified cytosolic ATP-dependent proteolytic pathways in skeletal muscle, which can be distinguished by their requirement for ubiquitin [10,12]. One is the ATP-dependert and ubiquitin-requiring proteolytic pathway, while the second is the ATP-dependent but ubiquitin-independent proteolytic pathway. The ATP-ubiquitin-dependent proteolytic pathway has been shown to be mainly responsible for the increased skeletal muscle protein degradation observed during short-term fasting [10]. However, the significance of these proteolytic systems in skeletal muscle under physiological and pathological conditions is also poorly understood and remains to be

determined [8-12].

3. Factors that regulate protein turnover in skeletal muscle

A relatively small number of workers have devoted their effort towards studies of protein turnover in skeletal muscle due to the technical problems involved (see Section C) [13]. Nevertheless, an awareness of some of the factors which regulate protein synthesis and degradation in skeletal muscle has been achieved during the last fifteen years. These factors are summarised in Table 1.1, and are briefly outlined below.

3.1. Hormones

Insulin [14,15], insulin-like growth factor-I and II [10], growth hormone [14] and prostaglandin $F_{2\alpha}$ [16] have an anabolic effect on skeletal muscle protein metabolism. By contrast, glucagon [17], glucocorticoids [18], high concentrations of thyroid hormones [11] and prostaglandin E_2 [16] have a catabolic effect on muscle protein metabolism. A number of reviews of the effect of hormones on protein turnover in skeletal muscle have been published [10,13,14].

3.2. Amino acids

3.2.1. Branched-chain amino acids (BCAA)

Branched-chain amino acids (BCAA), particularly leucine, are known to increase protein synthesis and decrease protein degradation in skeletal muscle in vitro [19,20]. Recently, leucine has been reported to mediate the effect of a mixture of amino acids on protein synthesis in rat skeletal muscle in vivo [21]. The anabolic effect of leucine has prompted extensive research into its clinical application to improve nitrogen balance during catabolic states [22].

The transamination product of leucine, α-ketoisocaproic acid (KIC), has been shown to mediate the inhibiting effect of leucine on protein degradation in rat skeletal muscle in vitro [19,20], which may explain why the administration of KIC in humans in catabolic conditions decreases the release of urinary nitrogen and 3-methyl-histidine [22]. On the other hand, the α-keto acids of isoleucine and valine have no effect on either protein synthesis or protein degradation in skeletal muscle, indicating a unique role for KIC in the regulation of muscle protein breakdown [20].

3.2.2. Glutamine

Glutamine is the most abundant free amino acid in plasma and skeletal muscle [1]. It plays an important role in interorgan metabolism of nitrogen and carbon [4] as well as in the regulation of cellular metabolism. For example, MacLennan et al. [23] have recently demonstrated that glutamine at physiological concentration ranges increases protein synthesis in perfused rat skeletal muscle. They have also found by using a ^{15}N-phenylalanine dilution technique that 15 mM glutamine (about 15 times its plasma concentration [24]) decreases protein degradation in perfused rat skeletal muscle [25]. A positive relationship has also been demonstrated between the rate of protein synthesis and the concentration of intracellular glutamine in rat skeletal muscle under a variety of experimental conditions such as starvation, injury, and following administration of bacterial endotoxin [26]. In this regard, glutamine has a similar effect on protein turnover in skeletal muscle as BCAA and KIC. However, unlike the BCAA, glutamine is extensively taken up by skeletal muscle and accumulated in this tissue [27], making it an attractive therapeutic

agent for improving nitrogen balance in catabolic states.

The first report on the effect of increasing intracellular glutamine concentrations on individuals in a catabolic state has been recently published [28]. In this study, the administration of 54 mg L-alanyl-L-glutamine-N/kg per day to patients following major surgery resulted in prevention of postoperative decreases in intramuscular glutamine concentrations and in more than a 50% decrease in urinary nitrogen loss. This anabolic effect of glutamine on nitrogen deposition was assumed to result from the action of glutamine on protein synthesis and degradation in skeletal muscle [28].

However, the role of glutamine in regulating skeletal muscle protein synthesis in vivo has been questioned by Garlick and Grant [21], as they found that infusion of glutamine failed to increase skeletal muscle protein synthesis in the rat. Their results are difficult to interpret since the glutamine infusion may not have significantly increased plasma glutamine concentration. As a result, the observations of Garlick and Grant [21] may not necessarily negate a role of glutamine in regulating skeletal muscle protein synthesis. Whether glutamine, at physiological concentration ranges, regulates skeletal muscle protein turnover needs to be clarified [29]. This question is addressed in Chapter 6.

3.3. Ketone bodies

Ketone bodies are the product of incomplete fatty acid oxidation in the liver. They have been implicated to play important roles as oxidative substrates and as metabolic signals in nonhepatic tissues [30]. For example, an increase in plasma concentrations of ketone bodies during fasting signals a reduced carbohydrate supply and

stimulates the brain to use them as an alternative energy source. The plasma concentrations of ketone bodies are increased to a greater extent than any other metabolite during a variety of physiological and pathological conditions such as fasting, feeding high fat diets, late pregnancy, exercise, diabetes, severe injury [30] and early lactation in dairy cows [31]. Thus, the study of the metabolic regulatory roles of ketone bodies in animals and humans is of particular interest to scientists studying a variety of physiological and pathological conditions.

Although the role of ketone bodies in the regulation of carbohydrate metabolism in animal tissues including skeletal muscle is well established, their role in regulating protein and amino acid metabolism is poorly understood [30]. It has been suggested that an increase in plasma ketone body concentrations results in a decrease in skeletal muscle protein degradation during long term fasting [32] and following severe injury [33]. However, many conflicting results concerning the effect of ketone bodies on muscle protein degradation have been reported in the literature. For example, ketone bodies have been shown to either inhibit [34] or not influence [35] protein degradation in the fasted rat diaphragm incubated in the absence of amino acids. Interpretation of these results [eg. 34] is ambiguous since they are based on measurement of tyrosine release without simultaneous measurement of protein synthesis, and there are many problems associated with the use of this approach to measure skeletal muscle protein degradation (see Section C). Thus, it is important to develop a reliable method for studying the effect of ketone bodies on protein degradation in skeletal muscle, which is reported in Chapter 7

of this thesis..

Both short-term and long-term fasting are characterised by a decrease in skeletal muscle protein synthesis [10], which may be partly due to decreased plasma concentrations of insulin and increased plasma concentrations of glucagon [10]. However, it is attractive to hypothesize that high plasma concentrations of ketone bodies may also contribute to the decrease in muscle protein synthesis observed during fasting. A preliminary study from our laboratory has shown that ketone bodies inhibit the rate of protein synthesis in chick skeletal muscles incubated in vitro [36]. By contrast, ketone bodies have been reported to have no effect on protein synthesis in fasted rat diaphragm [19]. This study, however, was performed in the absence of amino acids from the incubation media which may limit the rate of protein synthesis, thereby decreasing the sensitivity of muscle protein synthesis to ketone bodies. It is important that the effect of ketone bodies on protein turnover in skeletal muscle is studied in the presence of physiological concentrations of amino acids as discussed in chapter 7.

B. Amino acid metabolism in skeletal muscle

The free amino acids in skeletal muscle can be classified into four groups on the basis of their metabolic fate in this tissue (Table 1.2):

(1) amino acids which are extensively degraded but not synthesized;

(2) amino acids which are extensively synthesized but not degraded;

(3) amino acids that are both extensively degraded and synthesized;

(4) amino acids that are neither synthesized nor degraded.

1. Amino acids which are extensively degraded but not synthesized in skeletal muscle.

1.1. Branched-chain amino acids (BCAA)

In 1961, Johnson et al. [37] first reported that $^{14}CO_2$ is produced from [1-^{14}C]leucine in the rat diaphragm. Subsequently, BCAA were shown to be extensively transaminated and decarboxylated in skeletal muscle from many species including rats [38], humans [39], sheep [40] and chicks [41]. Oxidation of the BCAA carbon skeleton is incomplete in skeletal muscle [38,40,41]. Based on the proportion of skeletal muscle in the body and the relatively high activity of BCAA transaminase in skeletal muscle, it has been suggested that this tissue is the major site for BCAA transamination in the body [42]. The discovery that BCAA are degraded in skeletal muscle has led to reevaluation of an important role of this tissue in amino acid metabolism in animals.

The degradation of BCAA is initiated by transamination with α-ketoglutarate to produce branched-chain α-ketoacids (BCKA) and glutamate by BCAA aminotransferase (Fig. 1.2). The BCKA are then decarboxylated by the flux-generating BCKA dehydrogenase (BCKAD). This enzyme is inhibited by phosphorylation catalysed by BCKAD kinase and activated by dephosphorylation catalysed by BCKAD phosphatase [43]. The glutamate formed during BCAA transamination is coupled with *de novo* synthesis of alanine and glutamine (Fig. 1.2), which account for 40-50% of the amino acids released from skeletal muscle. Thus, degradation of BCAA in skeletal muscle not only supplies energy for muscle metabolism but also provides amino groups for the synthesis of alanine and glutamine.

1.2. Methionine

Like BCAA, methionine is not synthesized in animals in the absence of the appropriate carbon skeleton and therefore it is an essential amino acid [44]. Ten years ago, Mitchell and Benevenga [45] reported that methionine is as extensively transaminated as BCAA in homogenates of rat tissues including skeletal muscle, suggesting a major role for a transamination pathway in methionine degradation. Recently, Davis and his associates [46] have demonstrated extensive degradation of methionine in rat hindquarter preparations via a transamination pathway. However, these experiments and those of Mitchell and Benevenga were performed in the presence of 10-20 mM methionine (100-200 times normal plasma concentration) and in the absence of other amino acids. As a result, the physiological significance of a methionine transamination pathway in animal tissues including skeletal muscle remains unclear [44]. An assessment of methionine transamination in skeletal muscle in the presence or absence of amino acids is reported in Chapter 5.

2. Amino acids which are extensively synthesized but not degraded in skeletal muscle

On the basis of the available literature, glutamine appears to be the only amino acid which is extensively synthesized but not degraded in skeletal muscle [47]. Glutamine is released from skeletal muscle in relatively greater amounts than would be expected from the amino acid composition of muscle protein [48,49]. Thus, much attention has been directed towards the *de novo* synthesis of glutamine in skeletal muscle. Studies by Goldberg and his coworkers [5,47] have revealed

that BCAA donate the α-amino group for glutamine synthesis in rat skeletal muscle (Fig. 1.2). The free ammonia required for glutamine synthesis in skeletal muscle may arise from the purine nucleotide cycle [50] and extramuscular sources via the plasma [51]. On the other hand, the carbon skeleton of glutamine has been shown to arise, in part, from glutamate, aspartate, isoleucine, valine and asparagine [52]. As a result, the synthesis of glutamine draws α-ketoglutarate from the Krebs cycle, indicating the close relationship between amino acid metabolism and the operation of the Krebs cycle in this tissue.

There is some evidence in the literature for a species difference in glutamine synthesis in skeletal muscle. In 1963, Wu [53] reported that chick skeletal muscle contains no glutamine synthetase activity [53]. This observation, however, cannot explain the recent findings of Tinker et al. [54] that a large amount of glutamine is released from chick skeletal muscle. It is likely that chick skeletal muscle is capable of synthesising both alanine and glutamine as previously shown in mammalian skeletal muscle [47]. This hypothesis was therefore tested and reported in Chapter 2.

Although glutamine has been shown to be extensively synthesised in skeletal muscle, degradation of glutamine in this tissue is considered to be negligible [5,47] on the basis of reports that glutaminase activity is negligible or absent in rat skeletal muscle [55,56]. This view is widely held by investigators in interpretation of their experimental results [eg. 24,25]. However, degradation of glutamine can be initiated by glutamine transaminases, which are widely distributed in animal tissues including skeletal muscle [57]. Since

glutamine has been shown to be extensively oxidized in a wide range of other tissues and cells such as the small intestine, kidneys, liver, brain, reticulocytes, tumor cells, and lymphocytes [58], it is of importance to determine whether glutamine is also extensively oxidized in skeletal muscle. In addition, there is no information concerning glutaminase and glutamine aminotransferase activities in avian skeletal muscle. The possibility of glutamine degradation in skeletal muscle is therefore examined in Chapter 4.

3. Amino acids which are both extensively synthesized and degraded in skeletal muscle

3.1. Alanine

Like glutamine, alanine is preferentially released from skeletal muscle in relatively much greater amounts than would be expected from the amino acid composition of muscle protein [48,49]. A relatively large number of studies have been carried out to investigate the mechanism of alanine synthesis in skeletal muscle. Goldberg and his coworkers [47] have demonstrated that BCAA donate the amino group for alanine synthesis in rat skeletal muscle (Fig. 1.2). However, whether the carbon skeleton of alanine (pyruvate) mainly comes from glycolysis or glucogenic amino acids remains an open question, as discussed in the following two models.

3.1.1. Glycolysis provides the carbon skeleton for alanine synthesis

On the basis of the large amount of alanine released from skeletal muscle and the key role of alanine in hepatic gluconeogenesis, Felig et al. [59] and Marlette et al. [24] independently proposed the existence of the glucose-alanine cycle. According to this model, the

carbon skeleton of alanine is derived from glucose and glycogen in skeletal muscle. Alanine is then released from skeletal muscle and carried in the circulation to the liver for conversion to glucose, which returns to the muscle. This cycle implies a contribution of skeletal muscle glycogen stores to alanine synthesis and blood glucose homeostasis. Subsequent studies have provided convincing evidence for the important role of glycolysis in alanine synthesis in skeletal muscle [49,60,61]. For example, exogenous glucose increases the release of alanine from rat skeletal muscle [eg. 49,60,61], but inhibitors of glycolysis dramatically decrease alanine production [eg. 49,60]. It has been estimated that glycolysis provides over 95% of the pyruvate for muscle alanine synthesis [60].

The glucose-alanine cycle functions in carrying amino groups produced from the extensive degradation of BCAA in a non-toxic form (alanine) from skeletal muscle to the liver. It may also help to decrease lactate accumulation which may result in metabolic acidosis. In addition, alanine released from the skeletal musculature regulates the rate of hepatic protein synthesis [62] and protein degradation [63], and thus the availability of amino acids for hepatic gluconeogenesis. Therefore, although there is not a net increase in the amount of glucose synthesised via the glucose-alanine cycle, it may indirectly contribute to net glucose synthesis by regulating substrate availablity.

3.1.2. Amino acids provide the carbon skeleton for alanine synthesis

Garber et al. [64,65], Goldstein and Newsholme [66] and Ozand et al. [67] proposed an alternative pathway for the origin of alanine carbon, in which pyruvate is mainly derived from glucogenic amino

acids released during muscle protein degradation. Garber et al. [64,65] also suggested that leucine can supply the carbon skeleton for alanine synthesis in skeletal muscle, which is unlikely since leucine is purely a ketogenic amino acid [42]. This alternative model recognizes that alanine from skeletal muscle would directly lead to net glucose production. This theory, however, was largely based on the findings that glucose had no effect on alanine release or its tissue concentration in rat skeletal muscle [64,65,67] in direct contrast to those of Goldberg and other investigators [49,60,61] under similar expermental conditions. Thus, Garber et al. [64,65] and Ozand et al. [67] concluded that glycolysis was not related to alanine synthesis in skeletal muscle.

It follows that the relative importance of these two models in alanine synthesis remains to be determined. This question is further explored in avian skeletal muscle in Chapter 3.

3.2. Glutamate, aspartate and asparagine

Glutamate can be synthesised by transamination of BCAA, alanine or aspartate with α-ketoglutarate in skeletal muscle [47]. On the other hand, aspartate can be synthesised by transamination of glutamate with oxaloacetate while asparagine is synthesized from glutamine and aspartate.

Glutamate, aspartate and asparagine are not only actively synthesized in skeletal muscle, but also are extensively oxidized in this tissue [47]. The oxidation of these amino acids, however, is incomplete in skeletal muscle [47,52,60].

4. **Amino acids which are neither degraded nor synthesized in skeletal muscle**

None of the essential amino acids except for BCAA and methionine have been reported to be oxidised to any extent in skeletal muscle. These non-metabolizable amino acids are glycine, cysteine, serine, threonine, proline, lysine, arginine, histidine, phenylalanine, tyrosine and tryptophan [5,47] (Table 1.2).

5. **Interorgan metabolism of alanine and glutamine**

Large amounts of both alanine and glutamine are preferentially released into the circulation from skeletal muscle of many species including man [39,68,69], rats [48,49] and chicks [54]. Alanine and glutamine released from skeletal muscle have different fates in the body. Alanine is actively taken up by the liver where it is the most important glucogenic amino acid [24,59]. On the other hand, glutamine is taken up by the small intestine, where it is the major oxidative substrate during the postabsorptive state [70], and by the kidney particularly in acidosis, where it plays a vital role in regulating acid/base balance and contributes to renal gluconeogenesis [71]. Both the small intestine and the kidneys convert a significant amount of glutamine to alanine and serine [72]. During short-term fasting, the increased demand for glutamine by the gut and the kidneys is mainly met by an increase in glutamine release from skeletal muscle and partly met by a switch in the balance of glutamine from net uptake to net production in the liver [72]. Similarly, in acidotic mammals including rats, dogs, humans and sheep, increased release of glutamine from skeletal muscle at the expense of alanine increases the amount of

glutamine for extraction by the kidney in response to the metabolic challenge [72].

6. Regulation of amino acid metabolism by ketone bodies in skeletal muscle

It is well documented that alanine release from human skeletal muscle during long-term fasting is dramatically reduced [32]. A decrease in alanine release from skeletal muscle has also been found following the infusion of ketone bodies in man [73]. These in vivo studies suggest a role for ketone bodies in regulating amino acid metabolism in skeletal muscle [30,69,73]. In vitro studies have also demonstrated that ketone bodies decrease alanine release from incubated rat skeletal muscle [34], cultured rat skeletal muscle cells [74] and perfused rat hindquarters [75]. In addition, ketone bodies have been shown to markedly inhibit BCAA degradation in skeletal muscles from rats [76] and chicks [41]. However, the mechanism by which ketone bodies regulate metabolism of amino acids in skeletal muscle remains unknown.

Based on their observation that pyruvate prevented the inhibiting effect of ketone bodies on alanine synthesis, Palaiologos and Felig [34] suggested that ketone bodies decrease alanine synthesis by inhibiting glycolysis in skeletal muscle. This suggestion is consistent with the glucose-alanine cycle in which the carbon skeleton of alanine is derived from glycolysis. However, other authors [74,75] have questioned the role of muscle glycolysis in alanine synthesis, as they found that ketone bodies decrease alanine synthesis without decreasing glycolysis or pyruvate concentration in their skeletal

muscle preparations. These investigators thus suggested that ketone bodies decreased alanine synthesis by inhibiting BCAA degradation [74,75]. This suggestion appears to be supported by the our recent findings that ketone bodies inhibit BCAA transamination in skeletal muscle from the fasted-chick [41]. Whether ketone bodies inhibit alanine synthesis in the muscle by inhibiting glycolysis or BCAA degradation is investigated in Chapter 3.

C. Methodology of protein turnover and amino acid metabolism in skeletal muscle

The development in our understanding of protein turnover is best with methodological problems [10,13]. For example, there have been no satisfactory techniques for measuring protein degradation in any tissues including skeletal muscle. Also, experimental conditions vary greatly among different laboratories, resulting in difficulties in evaluating the literature. For example, in many *in vitro* studies of protein turnover and amino acid metabolism, plasma amino acids have not been included in the incubation medium [eg. 19,34,45,46]. Therefore, considerable effort must be directed towards increasing the physiological relevance of methods used to measure protein and amino acid metabolism in skeletal muscle.

1. In vivo studies of protein turnover and amino acid metabolism in skeletal muscle

1.1. Protein turnover in skeletal muscle

The rate of protein synthesis in skeletal muscle in vivo has been estimated by constant infusion [77], simple flooding infusion [78] and feeding amino acids containing stable or radioactive isotopes [79].

The rate of protein degradation in skeletal muscle is then estimated from the difference between the rate of protein synthesis and the rate of protein accretion. One useful method for estimating the rate of myofibrillar protein degradation in vivo is the measurement of 24-h urinary excretion of 3-methylhistidine [80]. This approach has recently been shown to be valid in adult rats [81], humans [82], cattle [83] and chicks [84].

1.2. Amino acid metabolism in skeletal muscle

Measurement of the arterioveinous (A-V) differences in amino acid concentrations across the forearm or hindlegs are commonly used to estimate which amino acid may be synthesised or degraded in skeletal muscle [eg. 39,54,68,73]. For example, large negative A-V differences in glutamine concentration across the human forelimb indicate that glutamine is preferentially released and may be synthesised in human skeletal muscle [39]. On the other hand, the large positive A-V difference in BCAA concentration across the human forelimb indicates that human skeletal muscle extensively degrades BCAA [39]. To the best of my knowledge, there have been no studies of amino acid degradation in skeletal muscle in vivo using radioactive or stable isotopes, probably due to methodological problems.

1.3. Advantages and disadvantages of in vivo studies

Since in vivo studies are conducted in intact animals, there is great opportunity to derive physiologically relevent results. Also, in vivo studies provide definite evidence for verification of hypotheses or findings from in vitro experiments, which may be of biological significance. However, interference of skeletal muscle metabolism by other organs and tissues makes it difficult to assess the function of

this tissue. For example, it is very difficult to measure total protein degradation or amino acid degradation in skeletal muscle in vivo. In addition, the interpretations of results from in vivo studies are not without any limitations since many factors in vivo may affect protein or amino acid metabolism directly or indirectly. Moreover, the number of animals studied is neccessarily limited due to both the expense of animals and chemicals used and the availability of facilities. This is particularly true for large animals. These factors, taken together, decrease the sensitivity of statistical analysis, and thus may mask the real biological effect of treatments.

2. In vitro studies of protein turnover and amino acid metabolism in skeletal muscle

2.1. Protein turnover in skeletal muscle

The incorporation of ^{3}H-phenylalanine into the acid precipitable fraction of muscle has been widely used to estimate the rate of protein synthesis in skeletal muscle in vitro [eg. 10,35]. ^{3}H-phenylalanine is preferentially chosen since it is economical and is a non-metabolizable amino acid in skeletal muscle (Table 1.2). Due to technical problems in measuring the specific radioactivity of tRNA-bound phenylalanine, the intracellular specific radioactivity of phenylalanine is usually used to calculate the rate of protein synthesis [eg. 35].

On the other hand, the release of tyrosine from skeletal muscles incubated in the absence of tyrosine has been used to measure either the net rate of protein degradation in the absence of an inhibitor of protein synthesis or the total rate of protein degradation in the

presence of an inhibitor of protein synthesis (eg. L-cycloheximide) [eg. 10,35]. A method of simultaneous measurement of protein synthesis and degradation has been reported by Tischler et al. [19], in which the total rate of protein degradation is calculated as the sum of the rate of tyrosine release plus the rate of tyrosine incorporation calculated by multiplying the rate of ^{3}H-phenylalanine incorporation by the ratio of tyrosine to phenylalanine incorporated into muscle proteins. Tyrosine is preferentially chosen since it is neither synthesised in the absence of appropriate carbon skeleton nor degraded in this tissue and it is easily measured with high sensitivity by fluorimeter [35].

It should be pointed out that the release of tyrosine from skeletal muscles incubated in the absence of an inhibitor of protein synthesis represents only the net rate of protein degradation, which depends on the balance between the rate of protein synthesis and the rate of protein degradation. On the other hand, the presence of L-cycloheximide in the incubation medium for measuring tyrosine release as an indicator of total protein degradation may disturb muscle metabolism, as it has been shown to inhibit muscle protein degradation [85].In addition, in the method described by Tischler et al. [19] it is assumed that the ratio of tyrosine to phenylalanine incorporated into muscle proteins is constant, which may not be valid since proteins with different ratios of tyrosine to phenylalanine may turn over at different rates and their degradation (eg. myofibrillar and non-myofibrillar proteins) may be regulated differently. Also, the absence of tyrosine and other amino acids from incubation media used in previous studies [eg. 19,34] may have limited the rate of muscle

protein synthesis, thereby decreasing the sensitivity of muscle protein synthesis to certain treatments. Thus, it appears important to develop a reliable technique for measuring protein degradation in skeletal muscle. An improved technique is described in Chapter 6.

2.2. Amino acid metabolism in skeletal muscle

^{14}C-labelled amino acids have been widely used to study their metabolism in isolated skeletal muscle preparations [eg. 37,38,40,41]. For example, the production of $^{14}CO_2$ from [1-^{14}C]leucine in skeletal muscle of rats [37,38] and chicks [36] indicates that the BCAA are decarboxylated in this tissue. On the other hand, the accumulation of amino acids in the incubation medium has been used to measure the rate of their net synthesis. For example, the release of large amounts of alanine and glutamine reflects the ability of skeletal muscle to synthesise these two amino acids [48,49].

2.3. Advantages and disadvantages of in vitro muscle preparations

Isolated skeletal muscle preparations from many species have been used to study oxygen consumption, protein turnover, and the metabolism of amino acids, fatty acids and carbohydrates [eg. 10,41,48,49,86-92]. In vitro models offer many advantages for studying intermediatery metabolism. For example, the preparation and incubation of isolated skeletal muscle is simple and rapid, making it possible to study a relatively large number of muscles in one day. In addition, the use of contralateral muscles allows direct comparisons between control and treatment muscles from the same animal, thereby decreasing experimental errors due to inter-animal differences. As a result, a paired experimental design can be effectively taken advantage of to detect small but significant effects of certain treatments. In vitro

models are also valuable in that experimental conditions can be easily manipulated to quickly test a large number of hypotheses of biological significance. Also, the metabolic differences in either amino acid or protein degradation in different muscle fiber types can be investigated using isolated muscles containing predominantly red or white muscle fibers, which would be impossible in in vivo studies. Moreover, isolated muscle preparations are free of interference from other tissues, thus allowing studies of the direct effects of factors on skeletal muscle metabolism.

In vitro muscle preparations are not without problems. A major concern with incubated muscles is whether the supply of oxygen, which depends on the diameter of the muscle and its metabolic rate, is adequate [86]. It has been shown that the supply of oxygen to the central core of the incubated muscle at 37^{0}C may be inadequate [93]. This may partly explain why the rate of protein synthesis in the central core of the incubated skeletal muscle is lower than that in the muscle periphery [93]. In addition, incubated skeletal muscles even from young growing animals such as rats is usually in negative protein balance [eg. 19,35,92]. Also, in vitro experimental results cannot necessarily be extrapolated to in vivo situations because of the absence of mechanisms responsible for maintaining homeostasis in the intact animal such as the neuroendocrine and circulatory systems. These limitations must be born in mind when interpreting experiment data from in vitro studies.

2.4. Validity of in vitro skeletal muscle preparations

There is no doubt that in vitro studies have certain limitations, and therefore they must be validated with care in terms of providing

physiologically useful information on muscle metabolism. First of all, does the development of the central core in incubated skeletal muscle negate any valuable conclusions? This appears unlikely as it has been recently shown that sepsis decreases protein synthesis in skeletal muscle in vivo and in both the periphery and the central core of incubated rat skeletal muscle [94]. In addition, fasting has been shown to decrease protein synthesis in skeletal muscle both in vivo and in vitro [10]. These findings suggest that results from in vitro studies can be meaningful despite the presence of the central core. Furthermore, if the control and treatment muscles are similar in sizes, the central core could be similar for both muscles and the results can be directly compared to provide physiologically relevant information [89].

Secondly, a number of biochemical criteria can be used to assess the viability of in vitro muscle preparations. It has been argued by Newsholme et al. [91] that mitochondria from incubated skeletal muscles are intact for at least 1-2 h. This can be supported by the finding that the oxidative decarboxylation of leucine, which is catalysed by the mitochondrial BCKA dehydrogenase [43], is linear in incubated skeletal muscles from rats [38] and chicks [95] for at least 2 h. Also, the tissue concentrations of ATP, phosphocreatine and glycogen in incubated skeletal muscle from chicks [89] and rats [86,91] are similar to their in vivo values following 2 h of incubation. In addition, the rates of protein synthesis and degradation, prostaglandin E_2 production, and amino acid release in incubated chick skeletal muscle are linear for at least 2 h [89]. Similarly glucose transport, glycolysis and glycogen synthesis are

also linear in incubated rat skeletal muscle for at least 1 h [91]. These biochemical criteria suggest that in vitro muscle preparations are valid for studies of intermediary metabolism.

Thirdly, are the results from in vitro muscle preparations physiologically relevant? This question can be answered by the great contributions that in vitro muscle preparations have made to the advancement of modern biochemistry. For example, incubated frog skeletal muscle was used to discover the formation of glucose-1-phosphate from glycogen [87] and the synthesis of glycogen from glucose-1-phosphate [88], which was the foundation of the Nobel prize-winning work of Cori and Cori [96]. In addition, the elucidation of the citric acid cycle, which is a milestone in modern biochemistry and a Nobel-prize winning achievement [97], was worked out by Krebs using in vitro pigeon breast muscle preparations [98]. Many other novel findings of physiological significance have also been obtained using in vitro muscle preparations, such as hormonal regulation of carbohydrate metabolism [90] and protein turnover [10,14], oxidation of branched-chain amino acids [37,47] and utilization of ketone bodies [90] in skeletal muscle.

During the last three years, considerable effort has been made to attempt to eliminate the central core of incubated muscles and to decrease the negative protein balance that occurs during incubation. For example, incubation of muscles at 33^{o}C can eliminate the central core in chick muscles [89]. Also, maintenance of normal length markedly improves protein balance and energy status in isolated skeletal muscle [92]. Moreover, continuous gassing of the incubation medium throughout the incubation period can also significantly improve

protein balance in incubated chick skeletal muscle [89]. In our laboratory, nitrogen balance is essentially maintained in incubated chick muscles from 9 or 10-days-old chicks which are held at their resting length and continuously oxygenated during incubation [89].

D. Objectives of this thesis

The objectives of this thesis are summarised as follows:

1. to determine whether chick skeletal muscle can synthesise alanine and glutamine;
2. to investigate how ketone bodies regulate the metabolism of amino acids in skeletal muscle;
3. to determine whether glutamine is oxidised in skeletal muscle;
4. to determine whether methionine is degraded via a transamination pathway in skeletal muscle;
5. to develop a new technique to measure protein degradation in skeletal muscle;
6. to determine whether glutamine regulates protein turnover in skeletal muscle;
7. to determine whether ketone bodies regulate protein turnover in skeletal muscle.

Isolated skeletal muscle preparations were used throughout much of the thesis. Chicks were the primary animal model used while rats were also used in some experiments.

Table 1.1. Factors reported in the literature that influence protein turnover in skeletal muscle

Factors	Protein synthesis	Protein degradation	Net effect
Insulin	increase	decrease	anabolic
Insulin-like growth factor I and II	increase	decrease	anabolic
Growth hormone	increase	no effect	anabolic
Testosterone & Estradiol	increase	decrease	anabolic
Triiodothyronine			
Physiological dose	increase	increase	no change
Hyperthyroid dose	increase	increase	catabolic
Glucagon	no effect	increase	catabolic
Glucocorticoids			
Fed state			
Low dose	decrease	no effect	catabolic
High dose	decrease	increase	catabolic
Fasted state	decrease	increase	catabolic
Prostaglandin E_2	no effect	increase	catabolic
Prostaglandin $F_{2\alpha}$	increase	no effect	anabolic
Muscle contraction	increase	increase	anabolic
Starvation			
Short term	decrease	increase	catabolic
Long term	decrease	decrease	preserve N
Calcium	no effect	increase	catabolic
Magnesium	no effect	decrease	anabolic
Leucine	increase	decrease	anabolic
α-ketoisocaproate	no effect	decrease	anabolic
Glucose	no effect	decrease	anabolic
Glutamine	increase?	decrease?	anabolic?
Ketone bodies	decrease?	decrease?	preserve N?

Table 1.2. Metabolic fates of amino acids in skeletal muscle reported in the literature

Amino acids	Metabolic fates
1. Leu, Ile, Val, Met	Extensively degraded but not synthesized
2. Gln	Extensively synthesized but not degraded.
3. Ala, Glu, Asp, Asn	Both extensively synthesized and degraded
4. Gly, Cys, Ser, Thr, Pro, Lys, Arg, His, Phe, Tyr, Trp	Neither synthesized nor degraded

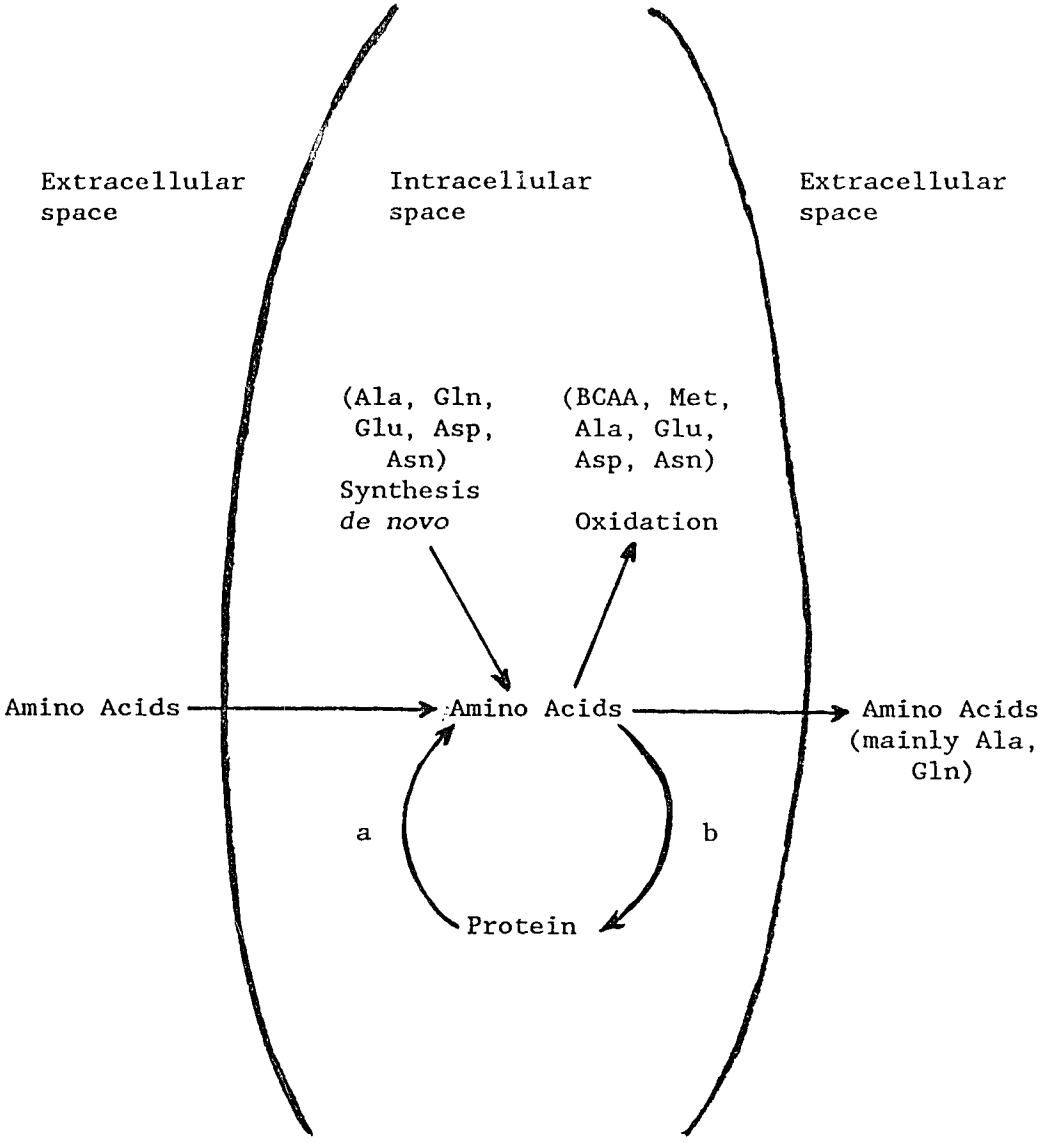

Fig. 1.1. Overview of protein turnover and amino acid metabolism in skeletal muscle as reported in the literature

a, protein degradation ; b, protein synthesis; BCAA, branched-chain amino acids.

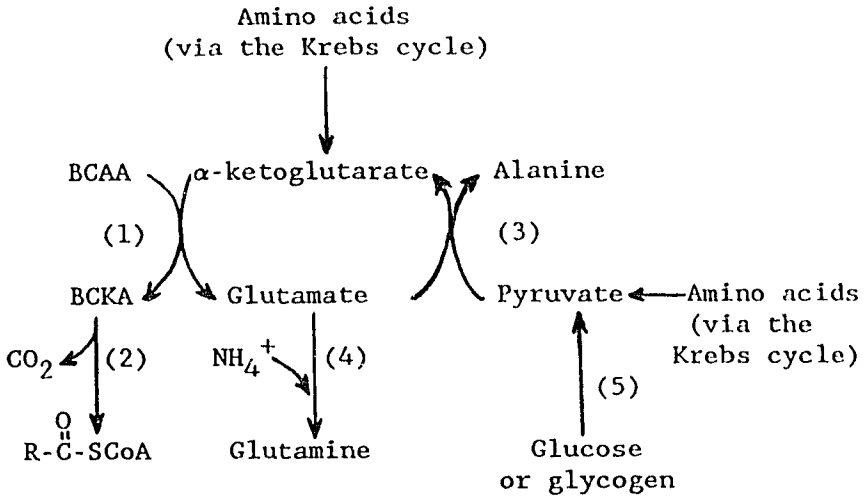

Fig. 1.2. BCAA Transamination coupled with *de novo* synthesis of alanine and glutamine in skeletal muscle

BCAA, branched-chain amino acids; BCKA, branched-chain α-ketoacids
1. BCAA aminotransferase; 2. BCKA dehydrogenase complex;
3. Alanine aminotransferase; 4. Glutamine synthetase; 5. Glycolysis.

E. References

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II. Formation Of Alanine And Glutamine In Chick Skeletal Muscle[1]

A. Introduction

Large amounts of alanine and glutamine are released from skeletal muscles of mammals such as rats [1-3], dogs [4], sheep [5] and humans [6]. Recently, Tinker et al. [7] demonstrated that large amounts of alanine and glutamine are also released from skeletal muscle in domestic fowl. Alanine and glutamine released from skeletal muscle play an important role in the interorgan metabolism of carbon and nitrogen [8,9]. Alanine is taken up by the liver where it is an important glucogenic precursor [6], and a regulator of protein synthesis [10] and ketone body production [11]. On the other hand, glutamine is extracted by the gut, where it serves as a major oxidative substrate [12], and by the kidneys, where it plays a vital role in the regulation of acid/base balance [9,13].

It has been shown that alanine and glutamine are synthesized in rat skeletal muscle by the transamination of pyruvate with glutamate and by the amidation of glutamate with NH_4^+, respectively [1,2,14]. Relatively large amounts of glutamate are formed by the transamination of the branched-chain amino acids (BCAA) with α-ketoglutarate in rat skeletal muscle [15]. Since the branched-chain amino acids are extensively degraded in chick skeletal muscle [16], they may also provide the amino groups for synthesis of alanine and glutamine in this tissue. However, no information is

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available on the formation of alanine and glutamine in avian muscles. In fact, glutamine synthetase has been reported to be absent in chick skeletal muscle [17], suggesting species differences in the capacity of skeletal muscle to synthesize glutamine [14,17]. The present study was therefore conducted to investigate whether alanine and glutamine are synthesized in chick skeletal muscle.

B. Materials and Methods

Bovine insulin, D-glucose, amino acids, α-ketoisocaproate (KIC), L-cycloserine, NH_4Cl, Hyamine hydroxide and other chemicals used were purchased from Sigma Chemical Co. St Louis, Mo., U.S.A. L-[1-^{14}C]leucine and L-[1-^{14}C]valine were purchased from ICN Radiochemicals, Montreal, Que. Day-old male broiler chicks (Hubbard strain, *Gallus domesticus*) were obtained from a local hatchery and were allowed free access to food and water.

Ten-day-old chicks were anesthetised with halothane and the extensor digitorum communis (EDC) muscles (19.4 ± 0.6 mg, mean ± SEM, n=20) were dissected and inserted into stainless steel supports [16]. Muscles were preincubated for 30 min and then incubated for 2 h at 37°C in 3.5 ml Krebs-Ringer bicarbonate buffer (119 mM NaCl, 25 mM $NaHCO_3$, 4.82 mM KCl, 1.26 mM $CaCl_2$, 1.25 mM $MgSO_4$, 1.24 mM NaH_2PO_4) saturated with 95% O_2:5% CO_2 (pH 7.4) containing 0.01 U/ml insulin, 12 mM glucose and 2 mM Hepes (Basal medium) [16]. Amino acids were either present or absent from the basal media depending on the experimental design. In all experiments, one EDC muscle was used for the control while the contralateral muscle was used for the treatment.

A preliminary experiment was performed to determine the amino acid composition of the free intracellular pool, skeletal muscle protein and incubation media after 2 h final incubation in the absence of exogenous amino acids. Subsequent experiments were designed to determine the effect of BCAA, KIC, L-cycloserine and NH_4Cl on the release and intracellular concentrations of glutamate, glutamine, alanine and tyrosine. In these experiments, the basal media contained plasma concentrations of all amino acids [18] (Appendix 1) except glutamate, glutamine, alanine, tyrosine, histidine and BCAA. BCAA were either present or absent from the media as indicated in Tables 1-6. At the end of the 2-h final incubation, muscles were placed in 2% trichloroacetic acid. Both muscle and incubation media were stored at -70^0C until analyzed within ten-days by HPLC [16,19]. Tyrosine released to the incubation media and present in the tissue at the end of the 2-h final incubation was used to estimate net protein degradation [20].

For measurement of the net rate of BCAA transamination, 0.5 mM L-[1-^{14}C]leucine (300 dpm/nmol) or 0.5 mM L-[1-^{14}C]valine (400 dpm/nmol) was included in the incubation media. $^{14}CO_2$ generated following decarboxylation of the oxo-acids produced during transamination of L-[1-^{14}C]leucine or L-[1-^{14}]valine was collected in Hyamine hydroxide and the net rates of transamination of leucine or valine were calculated based on the specific radioactivity of L-[1-^{14}C]leucine or L-[1-^{14}C]valine in the incubation media as previously described [16].

Results, given as mean ± S.E.M., were analyzed by the paired t-test or one-way variance analysis [21].

C. Results

In the preliminary experiment in which EDC muscles were incubated in the absence of all amino acids, glutamate, glutamine, alanine and tyrosine accounted for 3.0±0.2, 31.2±0.6, 19.0±0.4 and 1.8±0.2% (n=10), respectively, of the amino acids released to the incubation media. These four amino acids also accounted for 11.5±0.8, 25.2±1.0, 17.3±0.9 and 1.2±0.3% (n=10), respectively, of the amino acids in the intracellular free pool. In the EDC muscle protein, glutamate plus glutamine, alanine and tyrosine accounted for 13.0±0.1, 10.5±0.1 and 2.0±0.1% (n=10), respectively, of amino acids present. After the 2 h incubation period, the ratios of (glutamate plus glutamine):tyrosine and alanine:tyrosine released to the incubation medium and present in the intracellular free pool were 20.0±1.5 and 10.7±0.6 (n=10), and 30.5±3.3 and 14.6±0.9 (n=10), respectively, which were much higher ($P<0.01$) than the values for the same ratios in EDC muscle proteins which were 6.3±0.4 and 5.2±0.3 (n=10), respectively.

The addition of 0.5 mM leucine, isoleucine or valine (Table 2.1), or all BCAA together (0.5 mM leucine, 0.25 mM isoleucine and 0.5 mM valine) (Table 2.2) increased ($P<0.01$) both the release and the intracellular concentrations of glutamate, glutamine and alanine. The increase in the amount of glutamate, glutamine and alanine (Table 2.1) was similar to the net amount of the branched-chain amino acids transaminated during the incubation period (Table 2.3).

L-Cycloserine (1.5 mM), an inhibitor of aminotransferases [22], inhibited ($P<0.01$) the net rate of transamination of leucine and valine by approximately 60% (Table 2.3) which was reflected by increased ($P<0.01$) intracellular concentrations of BCAA and decreased

($P<0.01$) release and intracellular concentrations of glutamate, glutamine and alanine (Table 2.2). The release and intracellular concentration of free tyrosine in EDC muscles was not influenced ($P>0.05$) by the addition of 1.5 mM L-cycloserine to the incubation medium (data not shown).

The addition of 0.1 and 0.5 mM KIC increased ($P<0.01$) the intracellular concentration of leucine (Tables 2.4 and 2.5) and its release from EDC muscles (Table 2.5) while it decreased ($P<0.05$) the release and intracellular concentrations of glutamate, glutamine and alanine in EDC muscles (Tables 2.4 and 2.5). Addition of KIC to the incubation media decreased ($P<0.01$) the intracellular concentration of tyrosine but had no effect ($P>0.05$) on its release from EDC muscles (Tables 2.4 and 2.5). The addition of 0.5 mM KIC had no effect ($P>0.05$) on the release and intracellular concentrations of other amino acids (data not shown). The increase in the formation of leucine was similar to the decrease in the amount of the other metabolizable amino acids (Table 2.5).

The net rates of transamination of leucine and valine were not influenced ($P>0.05$) by the addition of 0.3 mM NH_4Cl to the incubation medium (Table 2.3). NH_4Cl at 0.3 mM decreased ($P<0.01$) the release and intracellular concentration of glutamate plus alanine but increased ($P<0.01$) the release and intracellular concentration of glutamine in EDC muscles (Table 2.6). The decrease in the amount of glutamate and alanine was essentially equal to the increase in the amount of glutamine (Table 2.6). The release and intracellular concentration of tyrosine in the EDC muscle was not influenced by 0.3 mM NH_4Cl (Table 2.6).

D. Discussion

Alanine and glutamine are released from chick skeletal muscle to a greater extent than would be expected from their abundance in muscle proteins, suggesting that they are synthesized de novo in this tissue. This suggestion is supported by the following observations. (1) The ratios of alanine:tyrosine (an amino acid not metabolized in chick skeletal muscle, our unpublished observations) and (glutamate plus glutamine):tyrosine released to the incubation medium were much higher than those present in muscle proteins. (2) Addition of BCAA to the incubation medium markedly increased the net formation of glutamate, glutamine and alanine (Table 2.1). (3) L-Cycloserine (1.5 mM) inhibited BCAA transamination (Table 2.3) and decreased the formation of glutamate, glutamine and alanine (Table 2.2). (4) KIC (0.1 and 0.5 mM) increased leucine formation, but decreased glutamate, alanine and glutamine formation (Tables 2.4 and 2.5). Indeed, the increase in the formation of leucine by 0.5 mM KIC is almost equal to the decrease in the total formation of glutamate, glutamine, alanine, aspartate, plus asparagine (Table 2.5).

Leucine is strictly ketogenic and does not make a net contribution of carbon skeletons to the synthesis of alanine and glutamine [15,23]. Thus, leucine increases the formation of glutamate, alanine and glutamine by donating amino groups. Although valine and isoleucine may contribute 4-carbon intermediates to the Krebs cycle, they do not increase the formation of alanine and glutamine to a greater extent than does leucine (Table 2.1). These results suggest that valine and isoleucine as well as leucine make a net contribution of nitrogen rather than carbon to the synthesis of

alanine and glutamine in chick skeletal muscle. Similarily, the net transamination rate of valine is lower than that of leucine (Table 2.3) which is closely reflected in the lower rate of formation of alanine plus glutamine from valine than from leucine (Table 2.1).

It has been shown in rat skeletal muscle that glutamate formed by transamination of BCAA with α-ketoglutarate is the common precursor for *de novo* synthesis of alanine and glutamine [15]. Glutamate also appears to be the common precursor in chick skeletal muscle as indicated by the following findings from this study. (1) KIC (0.1 and 0.5 mM) increased transamination of glutamate to form leucine, resulting in decreased intracellular concentration of glutamate (Tables 2.4 and 2.5). This decrease in intracellular glutamate concentration reduced the synthesis of alanine and glutamine (Tables 2.4 and 2.5). The ability of KIC to markedly decrease the release and intracellular concentrations of glutamate, alanine and glutamine is not simply due to an inhibition of net muscle protein degradation since 0.1 mM KIC had no significant effect on the net rate of protein degradation in EDC muscles as indicated by no change in intracellular tyrosine concentration or tyrosine release (Table 2.4). (2) NH_4Cl (0.3 mM), which had no effect on either the net rate of BCAA transamination (Table 2.3) or on the net rate of protein degradation in EDC muscle, increased the release and intracellular concentration of glutamine while concomitantly decreasing the release and intracellular concentrations of alanine and glutamate (Table 2.6). The decrease in the intracellular concentration of glutamate likely results from the increased conversion of glutamate to glutamine in the presence of elevated

concentrations of NH_4^+, which would also result in a decreased availability of glutamate for alanine synthesis. Similar effects of NH_4Cl have also been shown in the perfused rat hindquarter [24] and in the isolated rat skeletal muscle [1].

In conclusion, the present study clearly shows that large amounts of alanine and glutamine are synthesized de novo in chick skeletal muscle, thus accounting for their release from hindquarters of domestic fowl [7]. Our results demonstrate that BCAA provide the amino groups for *de novo* synthesis of alanine and glutamine and that glutamate is the common precursor for this process in chick skeletal muscle as previously shown in mammalian skeletal muscle [15]. These findings are in contrast to the early observations of Wu [17] that avian skeletal muscle does not contain glutamine synthetase.

Table 2.1. Effect of leucine, isoleucine and valine on the release and intracellular concentrations of glutamate, glutamine and alanine in fed chick EDC muscle.

EDC muscles were incubated in Krebs-Ringer bicarbonate buffer containing plasma concentrations of all amino acids except glutamate, glutamine, alanine, tyrosine, histidine and BCAAs, unless indicated. The results are expressed as mean ± SEM. ** treatment means significantly (P<0.01) different from control means obtained from the contralateral muscles as tested by the paired t-test. † mean significantly different (P<0.01) from those for 0.5 mM leucine or isoleucine treatments as tested by one-way variance analysis. n=10.

Additions	Release (nmol/mg tissue/2h)			Intracellular concentration (nmol/mg tissue)			Net formation (Treatment-Control) (nmol/mg tissue/2h)
	Glu	Gln	Ala	Glu	Gln	Ala	Glu + Gln + Ala
Control	0.14 ±0.02	1.66 ±0.12	1.15 ±0.09	1.19 ±0.15	1.79 ±0.21	1.27 ±0.17	-
0.5 mM Leucine	0.32** ±0.03	2.40** ±0.15	1.56** ±0.22	1.53** ±0.11	2.44** ±0.19	1.52** ±0.12	+ 2.57±0.38
Control	0.16 ±0.04	1.71 ±0.31	1.16 ±0.17	1.10 ±0.09	1.93 ±0.24	1.32 ±0.21	-
0.5 mM Isoleucine	0.29** ±0.05	2.39** ±0.36	1.43** ±0.19	1.47** ±0.23	2.58** ±0.15	1.66** ±0.31	+ 2.44±0.25
Control	0.17 ±0.02	1.82 ±0.17	1.24 ±0.08	1.05 ±0.16	1.64 ±0.29	1.15 ±0.16	-
0.5 mM Valine	0.30** ±0.03	2.10** ±0.31	1.56** ±0.22	1.32** ±0.28	2.02** ±0.36	1.42** ±0.25	+ 1.65±0.29†

Table 1.2. Effect of the branched-chain amino acids and L-cycloserine on the release and intracellular concentrations of glutamate, glutamine and alanine in fed chick EDC muscles.

EDC muscles were incubated in Krebs-Ringer bicarbonate buffer containing plasma concentrations of all amino acids except glutamate, glutamine, alanine, serine, histidine and BCAA. BCAA (0.5 mM leucine, 0.25 mM isoleucine and 0.5 mM valine) or BCAA plus 1.5 mM L-cycloserine (CS) were added to the incubation media as indicated. The results are expressed as mean ± SEM. ** treatment means significantly (P<0.01) different from the control means obtained from the contralateral muscles as tested by the paired t-test. n=10

Additions	Release (nmol/mg tissue/2h)			Intracellular concentration (nmol/mg tissue)					
	Glu	Gln	Ala	Glu	Gln	Ala	Leu	Ile	Val
None	0.13 ±0.04	1.87 ±0.28	1.18 ±0.24	1.13 ±0.14	2.01 ±0.34	1.27 ±0.27	0.07 ±0.01	0.04 ±0.01	0.08 ±0.01
BCAA	0.34** ±0.03	2.47** ±0.21	1.53** ±0.30	1.60** ±0.16	2.66** ±0.34	1.62** ±0.25	0.47** ±0.02	0.26** ±0.01	0.51** ±0.01
BCAA	0.39 ±0.02	2.64 ±0.19	1.65 ±0.13	1.75 ±0.29	2.81 ±0.18	1.73 ±0.10	0.49 ±0.02	0.26 ±0.01	0.54 ±0.03
BCAA +CS	0.30** ±0.05	2.23** ±0.36	1.25** ±0.19	1.46** ±0.23	2.47** ±0.15	1.37** ±0.31	0.67** ±0.02	0.37** ±0.02	0.71** ±0.04

Table 2.3. Effect of NH_4Cl and L-cycloserine (CS) on net transamination of leucine and valine in fed chick EDC muscles.

See Tables 1 and 2 for details of experimental design. [1-^{14}C]leucine (208.3 dpm/nmol) or [1-^{14}C]valine (186 dpm/nmol) was added to the incubation medium as described in the text. The results are expressed as mean ± SEM. ** treatment means within the column significantly ($P<0.01$) different from control means obtained from the contralateral muscles as tested by the paired t-test. † mean values for valine significantly different ($P<0.01$) from means for leucine within a row as tested by the one-way variance analysis. n=10

Additions	Leucine		Valine	
	intracellular specific radioactivity (dpm/nmol)	Net transamination (nmol/mg tissue/2 h)	intracellular specific radioactivity (dpm/nmol)	Net transamination (nmol/mg tissue/2 h)
0.5 mM Leucine	151.2±2.3	2.35±0.22		
0.5 mM Valine			127.7±2.9	1.41±0.15†
BCAA	170.8±3.7	1.12±0.08	159.2±2.7	0.54±0.04†
BCAA+0.3 mM NH_4Cl	161.2±4.5	1.08±0.10	150.1±3.5	0.53±0.05†
BCAA	172.3±3.6	1.09±0.06	148.7±3.1	0.56±0.07†
BCAA+1.5 mM CS	185.8±5.3	0.40±0.03**	163.2±4.1	0.24±0.01**†

Table 2.4. Effect of KIC on the release and intracellular concentrations of glutamate, glutamine, alanine and tyrosine in fed chick EDC muscle. EDC muscles were incubated in Krebs-Ringer bicarbonate buffer containing 0.5 mM leucine, 0.25 mM isoleucine, 0.5 mM valine and physiological concentrations of other amino acids except glutamate, glutamine, alanine, tyrosine and histidine. KIC was present or absent from incubation media as indicated. The results are expressed as mean ± SEM. *P<0.05 and **P<0.01: treatment means significantly different from control (0 mM KIC) means obtainnd from the contralateral muscles as tested by the paired t-test. n=10.

Medium [KIC] (mM)	Release (nmol/mg tissue/2h)				Intracellular concentration (nmol/mg tissue)				
	Glu	Gln	Ala	Tyr	Glu	Gln	Ala	Tyr	Leu
0	0.40 ±0.03	[illegible] ±0.12	1.57 ±0.12	0.030 ±0.005	1.87 ±0.11	2.86 ±0.22	1.73 ±0.16	0.059 ±0.007	1.44 ±0.07
0.1	0.35** ±0.04	2.55* ±0.20	1.31* ±0.13	0.034 ±0.005	1.57* ±0.09	2.51* ±0.27	1.48* ±0.13	0.053 ±0.008	0.[illegible]** ±0.02
0	0.44 ±0.04	2.79 ±0.27	1.88 ±0.14	0.037 ±0.007	1.65 ±0.08	2.79 ±0.38	1.62 ±0.15	0.060 ±0.009	0.46 ±0.02
0.5	0.33** ±0.03	2.35** ±0.21	1.36** ±0.10	0.032 ±0.006	1.25** ±0.06	1.83** ±0.25	1.17** ±0.05	0.037** ±0.006	1.03** ±0.04

Table 2.5. Effect of KIC on the release and intracellular concentrations of metabolizable amino acids and tyrosine in fed chick EDC muscles.

EDC muscles were incubated in Krebs-Ringer bicarbonate buffer containing no amino acids. KIC was absent or present at 0.5 mM in the incubation media as indicated. The results are expressed as mean ± SEM. TAA : Asp+Glu+Asn+Gln+Ala. *P<0.05 and **P<0.01: treatment means significantly different from control (0 mM KIC) means obtained from the contralateral muscles as tested by the paired t-test. n=10.

Amino acid	Control (0 mM KIC)		0.5 mM KIC	
	Release (nmol/mg tissue/2h) (C_r)	Intracellular (nmol/mg tissue) (C_i)	Release (nmol/mg tissue/2h) (T_r)	Intracellular (nmol/mg tissue) (T_i)
Asp	0.03±0.00	0.63±0.03	0.03±0.00	0.46±0.02**
Glu	0.13±0.02	1.29±0.04	0.11±0.02*	0.94±0.03**
Asn	0.08±0.01	0.25±0.02	0.09±0.01	0.21±0.01**
Gln	1.38±0.13	1.76±0.12	1.02±0.12**	1.32±0.11**
Ala	0.53±0.05	1.21±0.11	0.37±0.04**	0.82±0.01**
Leu	0.05±0.01	0.08±0.00	1.53±0.12**	0.36±0.01**
Tyr	0.07±0.01	0.09±0.01	0.07±0.01	0.07±0.01**
Net change $[(T_r+T_i)-(C_r+C_i)]$				
Leu	+1.77±0.24			
TAA	-1.95±0.29			

Table 2.6. Effect of NH_4Cl on the release and intracellular concentrations of glutamate, glutamine, alanine and tyrosine in fed chick EDC muscles.

See Table 4 for details of experimental design. The results are expressed as mean ± SEM. ** treatment means significantly ($P<0.01$) different from the control (0 mM NH_4Cl) means obtained from the contralateral muscles as tested by the paired t-test. n=10.

Medium [NH_4Cl] (mM)	Release (nmol/mg tissue/2h)				Intracellular concentrations (nmol/mg tissue)				Net change (nmol/2 h/ mg tissue)	
	Glu	Gln	Ala	Tyr	Glu	Gln	Ala	Tyr	Gln	Glu+Ala
0	0.39 ±0.04	2.66 ±0.22	1.69 ±0.14	0.029 ±0.007	1.70 ±0.06	2.98 ±0.19	1.84 ±0.29	0.045 ±0.009	- -	- -
0.3	0.25** ±0.02	3.67** ±0.16	1.18** ±0.08	0.033 ±0.009	1.08** ±0.11	3.94** ±0.28	1.27** ±0.13	0.053 ±0.007	+1.97 ±0.23	-1.86 ±0.28

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III. Effect Of Ketone Bodies On Alanine And Glutamine Metabolism In Chick Skeletal Muscle [1]

A. Introduction

Alanine and glutamine are the predominant amino acids released by skeletal muscles of rats [1-3], humans [4] and chicks [5] and play an important role in the interorgan metabolism of nitrogen and carbon during fasting and acidosis [4,6,7]. It has been demonstrated that branched-chain amino acids (BCAA) provide amino groups for de novo synthesis of alanine and glutamine in skeletal muscles of rats [8-14] and chicks (Chapter 2). However, it remains unclear whether the major source of carbon for alanine synthesis is glycolysis [8,12,13] or amino acids which generate four carbon intermediates of the tricarboxylic acid cycle [2,3,14].

Ketone bodies have been implicated to play a role in alanine and glutamine metabolism in skeletal muscle [15-18]. The infusion of DL-ß-hydroxybutyrate has been found to decrease plasma concentrations of alanine in fasted man [19] and dogs [20], suggesting decreased production and release of alanine from skeletal muscle [19,20]. Similarly, studies in vitro have demonstrated that acetoacetate and DL-ß-hydroxybutyrate suppress the formation of alanine [15,17,18] and glutamine [15] in skeletal muscles from fasted rats. In addition, ketone bodies have been reported to decrease alanine release from cultured rat skeletal muscle cells [21] and from perfused rat hindquarters [22]. The mechanisms by which ketone bodies regulate

1. A version of this chapter has been published. Wu, G. and Thompson, J.R.(1988) Biochem. J. 255, 139-144.

amino acid metabolism have not been elucidated.

It has been suggested that ketone bodies decrease alanine formation in fasted rat diaphragms in vitro [15] and in fasted dogs in vivo [20] by decreasing tissue concentration of pyruvate due to inhibition of glycolysis [15,20]. But, neither the rate of glycolysis nor the intracellular concentration of pyruvate was measured in these studies. The possible role of glycolysis has been questioned since ketone bodies have been found to decrease alanine release from cultured rat skeletal muscle cells [21] and perfused rat hindquarters [22] without influencing either the rate of glycolysis or intracellular pyruvate concentration in these preparations [21,22]. Thus, since ketone bodies have been shown to inhibit BCAA metabolism in perfused hindquarters [23] and incubated diaphragms [24] of rats and to decrease intracellular glutamate concentration in cultured rat skeletal muscle cells [21], a number of researchers have suggested that ketone bodies decrease alanine synthesis in skeletal muscle by directly inhibiting BCAA transamination [17,21]. This suggestion appears to be supported by our recent findings that ketone bodies markedly inhibit BCAA transamination in skeletal muscle from fasted chicks [25]. The present study was therefore conducted to test this hypothesis in an attempt to determine how ketone bodies regulate alanine and glutamine metabolism in skeletal muscles from fasted chicks.

B. Materials and Methods

Chemicals.

L-[1-^{14}C]leucine, L-[1-^{14}C]valine and L-[U-^{14}C]alanine

were purchased from ICN Radiochemicals, Montreal, Que. D-[5-^{3}H]glucose and 3-*O*-[methyl-^{14}C]methylglucose were purchased from American Radiolabeled Chemicals Inc., St. Louis, MO. L lactic dehydrogenase and all other chemicals used were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Animals.

Day-old male broiler chicks (Hubbard strain, *Gallus domesticus*) were obtained from a local hatchery and allowed free access to food and water. At 9 days of age, chicks weighing 115-125 g were fasted for 24 h before use.

Muscle preparations and incubation.

Individual fasted chicks were anesthetised with halothane. The extensor digitorum communis (EDC) muscles (15.8 ± 0.2 mg, mean ± S.E.M., n=20) were dissected and placed in 3.5 ml Krebs-Ringer bicarbonate buffer saturated with O_2:CO_2 (95%:5%) [25]. The buffer also contained insulin (0.01 U/ml) and 2 mM Hepes (basal medium). Glucose and amino acids were present or absent in the incubation media depending on the experimental design. In all experiments, one EDC muscle was used for the control while the contralateral muscle was used for the treatment. The muscles were preincubated for 30 min and then incubated for 2 h in fresh media at 37^{0}C [25], with additions as indicated in Tables 3.1-3.5.

Measurement of the release of glutamate, glutamine and alanine and intracellular concentrations of amino acids

In experiments designed to measure the amounts of alanine, glutamate, glutamine and tyrosine in the muscle proteins, in the intracellular free pool and released to the incubation media, EDC

muscles were incubated in the basal medium containing 12 mM glucose as described above. In experiments designed to determine the effect of pyruvate and ketone bodies on amino acid metabolism, the basal media contained 12 mM glucose, 0.5 mM leucine, 0.5 mM valine, and all other amino acids at plasma concentrations [26] except for alanine, glutamine, glutamate, tyrosine and histidine which were absent (medium B). Medium B containing either 0 or 12 mM glucose was used in studies of the effect of glucose on amino acid metabolism. At the end of the final incubation period, muscles were placed in ice-cold 2% trichloroacetic acid. The incubation media and the muscles were stored at $-70^{0}C$ and amino acids were analyzed within 2 weeks by h.p.l.c. as described previously [25,27].

Measurement of branched-chain amino acid transamination.

EDC muscles were incubated in medium B containing 0.5 mM L-[1-^{14}C]leucine (400 dpm/nmol) or 0.5 mM L-[1-^{14}C]valine (400 dpm/nmol) as described above. At the end of the 2-h final incubation, $^{14}CO_2$ was collected in Hyamine hydroxide (11,12,25) and the net transamination rates of leucine and valine were calculated as described previously [25,28].

Measurement of alanine oxidation

EDC muscles were incubated in medium B containing 1.0 mM L-[U-^{14}C]alanine (106 dpm/nmol) as described above. At the end of the final incubation, muscles were placed in 1 M $HClO_4$ and $^{14}CO_2$ was collected from the incubation media in Hyamine hydroxide [25]. Neutralized muscle extracts were passed through a column (1.0 x 13 cm) containing Dowex 1-10X resin (formate form) to separate alanine from the other metabolizable amino acids and

Krebs-cycle intermediates [29-31]. The fractions collected contained alanine, glutamine and asparagine but not glutamate, aspartate, lactate or pyruvate as verified by h.p.l.c. for amino acids [25,27] and by an enzymatic fluorimetic assay for lactate and pyruvate [32]. To convert glutamine and asparagine to glutamate and aspartate, respectively, each fraction was incubated with 0.5 ml 6 N HCl under N_2 at 110^0C for 10 h. The neutralized solution was passed through a Dowex 1-10X (formate form) column as described above. One ml of the fraction containing alanine was counted for radioactivity [25], while 0.2 ml was assayed for alanine by h.p.l.c. [25,27]. The specific activity of intracellular L-[U-^{14}C]alanine was calculated after correcting for that of alanine in the extracellular space [25] and was used to calculate the rate of alanine oxidation.

Measurement of glucose uptake and glycolysis.

Glucose uptake was measured in EDC muscles incubated in medium B containing 5 mM 3-*O*-[methyl-^{14}C]methylglucose (38 dpm/nmol) [33-35]. At the end of the 2-h incubation, each EDC muscle was well rinsed with nonradioactive incubation media, solubilized in 0.5 ml Soluene and counted for radioactivity [25]. The intracellular radioactivity of 3-*O*-[methyl-^{14}C]methylglucose was calculated after correcting for that in the extracellular space [25].

Glycolysis was measured in EDC muscles incubated in medium B containing 12 mM D-[5-^{3}H]glucose (50 dpm/nmol) [8]. At the end of the 2-h incubation, [^{3}H]-water accumulated in the incubation media was separated from D-[5-^{3}H]glucose and the rate of glycolysis was calculated as described by Chang and Goldberg [8].

Measurement of the intracellular concentration and release of pyruvate

EDC muscles were incubated in medium B as described above. At the end of the 2-h final incubation, each muscle was placed in 0.85 ml ice-cold 1.0 M $HClO_4$ and the incubation medium was acidified with 0.2 ml 1.0 M $HClO_4$. Neutralized muscle extracts and media were analyzed immediately for pyruvate [32]. The intracellular concentration of pyruvate was calculated after correcting for pyruvate in the extracellular space [25].

Statistical analysis of results

All results, given as mean ± S.E.M., were analyzed by comparing the mean value of the treatment muscles with that of the contralateral control muscles by the paired t-test as described by Steel and Torrie [36].

C. Results

Effects of ketone bodies, glucose and pyruvate on the intracellular concentration and release of glutamate, glutamine and alanine.

A relatively large amount of alanine and glutamine and a relatively small amount of glutamate are released from fasted chick EDC muscles during a 2-h final incubation period (Table 3.1; Appendix 2). The intracellular concentrations of these amino acids remain constant throughout the final incubation (Appendix 3). The ratio of alanine:tyrosine in the incubation media and in the intracellular free amino acid pool is 10.3 ± 0.4 (n=10) and 13.3 ± 0.7 (n=10), respectively, which is much higher ($P<0.001$) than that in EDC muscle proteins (5.0 ± 0.2, n=10). The ratio of (glutamate+glutamine):

tyrosine in the incubation media and in the intracellular free pool is 12.2 ± 0.8 (n=10) and 22.7 ± 1.0 (n=10), respectively, which is also much higher (P<0.001) than that in EDC muscle proteins (6.1 ± 0.3, n=10).

Glucose (12 mM) decreased (P<0.05) the intracellular concentration and release of glutamate and glutamine but increased (P<0.001) the intracellular concentration and release of alanine in EDC muscles incubated in the absence of ketone bodies (Table 3.1). In the presence of 4 mM ketone bodies, 12 mM glucose had no effect on the intracellular concentration and release of glutamine and alanine but increased (P<0.01) the concentration and release of glutamate (Table 3.1).

Ketone bodies (4 mM) markedly increased (P<0.01) the intracellular concentration and release of glutamate and glutamine, but decreased (P<0.001) the intracellular concentration and release of alanine in EDC muscles incubated with 12 mM glucose (Table 3.1). Similar results were obtained with 1 mM acetoacetate while 1 mM DL-ß-hydroxybutyrate slightly increased (P<0.05) the intracellular concentration and release of glutamate, glutamine and alanine (Appendix 4). However, ketone bodies (4 mM) did not influence (P>0.05) the intracellular concentration and release of glutamine and alanine in EDC muscles incubated with 5 mM pyruvate (Table 3.1).

Effect of ketone bodies on intracellular concentrations of aspartate and the branched-chain amino acids.

Acetoacetate (4 mM) and DL-ß-hydroxybutyrate (4 mM) decreased (P<0.001) the intracellular concentration of aspartate by 38% and 28%, but increased (P<0.001) the intracellular concentrations of

valine, isoleucine and leucine by 42%-47% and 25-27%, respectively, in EDC muscles incubated with 12 mM glucose (Table 3.2). However, 4 mM ketone bodies had no effect (P>0.05) on the concentration of these amino acids in EDC muscles incubated in the presence of 5 mM pyruvate (Table 3.2). In addition, 1 and 4 mM ketone bodies did not influence (P>0.05) the intracellular concentration of asparagine and amino acids not metabolized in fasted chick EDC muscles (data not shown).

Effect ketone bodies and pyruvate on BCAA transamination

Ketone bodies (4 mM) inhibited (P<0.001) the net transamination rates of leucine and valine by 40-52% in EDC muscles incubated in the absence of pyruvate (Table 3.3). The addition of 5 mM pyruvate (5 mM) to the incubation media prevented the inhibiting effect of ketone bodies on BCAA transamination (Table 3.3).

Effect of ketone bodies on glucose uptake, glycolysis, and the intracellular concentration and release of pyruvate

Acetoacetate and DL-ß-hydroxybutyrate (1 and 4 mM) did not influence (P>0.05) 3-*O*-[methyl-^{14}C]methylglucose uptake by EDC muscles (Table 3.4). However, acetoacetate (4 mM) and DL-ß-hydroxybutyrate (4 mM) decreased (P<0.001) the rate of glycolysis by 41% and 34%, the intracellular concentration of pyruvate by 61% and 50%, and the release of pyruvate by 64% and 52%, respectively, in EDC muscles incubated with 12 mM glucose (Table 3.4). Ketone bodies at 1 mM also decreased (P<0.01) the rate of glycolysis and the intracellular concentration and release of pyruvate (Table 3.4).

Effect of ketone bodies on alanine oxidation

Acetoacetate (4 mM) and DL-ß-hydroxybutyrate (4 mM) increased ($P<0.01$) the specific activity of intracellular L-[U-^{14}C]alanine by 75% and 19%, and decreased ($P<0.001$) the rate of alanine oxidation by 48% and 53%, respectively, in chick EDC muscles (Table 3.5). Ketone bodies at 1 mM also inhibited ($P<0.001$) the rate of alanine oxidation in EDC muscles incubated in the presence of 12 mM glucose (Table 3.5).

DISCUSSION

The present study demonstrates that alanine and glutamine are released by skeletal muscles from fasted chicks to a much greater extent than would be expected from their abundance in muscle proteins. Although alanine oxidation is inhibited by ketone bodies (Table 3.5), which has not been reported in other species, the intracellular concentration and the release of alanine in fasted chick EDC muscles are markedly decreased by 1 and 4 mM acetoacetate and 4 mM DL-ß-hydroxybutyrate (Table 3.1). Our results indicate that ketone bodies suppress alanine synthesis in chick skeletal muscle as previously shown in rat skeletal muscle [15,17,18].

It is unlikely that inhibition of BCAA transamination by ketone bodies limits intracellular glutamate availability for alanine synthesis since the intracellular glutamate concentration is markedly increased by ketone bodies in fasted chick EDC muscles (Table 3.1). Thus, ketone bodies probably decrease alanine synthesis in skeletal muscles by decreasing intracellular pyruvate concentration, which may also result in decreased BCAA transamination due to decreased

regeneration of α-ketoglutarate. This conclusion is supported by the following observations. (1) Ketone bodies have no effect on glucose uptake but markedly inhibit the rate of glycolysis in fasted chick EDC muscles, resulting in a dramatic decrease in the intracellular concentration and release of pyruvate (Table 3.4). (2) Glucose increases alanine formation in EDC muscles incubated in the absence of ketone bodies but has no effect on alanine formation in EDC muscles incubated in the presence of 4 mM ketone bodies (Table 3.1). (3) The addition of pyruvate, which does not affect BCAA transamination in fasted chick EDC muscles incubated in the absence of ketone bodies [28], prevents the inhibiting effect of ketone bodies on BCAA transamination in fasted chick EDC muscles (Table 3.3) and on alanine formation in both fasted chick EDC muscles (Table 3.1) and fasted rat diaphragms [15].

The effect of ketone bodies on glutamine metabolism is not well documented in skeletal muscle. In the present study, it is found that ketone bodies markedly increase both the intracellular concentration and release of glutamine from fasted chick EDC muscles (Table 3.1). Our results are in sharp contrast to those of Palaiologos and Felig [15] who reported that ketone bodies decrease glutamine release from fasted rat hemidiaphragms. An increase in the intracellular concentration and in the release of glutamine by ketone bodies may be attributed to an increased intracellular glutamate concentration (Table 3.1) which has been shown to increase glutamine synthetase activity in rat skeletal muscle cells [37]. It is also interesting to note that an increase in both glutamine synthetase activity and glutamine release has been reported in skeletal muscles of diabetic

ketoacidotic rats in which concentrations of ketone bodies are elevated [38,39].

Increased release of glutamine at the expense of alanine may be of physiological significance to animals with high plasma levels of ketone bodies. Decreased release of alanine from skeletal muscles contributes to decreased plasma concentrations of alanine [4,19], which may result in decreased hepatic protein synthesis during prolonged starvation [40] since alanine has been implicated to be rate-limiting for hepatic protein synthesis [40]. On the other hand, an increase in the release of glutamine from skeletal muscle provides more glutamine for removal by the kidneys where it plays a key role in the excretion of hydrogen ions during acidosis [41]. Also, an increase in the release of glutamine from skeletal muscle may help to inhibit lipolysis and hence hepatic ketogenesis during fasting [42,43], thereby preventing a further increase in plasma levels of ketone bodies.

Table 3.1. Effect of ketone bodies, glucose and pyruvate on the intracellular concentrations and the release of glutamate, glutamine and alanine in EDC mucles from 24-h fasted chicks.

EDC muscles from fasted chicks were incubated in media containing no glucose (None) or glucose (Gluc) (12 mM) as described in the text. Acetoacetate (AcAc) (4 mM), DL-ß-hydroxybutyrate (HB) (4 mM), or pyruvate (Pyr) (5 mM) was added to the incubation media of the treatment muscles as indicated. Results are given as mean ± S.E.M. for 10 observations per treatment and statistically analyzed by the paired t test. Treatment means marked with * (P<0.05), **(P<0.01) and *** (P<0.001) are different from the control means obtained from the contralateral muscles.

Additions to incubation medium	Intracellular concentration (nmol/mg tissue)			Release (nmol/mg tissue/2 h)		
	Glutamate	Glutamine	Alanine	Glutamate	Glutamine	Alanine
None	1.56±0.09	2.43±0.19	1.13±0.05	0.46±0.03	2.44±0.17	1.18±0.08
Gluc	1.20±0.06**	2.06±0.15**	2.13±0.13***	0.36±0.02*	2.12±0.18**	2.16±[illegible]***
None	1.53±0.12	2.43±0.24	1.19±0.08	0.44±0.04	2.30±0.1[illegible]	1.20±0.17
Gluc + AcAc	2.87±0.14***	2.51±0.18	1.32±0.12	0.55±0.03**	2.40±0.23	1.44±0.19
None	1.49±0.13	2.27±0.19	1.25±0.15	0.40±0.02	2.34±0.09	1.21±0.16
Gluc + HB	2.06±0.16***	2.45±0.17	1.43±0.17	[illegible]**	2.51±0.15	1.59±0.20
Gluc	1.35±0.07	2.14±0.12	2.32±0.16	[illegible]0.04	1.98±0.11	2.55±[illegible]
Gluc + AcAc	3.67±0.11***	2.59±0.20**	1.29±0.11***	[illegible]**	2.41±[illegible]	[illegible]
Gluc	1.23±0.04	2.18±0.10	2.16±0.08	0.36±0.04	2.51±0.17	1.47±0.12
Gluc + HB	[illegible]***	[illegible]***	1.51±0.11***	[illegible]**	[illegible]	[illegible]
Gluc	1.36±0.07	2.36±0.15	[illegible]	[illegible]	[illegible]	[illegible]
Gluc + Pyr	[illegible]***	[illegible]**	2.64±0.22	[illegible]	[illegible]	2.72±0.26***
Gluc + Pyr	[illegible]	[illegible]0.14	[illegible]	[illegible]	[illegible]	[illegible]
Gluc + Pyr + AcAc	[illegible]***	[illegible]0.12	[illegible]	[illegible]	[illegible]0.14	[illegible]0.17
Gluc + Pyr	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]0.14
Gluc + Pyr + HB	1.62±[illegible]	[illegible]0.17	2.77±[illegible]	[illegible]	[illegible]	[illegible]

Table 3.2. Effect of ketone bodies on the intracellular concentration of aspartate and BCAA in EDC mucles from 24-h fasted chicks.

EDC muscles from fasted chicks were incubated as described in the text. Glucose (12 mM) was present in the media. Acetoacetate (AcAc) (4 mM), DL-β-hydroxybutyrate (HB) (4 mM) or pyruvate (Pyr) (5 mM) was added to the incubation media of the treatment muscles as indicated. Results are given as mean ± S.E.M. for 10 observations per treatment and statistically analyzed by the paired t-test. Treatment means marked with ** (P<0.01) and *** (P<0.001) are different from the control means obtained from the contralateral muscles.

Additions to incubation medium	Aspartate	Valine	Isoleucine	Leucine
	(nmol/mg tissue)			
None	1.12±0.04	0.42±0.01	0.19±0.01	0.38±0.02
AcAc	0.69±0.02***	0.61±0.02***	0.28±0.01***	0.54±0.02***
None	1.08±0.06	0.44±0.01	0.21±0.01	0.41±0.01
HB	0.78±0.05**	0.55±0.02***	0.27±0.02***	0.52±0.03***
Pyr	0.95±0.02	0.45±0.04	0.20±0.02	0.39±0.03
Pyr + AcAc	0.94±0.06	0.47±0.06	0.21±0.03	0.40±0.02
Pyr	0.99±0.04	0.43±0.05	0.20±0.02	0.40±0.05
Pyr + HB	1.03±0.04	0.46±0.04	0.22±0.03	0.41±0.04

Table 3.3. The effect of pyruvate and ketone bodies on transamination of leucine and valine in EDC muscles from 24-h fasted chicks.

EDC muscles from fasted chicks were incubated with L-[1-^{14}C]leucine or L-[1-^{14}C]valine for 2 h as described in the text. Glucose (12 mM) was present in the incubation media. Pyruvate (Pyr) (5 mM), acetoacetate (AcAc) (4 mM) or DL-ß-hydroxybutyrate (HB) (4 mM) was added to the incubation media of the treatment muscles as indicated. Results are given as mean ± S.E.M. for 12 observations per treatment and statistically analyzed by the paired t-test. Treatment means marked with *** (P<0.001) are different from the control means obtained from the contralateral muscles.

Additions to incubation medium	Net rate of transamination (nmol/mg tissue/2 h)	
	Leucine	Valine
None	1.46±0.08	0.80±0.09
AcAc	0.66±0.04***	0.38±0.06***
None	1.40±0.14	0.76±0.07
HB	0.78±0.06***	0.44±0.04***
Pyr	1.34±0.11	0.72±0.04
Pyr + AcAc	1.30±0.08	0.68±0.04
Pyr	1.42±0.10	0.80±0.08
Pyr + HB	1.36±0.07	0.82±0.10

Table 3.4. The effect of ketone bodies on 3-*O*-methylglucose uptake, glycolysis, and on the intracellular concentration and release of pyruvate in EDC muscles from 24-h fasted chicks.

EDC muscles were incubated with 5 mM 3-*O*-[methyl-^{14}C]-methylglucose and 12 mM glucose to measure glucose uptake or with 12 mM D-[5-^{3}H]glucose to measure glycolysis as described in the text. Acetoacetate (AcAc) (1 or 4 mM) or DL-ß-hydroxybutyrate (HB) (1 or 4 mM) was added to the incubation media of the treatment muscles as indicated. The results are given as mean ± S.E.M. for 6 observations per treatment and statistically analyzed by the paired t-test. Treatment means marked with *** (P<0.001) are different from the control means obtained from the contralateral muscles.

Additions to incubation medium	Uptake of 3-*O*-[methyl-^{14}C]methyl-glucose (dpm/mg tissue/2h)	Rate of glycolysis (nmol glucose/mg tissue/2h)	Pyruvate	
			Intracellular Concentration (nmol/mg tissue)	Release (nmol/mg tissue/2h)
None	111.2±2.4	23.80±0.72	0.28±0.05	5.67±0.48
1 mM AcAc	108.9±2.3	17.38±0.68***	0.16±0.02***	3.01±0.23***
None	113.4±3.3	23.32±0.92	0.31±0.03	5.49±0.27
4 mM AcAc	111.6±1.5	13.68±0.80***	0.12±0.01***	1.95±0.16***
None	108.1±3.4	22.12±0.88	0.25±0.03	5.20±0.23
1 mM HB	109.6±2.8	19.40±0.68**	0.19±0.02***	4.07±0.25***
None	110.8±1.9	23.36±1.10	0.28±0.02	5.35±0.34
4 mM HB	114.2±2.8	15.40±0.82***	0.14±0.01***	2.55±0.32***

Table 3.5. The effect of ketone bodies on alanine oxidation in EDC muscles from 24-h fasted chicks.

EDC muscles from fasted chicks were incubated with 1.0 mM L-[U-^{14}C]alanine for 2 h as described in the text. Glucose (12 mM) was present in the incubation medium. Acetoacetate (AcAc) (1 or 4 mM) or DL-ß-hydroxybutyrate (HB) (1 or 4 mM) was added to the incubation media of the treatment muscles as indicated. The results are given as mean ± S.E.M. for 6 observations per treatment and statistically analyzed by the paired t-test. Treatment means marked with ** (P<0.01) and *** (P<0.001) are different from the control means obtained from the contralateral muscles.

Additions to incubation medium	Specific activity of intracellular alanine (d.p.m./nmol)	Alanine oxidation (nmol/mg tissue/2h)
None	59.5±3.8	1.35±0.20
1 mM AcAc	68.7±2.5**	0.86±0.12***
None	55.4±3.1	1.22±0.0
4 mM AcAc	69.0±2.5**	0.63±0.0/***
None	61.1±6.6	1.28±0.16
1 mM HB	63.9±5.5	0.88±0.09***
None	63.2±4.1	1.26±0.13
4 mM HB	75.3±3.8**	0.59±0.06***

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IV. Glutamine Metabolism In Skeletal Muscle[1]

A. Introduction

Glutamine is the most abundant free amino acid in plasma and skeletal muscle, and is a key precursor for synthesis of compounds such as proteins, ATP, nucleotides and NAD [1,2]. It is a major carrier of nitrogen for interorgan transport and for detoxification of ammonia and plays an important role in acid/base balance [2]. These diverse roles of glutamine have prompted extensive studies of its metabolism in a variety of mammalian tissues and cells [2-3]. Studies during the last ten years have shown that glutamine is not only extensively oxidised in the kidney [4], small intestine [5], reticulocytes [6], certain tumor cells [3], macrophages [7], lymphocytes [8] and cultured cell lines [9], but also is a major source of energy for these tissues and cells [3-9].

Glutamine degradation can be initiated by either glutaminase or glutamine aminotransferase [1]. (Fig. 4.1). The phosphate-activated glutaminase appears to be exclusively located within the mitochondria [3] and is known to play a major role in initiating glutamine metabolism in mammalian kidneys [4], small intestine [5], brain [10], liver [11], tumor cells [3] and lymphocytes [8]. A phosphate-independent, but maleate-activated glutaminase has been described in rat kidneys, brain, liver [12] and small intestine [13], but does not appear to be related to glutamine oxidation in these

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tissues [3-5,11,13]. On the other hand, glutamine aminotransferase K and L, which are distinguished on the basis of their relative activities towards phenylpyruvate and L-albizziin, have also been suggested to play an important role in glutamine metabolism in mammalian tissues such as the kidneys and the liver [1,14].

It is generally considered that the kidneys and intestine are the major sites for glutamine utilization while the brain and particularly skeletal muscle are the major sites for glutamine synthesis in the body [1-5]. Since skeletal muscle is the major source of glutamine in postabsorptive states [15], much effort has been directed towards studies of glutamine synthesis in this tissue [16]. On the other hand, glutamine oxidation in skeletal muscle is believed to be quantitatively unimportant [5,20] on the basis of early observations that glutaminase activity was negligible or absent in rat skeletal muscle [19-20]. Thus, little attention has been paid to glutamine oxidation in this tissue. However, glutamine degradation can be initiated by glutamine aminotransferase K and L to form α-ketoglutaramate, which is then hydrolysed to α-ketoglutarate by ω-amidase [1] (Fig. 4.1). Both glutamine aminotransferases K and L and ω-amidase are widely distributed in rat tissues including skeletal muscle [14,21,22]. Thus, such a view that glutamine oxidation in skeletal muscle is negligible [16-20] is open to reconsideration. In addition, there is no information concerning glutamine degradation, glutaminase or glutamine aminotransferase in avian skeletal muscle. The present study was therefore designed to investigate glutamine oxidation in skeletal muscles using the domestic chicken and the laboratory rat as

experimental animals.

Materials and Methods.

Chemicals

L-[1-^{14}C]leucine was obtained from ICN Radiochemicals, Montreal, Quebec. L-[1-^{14}C]glutamic acid and L-[U-^{14}C]glutamine were obtained from American Radiolabeled Chemicals Inc., St. Louis. The radiochemical purity of these compounds was determined to be greater than 99% by descending paper chromatography [23]. L-Albizziin, β-phenylpyruvate, α-keto-γ-methiolbutyrate and other chemicals were obtained from Sigma Chemicals Co., St. Louis, U.S.A.

L-[1-^{14}C]glutamine was prepared from L-[1-^{14}C]glutamic acid essentially as previously described [24], except that the glutamic acid concentration in the reaction mixtures was 0.45 mM and that the source of glutamine synthetase was chick pectoralis muscle cytosol obtained by centrifugation of muscle homogenates (1 g muscle in 4 ml 0.9% NaCl) at 15000 X g, 4^{0}C for 10 min. Almost all L-[1-^{14}C]glutamic acid was converted to L-[1-^{14}C]glutamine, which was separated from its labelled precursor by Dowex anion exchange chromatography [25]. The radiochemical purity of L-[1-^{14}C]glutamine was determined to be 98.7% by paper chromatography [23]. ^{14}C-glutamine and ^{14}C-leucine were purified immediately before use as previously described [25].

Animals

Nine-day-old male broiler chicks (*Gallus domesticus*) of the Hubbard strain (115-130 g) and young female Sprague Dawley rats of

the Buffalo strain (60-80 g) were used. Animals were offered feed and water *ad libitum*. Chicks were fed a broiler starter ration containing 23% crude protein. Rats were fed laboratory chow (Continental Grain Co., Chicago) containing 24% crude protein.

Skeletal muscle preparations and incubation.

Chick extensor digitorum communis (EDC) and pectoralis muscles [23], and rat extensor digitorum longus (EDL), soleus, quarter-diaphragm and gastrocnemius muscles [26] were dissected. Rat quarter-diaphragm, EDL, soleus, and chick EDC muscles were preincubated for 30 min in 3.5 ml Krebs-Ringer bicarbonate buffer saturated with O_2/CO_2 (95%:5%) [23] and then transferred to fresh medium of similar composition and incubated for 2 h at 37°C. The media for rat muscles also contained insulin (0.1 unit/ml), 2 mM HEPES (pH 7.4), 5 mM glucose and amino acids at concentrations found in rat plasma (Appendix 1). The media for chick muscles also contained insulin (0.01 unit/ml), 2 mM HEPES, 12 mM glucose and amino acids at concentrations found in chick plasma [23] (Appendix 1), unless otherwise specified in the Tables.

Measurement of glutamine and leucine degradation

For measurement of glutamine oxidation, L-[1-^{14}C]glutamine or L-[U-^{14}C]glutamine (150 dpm/μl) was included in the incubation media containing glutamine at concentrations as indicated in the Tables. At the end of the 2-h final incubation, muscles were placed in 0.75 ml 1.5 M $HClO_4$. $^{14}CO_2$ was collected from the incubation medium in Hyamine hydroxide [23]. Calculations of CO_2 production from glutamine oxidation were based on the specific radioactivity of ^{14}C-glutamine (dpm/nmol labelled C) in the medium or in the

intracellular space as indicated in the Tables. The intracellular specific radioactivity of ^{14}C-glutamine was measured as previously described [27] .

To compare the rate of oxidative decarboxylation of glutamine with that of leucine, which is known to be extensively transaminated and oxidatively decarboxylated in skeletal muscle [16,28], we measured the production of $^{14}CO_2$ from 0.5 mM L-[1-^{14}C]leucine and 1.0 mM L-[1-^{14}C]glutamine in rat and chick skeletal muscles as described previously [29]. These concentrations are similar to in vivo plasma concentrations (mM) of leucine, which are 0.27 ± 0.01 and 0.26 ± 0.01 (n=10) for chicks and rats, respectively, and of glutamine, which are 1.10 ± 0.08 and 0.71 ± 0.04 (n=10) for chicks and rats, respectively.

Preparation of skeletal muscle mitochondrial and cytosolic fractions

Isolation of skeletal muscle mitochondria was carried out as described by Lee and Martens [30], except that Nagarse and bovine serium albumin were not used. The supernatant from the 14000 X g centrifugation was used as the cytosol fraction. The mitochondrial pellet (3.7-4.5 mg protein) from one gram of muscle was suspended in 2.0 ml 0.25 M sucrose. The mitochondrial protein content was determined by the Bradford method [31].

Assay of phosphate-activated and maleate-activated glutaminase.

Glutaminase activity in the mitochondrial or cytosolic fractions (0.1 ml) was determined by measuring the conversion of ^{14}C-glutamine to ^{14}C-glutamate in the presence of an inhibitor of glutamate oxidation as described previously [32]. Blanks with

denatured mitochondrial or cytosolic proteins and other reaction components were run in each assay. The mitochondrial phosphate-activated glutaminase reaction was linear during the 60 min incubation period. The phosphate-independent, maleate-activated glutaminase activity was measured as described above, except that 20 mM maleate was included in the assay mixture without addition of phosphate [33].

Assay of glutamine aminotransferase K and L

Glutamine aminotransferase K and L activities were measured as described by Cooper and Meister [14], except that the products of transamination, phenylalanine (glutamine aminotransferase K) or methionine (glutamine aminotransferase L), were quantified by HPLC [23]. Blanks containing denatured mitochondrial or cytosolic proteins plus all other reaction components were run in each assay. Methionine and phenylalanine were not detected in the blanks of mitochondrial preparations while they were detected in negligible amounts in the blanks of cytosolic preparations. The glutamine aminotransferase K and L reactions were shown to be linear during the 60 min incubation period.

Statistical analysis

Results, given as means ± SEM, were statistically analysed by the paired t-test or analysis of variance [34], as stated in the Tables.

C. Results

Oxidation of glutamine and leucine in rat and chick skeletal muscle

Isolated skeletal muscles from both rats and chicks extensively decarboxylated 1.0 mM [1-^{14}C]glutamine (Table 4.1). The highest

rate of glutamine oxidative decarboxylation occurred in chick EDC muscles, followed by rat EDL, diaphragm and soleus muscles (Table 4.1). It is interesting to note the relatively high rate of glutamine oxidative decarboxylation in rat diaphragm despite the relatively low intracellular glutamine concentration (Table 4.1).

Leucine, when added at 0.5 mM to the incubation medium, was extensively decarboxylated in skeletal muscles from both rats and chicks (Table 4.1). The rate of oxidative decarboxylation of physiological concentrations (1.0 mM) of glutamine was much higher ($P<0.01$) than that of 0.5 mM leucine in all muscles studied. (Table 4.1).

As the concentration of glutamine in the incubation media was increased from 0.5 mM to 10.0 mM, intracellular glutamine concentrations increased in rat diaphragm and chick EDC muscle (Table 4.2). The rate of oxidative decarboxylation of L-[1-^{14}C]glutamine was also increased with increasing intramuscular glutamine concentration (Table 4.2). The ratio of CO_2 produced from glutamine carbons 1-5 to that from glutamine carbon 1 alone ranged from 2.41 ± 0.16 to 2.62 ± 0.08 (Table 4.3).

Activity of the mitochondrial and cytosolic glutaminase

There was no detectable cytosolic glutaminase activity in any of the muscles studied in the presence or absence of phosphate. In the absence of phosphate, mitochondrial phosphate-activated glutaminase activity was not detected in rat skeletal muscle while trace amounts of activity were detected in chick muscle (Table 4.4). In the presence of 150 mM phosphate, high phosphate-activated glutaminase activity was found in chick skeletal muscle mitochondria while lower

($P<0.01$) activity was measured in rat muscle mitochondria (Table 4.4). The mitochondrial phosphate-activated glutaminase activity may be underestimated for two reasons. (1) The recovery of mitochondria from skeletal muscles was less than 100% [30]. (2) Since the mitochondria were intact, glutamine transport into the mitochondria may have been limiting. The phosphate-independent, maleate activated glutaminase activity was not detected in rat skeletal muscle, while trace amounts of activity (0.12-0.13 nmol Glu/h/mg tissue) were found in chick muscle mitochondria incubated in the presence of 10 mM glutamine. The mitochondrial phosphate-activated glutaminase activity was increased with increasing glutamine concentration in all muscles studied (Fig. 4.2). The apparent Km values for glutamine were about 8.5-10 mM for rat and chick muscles as determined from Fig. 4.2.

Glutamine aminotransferase K activity

Glutamine aminotransferase K activity was higher ($P<0.01$) in the rat than in the chick skeletal muscle mitochondria and cytosol (Table 4.5). The activity of this enzyme was at least an order of magnitude greater in rat skeletal muscle cytosol than in all other samples (Table 4.5). At a constant concentration of 10 mM glutamine, the cytosolic glutamine aminotransferase K activity in all muscles studied did not significantly ($P>0.05$) change as phenylpyruvate concentration was increased from 25 μM to 100 μM (Table 4.5). The apparent Km value for phenylpyruvate was probably less than 25 μM (Table 4.5).

At a constant concentration of 50 μM phenylpyruvate, the cytosolic glutamine aminotransferase K activity in all muscles studied increased ($P<0.05$) slightly as glutamine concentration was

increased from 1.0 mM to 50 mM (Table 4.6). The apparent Km value for glutamine was probably less than 1.0 mM (Table 4.6). Similar results were also obtained for mitochondrial glutamine aminotransferase K activities in rat and chick skeletal muscles (Appendix 5).

Glutamine aminotransferase L activity

The distribution of glutamine aminotransferase L activity in rat and chick skeletal muscles (Table 4.7) followed a similar pattern to glutamine aminotransferase K. At a constant concentration of 10 mM L-albizziin, the cytosolic glutamine aminotransferase L activity in all muscles studied significantly ($P<0.01$) increased as α-keto-γ-methiolbutyrate concentration was increased from 10 μM to 50 μM (Table 4.7). The Km value of the cytosolic enzyme for α-keto-γ-methiolbutyrate was about 10 μM (Table 4.7). However, the mitochondrial glutamine aminotransferase L activity did not significantly change ($P>0.05$) with increasing concentrations of α-keto-γ-methiolbutyrate (Table 4.7).

At a constant concentration of 50 μM α-keto-γ-methiolbutyrate, the cytosolic glutamine aminotransferasee L activity in all rat and chick muscles studied only slightly increased ($P<0.05$) as L-albizziin concentration was increased from 1.0 mM to 50 mM. The Km value for L-albizziin was probably less than 1.0 mM (Table 4.6). Similar results were also obtained for the mitochondrial glutamine aminotransferase L activities (Appendix 5).

D. Discussion

Glutamine degradation in rat and chick skeletal muscle

It is generally believed that only six amino acids are

significantly degraded in skeletal muscle, alanine, glutamate, aspartate, leucine, isoleucine and valine [16]. However, the present study clearly demonstrates, for the first time, that glutamine is also extensively degraded in skeletal muscles from rats and chicks when present at physiological concentrations (Table 6.1). Indeed, the rate of oxidative decarboxylation of glutamine in rat and chick skeletal muscle is much greater than that of leucine (Table 6.1), which is extensively decarboxylated in this tissue [28]. These findings thus challenge the traditional view that oxidation of glutamine in skeletal muscle is negligible [16-20] based on observations of negligible glutaminase activity in rat skeletal muscle [19-20].

The degradation of glutamine in skeletal muscle is not complete. The ratio of CO_2 produced from glutamine carbons 1-5 to CO_2 produced from glutamine carbon 1 alone ranged from 2.41 to 2.62 (Table 4.3), and did not approach the theoretical maximum value of 5 indicating complete oxidation of the glutamine carbon skeleton. Incomplete degradation of glutamine has also been reported in macrophages [7], lymphocyes [8] and cultured cell lines [9]. The complete metabolic fate of glutamine carbons 2-5 cannot be determined from the present study and merits further investigation.

The role of mitochondrial phosphate-activated glutaminase in glutamine degradation in chick skeletal muscle

The presence of high mitochondrial phosphate-activated glutaminase activity in chick skeletal muscle has not been previously reported. Both the rate of glutamine degradation (Table 4.2) and the mitochondrial glutaminase activity (Fig. 4.2) were increased with

increasing glutamine concentration. Glutaminase activity alone (Table 4.4) (Fig. 4.2) can fully account for the provision of glutamate for subsequent oxidation (Tables 4.1 and 4.2). Since glutamine aminotransferase K and L activities are very low in chick skeletal muscle (Tables 5-7), our results suggest that the mitochondrial phosphate-activated glutaminase may play the major role in initiating glutamine degradation in this tissue. The glutamate formed from glutamine may be transaminated with ketoacids by glutamate aminotransferase in skeletal muscle to form α-ketoglutarate, which is then oxidised via the Krebs cycle.

The role of cytosolic glutamine aminotransferases K and L in glutamine oxidation in rat skeletal muscle

In contrast to chick skeletal muscle, the relatively low mitochondrial glutaminase activity in rat skeletal muscle (Table 4.4) (Fig. 4.2) cannot fully account for the rate of glutamine oxidative decarboxylation in rat muscles (Tables 4.1 and 4.2). Our values for glutaminase activity are similar to those of Ardawi [35] who reported that the phosphate-activated glutaminase activity in rat hindlimb skeletal muscle was 0.5-0.8 nmol Glu/h/mg tissue. Thus, the cytosolic glutamine aminotransferases K and L appear to play a major role in initiating glutamine degradation in this tissue (Tables 5-7). Transamination of glutamine with ketoacids is driven by deamidation of the α-ketoglutaramate produced to α-ketoglutarate catalysed by ω-amidase [1,14,22] (Fig. 4.1). Relatively high ω-amidase activity has been found in rat skeletal muscle [21,22]. The α-ketoglutarate produced is then oxidised via the Krebs cycle. In view of the large proportion (40-45%) of the body

accounted for by skeletal muscle, our results suggest that this tissuse may be an important site for glutamine transamination in the rat. Since the Km of glutamine aminotransferase K and L for glutamine in skeletal muscle was less than 1.0 mM (Table 6), which is much lower than intracellular glutamine concentrations in skeletal muscle (Table 4.1), it is possible that glutamine aminotransferases catalyze a flux-generating reaction [36] in the oxidation of glutamine in rat skeletal muscle.

Recently, Cooper and Meister [14] reported glutamine transaminase K and L activities in rat skeletal muscle (1.5-1.7 nmol/h/mg tissue), which were much lower than our values (Tables 4.5 and 4.6). This difference may be due to the different techniques used to measure the enzyme activity. Glutamine transamination was measured by quantifying its products by HPLC in the present study, which is much easier and more sensitive than monitoring small changes in the concentration of its substrates (0.4 mM phenylpyruvate or 40 mM L-albizziin) by the method of Cooper and Meister [14]. As pointed out by Allison and Purich [37], determination of product formation, rather than substrate depletion, provides the most sensitivity and accuracy in measuring enzyme activity.

Relationship between muscle fiber type and glutaminase activity

The activity of mitochondrial phosphate-activated glutaminase appears to be related to skeletal muscle fiber type. For example, chick pectoralis muscle, which contains a greater proportion of fast-twitch glycolytic fibers than chick EDC muscles, 99% vs 63% [38,39], has much higher glutaminase activity than the latter (Table 4.4) (Fig. 4.2). Similarly, in the rat, the EDL muscle, which has a

greater proportion of fast-twitch glycolytic fibers than the soleus muscle, 79% vs 0% [40], has much higher glutaminase activity than the soleus muscle (Table 4.4) (Fig. 4.2). These findings suggest that the amount of glutaminase activity in skeletal muscle is related to the muscle fiber type.

Physiological significance of glutamine oxidation in skeletal muscle

Glutamine is a non-essential amino acid and is also the most abundant free amino acid in skeletal muscle [16,18]. The degradation of the relatively large amount of glutamine may provide a considerable amount of energy for skeletal muscle metabolism. In addition, the glutamine carbon skeleton may contribute to Krebs cycle intermediates such as oxaloacetate, which may facilitate oxidation of acetyl-CoA produced from fatty acids and ketone bodies in skeletal muscle. This point is substantiated by our findings that glutamine oxidation in chick skeletal muscle is markedly increased in the presence of elevated concentrations of ketone bodies (Chapter 7). Moreover, the degradation of glutamine may result in the production of considerable amounts of ammonia in skeletal muscle (Fig. 4.1). As a result, the view that the purine nucleotide cycle is the only significant source of ammonia released from skeletal muscle particularly during exercise [16,19] may be subject to further evaluation. In short, degradation of glutamine may provide energy, Krebs cycle intermediates and produce ammonia in skeletal muscle.

The ATP-requiring synthesis of glutamine from glutamate by glutamine synthetase [41] and the hydrolysis of glutamine to glutamate by glutaminase may permit the operation of a "futile cycle"

in skeletal muscle, ie. a glutamine-glutamate cycle. This cycle may be an important substrate cycle in amino acid metabolism in skeletal muscle. As pointed out by Newsholme [42], a substrate cycle operates to improve the sensitivity of metabolic control. Thus, this glutamine glutamate cycle could be important in regulating intramuscular concentrations of glutamine which have recently been shown to be positively related to the rate of protein synthesis in rat skeletal muscle under normal and catabolic conditions [18,43]. This cycle may also help to explain the dramatic decreases in intramuscular glutamine concentrations observed under a variety of physiological and pathological conditions such as exercise [44], starvation, infection and injury [18,45]. It seems of importance to examine in detail the kinetics and regulation of glutamine metabolism in skeletal muscle, in view of its high rate of oxidation as demonstrated here.

In conclusion, glutamine is degraded in skeletal muscles from both rats and chicks. However, there are striking species differences in the pathways of conversion of glutamine to α-ketoglutarate. It appears that the cytosolic glutamine aminotransferases K and L in rat skeletal muscle and mitochondrial phosphate-activated glutaminase in chick skeletal muscle play major roles in initiating glutamine degradation. Glutamine degradation in skeletal muscle may be of physiological significance in providing energy and Krebs cycle intermediates and regulating intramuscular glutamine concentrations.

Table 4.1. Oxidative decarboxylation of [1-^{14}C]glutamine and [1-^{14}C]leucine in rat and chick skeletal muscle.

Isolated skeletal muscles from rat and chicks were incubated in the presence of plasma concentrations of amino acids and glucose as described in the text. One muscle was incubated with 1.0 mM [1-^{14}C]glutamine (150 dpm/nmol) while the contralateral muscle was incubated with 0.5 mM [1-^{14}C]leucine (400 dpm/nmol). Intracellular specific radioactivity of [1-^{14}C]glutamine or [1-^{14}C]leucine was measured for calculation of their rates of decarboxylation. Means within a column followed by different superscripts are significantly ($P<0.01$) different as determined by analysis of variance. The rate of leucine oxidative decarboxylation is significantly less ($P<0.01$) than that of glutamine as analysed by the paired t-test. n=6.

Muscle	Glutamine		Leucine	
	Intracellular concentration (nmol/mg tissue)	Oxidative decarboxylation (nmol/h/mg tissue)	Intracellular concentration (nmol/mg tissue)	Oxidative decarboxylation (nmol/h/mg tissue)
Rat				
EDL	8.61±0.26^{a}	3.39±0.14^{a}	0.40±0.04^{a}	1.06±0.05^{a}
Soleus	5.35±0.39^{b}	1.34±0.16^{b}	0.65±0.03^{b}	0.21±0.04^{b}
Diaphragm	2.82±0.20^{c}	3.32±0.32^{a}	0.41±0.03^{a}	1.28±0.09^{c}
Chick				
EDC	6.16±0.17^{d}	3.92±0.43^{a}	0.41±0.02^{a}	0.84±0.07^{d}

Table 4.2. Oxidative decarboxylation of [1-^{14}C]glutamine in rat diaphram and chick EDC muscle

Rat quarter-diaphragm and chick muscles were incubated in the presence of L-[1-^{14}C]glutamine (150 dpm/ul), plasma concentrations of glucose and all amino acids except for glutamine which was added to the medium at concentrations as indicated. Intracellular specific radioactivity of [1-^{14}C]glutamine was used to calculate the rate of glutamine oxidation. Means within a column followed by different superscripts are significantly different ($P<0.01$) as determined by the paired t-test for rat diaphragms and by analysis of variance for chick EDC muscles. n=5.

Medium	Rat diaphragm		Chick EDC muscle	
[Gln] (mM)	Intracellular concentration (nmol/mg tissue)	Oxidative decarboxylation (nmol/h/mg tissue)	Intracellular concentration (nmol/mg tissue)	Oxidative decarboxylation (nmol/h/mg tissue)
0.5	1.89±0.22^{a}	2.21±0.13^{a}	4.16±0.37^{a}	2.35±0.16^{a}
1.0	2.68±0.16^{b}	3.29±0.11^{b}	6.30±0.29^{b}	3.46±0.07^{b}
2.0			10.27±0.32^{c}	4.39±0.27^{c}
4.0	4.85±0.26^{c}	6.11±0.19^{c}	14.12±0.47^{d}	4.98±0.57^{d}
6.0			17.30±0.51^{e}	6.05±0.34^{e}
10.0	9.96±0.44^{d}	8.97±0.48^{d}	21.18±0.96^{f}	7.30±0.69^{f}

Table 4.3. Oxidation of [U-^{14}C]glutamine and [1-^{14}C]glutamine in rat and chick skeletal muscle

Rat and chick muscles were incubated in the presence of plasma concentrations of amino acids and glucose as described in the text. One muscle was incubated with 1.0 mM [U-^{14}C]glutamine while the contralateral muscle was incubated with 1.0 mM [1-^{14}C]glutamine. The specific radioactivity (dpm/nmol labelled carbon) of [U-^{14}C]glutamine and [1-^{14}C]glutamine in the incubation medium was used for calculation of the rate of CO_2 production from glutamine carbons 1-5 and from glutamine carbon 1, respectively. Means within a column followed by different superscripts are significantly different ($P<0.01$) as determined by analysis of variance. n=6.

Muscle	CO_2 production from glutamine carbons 1-5 (A)	CO_2 production from glutamine carbon 1 (B)	A/B
	(nmol/h/mg tissue)		
Rat			
EDL	1.71±0.12[a]	0.76±0.15[a]	2.41±0.19
Soleus	1.26±0.11[b]	0.49±0.05[b]	2.59±0.06
Diaphragm	2.67±0.17[c]	1.04±0.06[c]	2.57±0.04
Chick			
EDC	3.49±0.29[d]	1.54±0.10[d]	2.27±0.08

Table 4.4. Mitochondrial phosphate-activated glutaminase activity in rat and chick skeletal muscle.

Phosphate-activated glutaminase activity was assayed in the presence of 10 mM glutamine and in the presence or absence of 150 mM phosphate as described in the text. Means within a column followed by different superscripts are significantly different ($P<0.01$) as determined by analysis of variance. ND: not detectable. n=8.

Muscle	Phosphate-activated glutaminase (nmol glutamate produced/ h/ mg tissue)	
	-Phosphate	+Phosphate
Rat		
EDL	ND	2.25±0.13[a]
Gastrocnemius	ND	0.87±0.04[b]
Diaphragm	ND	0.63±0.03[c]
Soleus	ND	0.34±0.06[d]
Chick		
Pectoralis	0.17±0.02	15.75±0.18[e]
EDC	0.15±0.01	8.24±0.18[f]

Table 4.5. Glutamine aminotransferase K activity in rat and chick skeletal mucles.

Glutamine aminotransferase K activity was assayed in the presence of 10 mM glutamine and 10-100 μM phenylpyruvate as described in the text. Means within a tissue fraction followed by different superscripts are significantly different ($P<0.01$) as determined by analysis of variance. The activity of this enzyme in rat muscles is higher ($P<0.01$) than that in chick muscles. The activity of this enzyme in rat diaphragm is higher ($P<0.05$) than that in the other rat muscles. n=3.

Muscle	Mitochondria				Cytosol			
	Phenylpyruvate concentration (μM)				Phenylpyruvate concentration (μM)			
	10	25	50	100	10	25	50	100
	(nmol Phe produced/h/mg tissue)							
Rat								
EDL	0.12^a ±0.01	0.19^b ±0.01	0.26^c ±0.02	0.31^c ±0.02	5.74^a ±0.09	14.24^b ±0.10	14.26^b ±0.13	14.52^b ±0.29
Soleus	0.13^a ±0.01	0.18^b ±0.02	0.27^c ±0.03	0.33^c ±0.01	5.56^a ±0.10	12.99^b ±0.19	13.32^b ±0.13	13.57^b ±0.08
Gastrocnemius	0.13^a ±0.01	0.20^b ±0.02	0.30^c ±0.03	0.34^c ±0.01	5.34^a ±0.11	12.95^b ±0.43	13.45^b ±0.17	13.21^b ±0.10
Diaphragm	0.18^a ±0.02	0.26^b ±0.02	0.34^c ±0.02	0.40^c ±0.03	6.87^a ±0.07	16.81^b ±0.05	17.32^b ±0.09	17.67^b ±0.24
Chick								
EDC	0.06^a ±0.01	0.08^b ±0.01	0.09^b ±0.01	0.14^c ±0.01	0.34^a ±0.01	0.40^b ±0.02	0.46^b ±0.02	0.52^b ±0.03
Pectoralis	0.06^a ±0.01	0.08^b ±0.01	0.09^b ±0.01	0.13^c ±0.01	0.28^a ±0.02	0.37^b ±0.03	0.42^b ±0.02	0.46^b ±0.04

Table 4.6. The activities of cytosolic glutamine aminotransferases K and L in rat and chick skeletal muscles.

Cytosolic glutamine aminotransferase K activity was assayed in the presence of 1-50 mM glutamine and 50 μM phenylpyruvate as desribed in the text. Cytosolic glutamine aminotransferase L activity was assayed in the presence of 1-50 mM L-albizziin and 50 μM α-keto-γ-methiolbutyrate as described in the text. Means within a row followed by different superscripts are significantly different ($P<0.05$) as determined by analysis of variance. n=3.

Muscle	Glutamine aminotransferase K (nmol Phe/h/mg tissue) Glutamine concentrations (mM)				Glutamine aminotransferase L (nmol Met/h/mg tissue) L-albizziin concentration (mM)			
	1.0	2.5	10	50	1.0	2.5	10	50
Rat								
EDL	10.01^{a} ±0.24	12.56^{b} ±0.38	14.61^{c} ±0.24	15.33^{c} ±0.21	5.27^{a} ±0.04	6.86^{b} ±0.12	6.65^{b} ±0.13	6.89^{b} ±0.07
Soleus	9.42^{a} ±0.07	11.55^{b} ±0.29	13.63^{c} ±0.12	13.65^{c} ±0.09	6.05^{a} ±0.08	7.45^{b} ±0.26	7.45^{b} ±0.08	7.28^{b} ±0.12
Gastrocnemius	9.21^{a} ±0.09	11.76^{b} ±0.06	13.40^{c} ±0.11	13.76^{c} ±0.12	5.15^{a} ±0.12	6.67^{b} ±0.08	6.98^{b} ±0.10	6.88^{b} ±0.14
Diaphragm	12.71^{a} ±0.32	15.71^{b} ±0.72	17.33^{c} ±0.03	17.39^{c} ±0.39	7.56^{a} ±0.23	8.63^{b} ±0.24	9.20^{b} ±0.22	9.96^{b} ±0.18
Chick								
EDC	0.35^{a} ±0.02	0.44^{b} ±0.02	0.51^{b} ±0.04	0.49^{b} ±0.03	0.32^{a} ±0.04	0.41^{b} ±0.01	0.43^{b} ±0.06	0.44^{b} ±0.06
Pectoralis	0.29^{a} ±0.02	0.38^{b} ±0.03	0.43^{b} ±0.02	0.41^{b} ±0.04	0.28^{a} ±0.03	0.36^{b} ±0.03	0.35^{b} ±0.03	0.37^{b} ±0.05

Table 4.7. Glutamine aminotransferase L activity in rat and chick skeletal muscles.

Glutamine aminotransferase L activity was assayed in the presence of 10 mM L-albizziin and 10-100 μM α-keto-γ-methiolbutyrate as described in the text. Means within a tissue fraction followed by different superscripts are significantly different ($P<0.01$) as determined by analysis of variance. The activity of glutamine aminotransferase L in rat muscles is higher ($P<0.01$) than in chick muscles and its activity in rat diaphragm is higher ($P<0.05$) than in the other rat muscles. n=3.

Muscle	Mitochondria				Cytosol			
	α-keto-γ-methiolbutyrate concentration (μM)				α-keto-γ-methiolbutyrate concentration (μM)			
	10	25	50	100	10	25	50	100
	(nmol Met produced/h/mg tissue)							
Rat								
EDL	0.08 ±0.01	0.07 ±0.01	0.09 ±0.01	0.09 ±0.01	1.68^a ±0.11	3.14^b ±0.18	6.41^c ±0.56	6.51^c ±0.42
Soleus	0.08 ±0.01	0.09 ±0.01	0.09 ±0.01	0.10 ±0.01	1.63^a ±0.06	3.97^b ±0.07	7.66^c ±0.07	7.71^c ±0.08
Gastrocnemius	0.08 ±0.01	0.08 ±0.01	0.09 ±0.01	0.09 ±0.01	1.59^a ±0.12	3.33^b ±0.27	6.98^c ±0.21	7.05^c ±0.32
Diaphragm	0.10 ±0.02	0.11 ±0.02	0.12 ±0.01	0.11 ±0.01	2.06^a ±0.13	4.54^b ±0.06	8.43^c ±0.13	8.96^c ±0.24
Chick								
EDC	0.05 ±0.01	0.05 ±0.01	0.06 ±0.01	0.05 ±0.01	0.19^a ±0.01	0.31^b ±0.01	0.43^c ±0.02	0.56^d ±0.02
Pectoralis	0.05 ±0.01	0.06 ±0.01	0.05 ±0.01	0.06 ±0.01	0.16^a ±0.01	0.25^b ±0.02	0.35^c ±0.01	0.46^d ±0.01

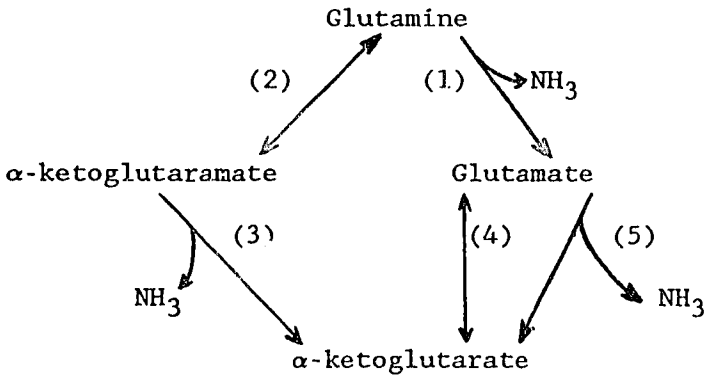

Fig. 4.1. Initiation of glutamine degradation in animal tissues.

(1) Glutaminase, (2) Glutamine aminotransferase K and L,
(3) ω-amidase, (4) Glutamate aminotransferases,
(5) Glutamate dehydrogenase.

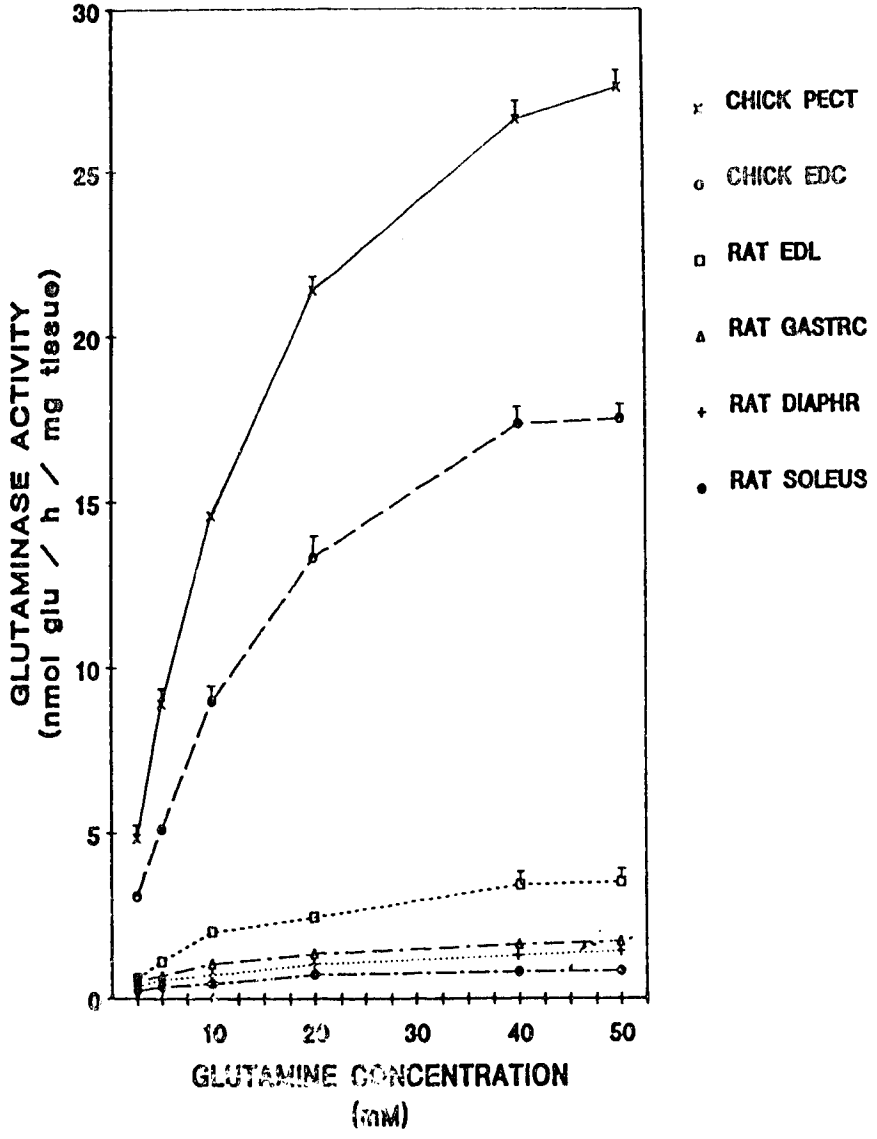

Fig. 4.2. Effect of glutamine concentrations on mitochondrial phosphate-activated glutaminase activity in rat and chick skeletal muscle.

Mitochondrial phosphate-activated glutaminase activity was measured in the presence of 150 mM phosphate and 2.5-50 mM glutamine as described in the text. Data represent mean ± SEM, n=3. The SEM values smaller than the legend size are not shown. ✕Chick pectoralis, ○Chick EDC, □Rat EDL, △Rat gastrocnemius, +Rat diaphragm, ●Rat soleus.

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V. Is Methionine Transaminated In Skeletal Muscle?[1]

A. Introduction

Skeletal muscle is generally considered not to degrade methionine via the transsulphuration pathway due to the very low activity or absence of the required enzymes [1,2]. An alternative pathway has been proposed which initially involves methionine transamination [3-6]. This proposal is largely based on the detection of methionine transamination in homogenates of skeletal muscle, liver and other tissues [3-5] and in extracts of rat skeletal muscle [6]. Recently, it has been reported that [U-^{14}C]methionine is metabolized to $^{14}CO_2$ and ^{14}C-labelled Krebs cycle intermediates via a transsulphuration-independent pathway in perfused rat hindquarter preparations [6]. Based on studies with muscle homogenates, Benevenga and his associates [4,5] have suggested that the skeletal musculature might be the major site for methionine transamination in animals. However, all of these studies were performed in the absence of amino acids other than methionine [4-6] and at very high methionine concentrations ranging from 10 mM [4,5] to 20 mM [6], which are about 100 to 200 times physiological plasma concentrations [7,8]. As a result, the physiological significance of methionine transamination, as shown in skeletal muscle homogenates and extracts, remains unclear [9]. The present study was therefore conducted to investigate methionine transamination in intact skeletal muscles from chicks and

1. A version of this chapter has been published. Wu, G. and Thompson, J.R. (1989) Biochem. J. 257, 281-284.

rats in the absence or presence of amino acids at plasma concentrations.

B. Materials and Methods

Chemicals and animals

L-[1-^{14}C]methionine was obtained from American Radiolabeled Chemicals Inc., St Louis, U.S.A. Its radiochemical purity was determined to be greater than 99% by descending paper chromatography using n-butanol:acetic acid:H_2O (2:1:1) as the solvent. The source of L-[1-^{14}C]leucine and all other chemicals used was reported previously [10].

Nine-day-old male broiler chicks (Hubbard strain, Gallus domesticus) and 21-day-old weaned male Buffalo rats were provided free access to food and water. Animals were anesthetized with halothane before the muscles were removed.

Methionine transamination in skeletal muscle homogenates of rats and chicks

Rat gastrocnemius (314.2±18.8 mg), diaphragm (122.9±8.4 mg), extensor digitorum longus (EDL) (24.9±2.6 mg) and soleus (20.3±1.6 mg) muscles, and chick extensor digitorun communis (EDC) muscles (26.4±0.8 mg) were homogenized in 0.25 M sucrose in ice [5] using a Potter-Elvehjem glass tube fitted with a glass pestle. Briefly, individual rat gastrocnemius muscles were homogenized in 2 ml 0.25 M sucrose. The rat diaphragm, EDL and soleus muscles from seven rats were pooled by muscle type and homogenized in 8 ml 0.25 M sucrose, and each homogenate was distributed equally into 4 tubes. Two EDC muscles from individual chicks were pooled and homogenized in 2 ml

0.25 M sucrose. The homogenates (2 ml) were incubated at 37°C for 2 h in a final volume of 5 ml in the presence of 10 mM L-[1-^{14}C]methionine (11 dpm/nmol), 10 mM pyruvate, 1 mM ATP, 5 mM $MgCl_2$ and 10 mM phosphate buffer (pH 7.4) saturated with O_2:CO_2 (19:1) as described by Mitchell and Benevenga [5]. BCAA were absent or present in the incubation media as indicated in Table 1. Incubation media without muscle homogenates were run to correct for background radioactivity values. Incubation was terminated by the injection of 0.2 ml 1.5 M $HClO_4$ into the media. $^{14}CO_2$ from [1-^{14}C]methionine decarboxylation and from chemical decarboxylation of [1-^{14}C]oxomethionine by 30% H_2O_2 was separately collected in Hyamine hydroxide [10,11]. The net rate of methionine transamination was calculated based on the medium specific radioactivity of [1-^{14}C]methionine [11].

Methionine transamination in intact rat and chick skeletal muscles

The rat quarter-diaphragm, EDL and soleus muscles and the chick extensor digitorum communis (EDC) muscles (19.5±0.6 mg) were dissected and incubated in Krebs-Ringer bicarbonate buffer saturated with O_2:CO_2 (19:1) [10,12,13]. The buffer also contained either 0.01 U/ml insulin, 2 mM Hepes, 12 mM glucose and 0.2 mM methionine (medium A) or 0.1 U/ml insulin, 2 mM Hepes, 5 mM glucose and 0.5 mM methionine (medium B). All muscles were preincubated for 30 min as recommended by Goldberg et al. [14] in either medium A or medium B and then incubated for 2 h in fresh media containing L-[1-^{14}C]methionine (60 d.p.m./ul), with or without the addition of BCAA or all amino acids other than methionine at plasma concentrations (Appendix 1). In all of these experiments, one muscle

was used for the control while the contralateral muscle was used for the treatment. Incubation media without muscles were run to correct for background radioactivity values. At the end of the final incubation period, muscles were removed from the incubation media and placed in 1 ml ice-cold 2% trichloroacetic acid. $^{14}CO_2$ from methionine decarboxylation and from chemical decarboxylation of [1-^{14}C]oxomethionine by 30% H_2O_2 was separately collected in Hyamine hydroxide [10,11]. The amount of $^{14}CO_2$ from chemical decarboxylation of [1-^{14}C]oxomethionine by 30% H_2O_2 averaged 2540 d.p.m. for chick EDC muscles incubated with 0.2 mM methionine in the absence of all other amino acids, accounting for approximately 1.2% of the added radioactivity and 24.2% of the total [1-^{14}C]methionine taken up by the muscle. The rate of methionine degradation was calculated based on the specific radioactivity of intracellular L-[1-^{14}C]methionine which was measured essentially as described for that of L-[1-^{14}C]leucine [10] following correction for the extracellular space [10,12,13]. In a preliminary experiment, the rate of methionine transamination was measured at 30 min intervals during the incubation period in the presence of 0.5 mM L-[1-^{14}C]methionine (medium A). Methionine transamination was linear over the 2 h final incubation period ($r=0.998$, $P<0.001$; $n=5$).

Leucine transamination in intact rat and chick skeletal muscle

Intact rat and chick skeletal muscles were dissected and incubated with 0.5 mM L-[1-^{14}C]leucine in the absence of other amino acids (Medium B) as described above. $^{14}CO_2$ released following transamination of L-[1-^{14}C]leucine was collected in Hyamine hydroxide and the net leucine transamination rate was

calculated based on the specific radioactivity of intracellular L-[1-^{14}C]leucine [10,12,13]. Under these conditions, it was previously demonstrated that the rate of leucine catabolism remains constant throughout the 2-h incubation period [15]. The amount of $^{14}CO_2$ released following transamination of L-[1-^{14}C]leucine by chick EDC muscles and rat soleus muscles averaged 13600 d.p.m. and 6600 d.p.m., accounting for approximately 3.6% and 1.7% of the added radioactivity and 82.4% and 77.6% of the total L-[1-^{14}C]leucine taken up by muscles, respectively.

Statistical Analysis

The results, given as mean ± SEM, were statistically analyzed by the t-test as described by Steel and Torrie [16].

C. Results and Discussion

Methionine was found to be extensively transaminated in homogenates of rat diaphragm, gastrocnemius, EDL and soleus muscles, and of chick EDC muscles incubated in the presence of 10 mM methionine in the absence of other amino acids (Table 5.1). These results are consistent with those reported for rat gastrocnemius muscle homogenates [5] and for rat skeletal muscle extracts [6,7]. The addition of BCAA markedly inhibited methionine transamination in skeletal muscle homogenates of rats and chicks (Table 5.1), suggesting that BCAA transaminase catalyzes methionine transamination.

In contrast to muscle homogenates, methionine transamination was not detected in intact rat quarter-diaphragm, EDL and soleus muscles incubated with 0.2 mM methionine in the presence or absence of plasma

concentrations of other amino acids. Likewise, methionine transamination was barely detected in intact rat quarter-diaphragms incubated with 0.5 mM methionine in the absence of other amino acids (Table 5.2) and was not detected in this tissue in the presence of other amino acids or the branched-chain amino acids alone at plasma concentrations. Similarly, methionine transamination was not detected in intact rat EDL and soleus muscles incubated with 0.5 mM methionine in the absence (Table 5.2) or presence of plasma concentrations of other amino acids or the branched-chain amino acids alone.

It is unlikely that our failure to detect methionine transamination in intact rat skeletal muscles is due to artifacts involved in muscle incubation since 0.5 mM L-[1-^{14}C]leucine is extensively transaminated in intact rat skeletal muscles incubated under the same conditions used for methionine transamination studies (Table 5.2). Thus, our results raise a serious question regarding the physiological significance of methionine transamination as shown in rat skeletal muscle homogenates and extracts in which 10 or 20 mM methionine was the only amino acid provided [4,5].

This question also arises from our measurements of methionine transamination in intact chick skeletal muscles incubated in the absence or presence of other amino acids. As shown in Tables 5.2 and 5.3, methionine is extensively transaminated in intact chick EDC muscles incubated with either 0.2 or 0.5 mM methionine in the absence of other amino acids. However, methionine transamination is barely detectable in chick muscles incubated in the presence of plasma concentrations of other amino acids or BCAA alone (Table 5.3). In

fact, the presence of plasma concentrations of BCAA is equally effective in inhibiting methionine transamination as the presence of all plasma amino acids. The inhibitory effect of BCAA may be accounted for in part by a decrease in methionine uptake (Table 5.3) and an inhibition of methionine transamir by BCAA (Tables 5.1 and 5.3).

It follows that there is a striking difference in methionine transamination between intact rat and chick skeletal muscles incubated in the absence of other amino acids. This species difference may be due to a difference in the activity of the BCAA transaminase for methionine between rats and chicks (Tables 5.1 and 5.2). Whatever the difference is, our results clearly demonstrate that transamination of methionine is negligible in intact rat and chick skeletal muscle in the presence of plasma concentrations of methionine and other amino acids or the branched-chain amino acids alone (Table 5.3). Recent studies in humans have also shown that transamination of methionine in normal subjects is quantitatively of little importance even after methionine loading [17].

Table 5.1. Methionine transamination in rat and chick skeletal muscle homogenates

Rat and chick skeletal muscle homogenates were incubated with 10 mM L-[1-^{14}C]methionine either in the absence of other amino acids (Control) or in the presence of 5 mM each of leucine, isoleucine plus valine (BCAA). Results, given as mean ± SEM, were statistically analyzed by the paired t-test for the rat gastrocnemius muscles and by the nonpaired t-test for other rat muscles and chick muscles. Treatment means with * and ** are significantly different from control means at $P<0.05$ and $P<0.001$, respectively. n=7 for gastrocnemius muscle, n=2 for other rat muscles and n=6 for chick EDC muscles. ND: not detectable.

Muscle	Methionine decarboxylation	Oxomethionine production nmol/mg tissue/2 h)	Net methionine transamination
Rat			
Gastrocnemius			
Control	0.013±0.001	0.77±0.04	0.78±0.04
+BCAA	0.010±0.001**	0.21±0.04**	0.22±0.04**
Diaphragm			
Control	0.034±0.002	1.05±0.03	1.09±0.03
+BCAA	0.024±0.002	0.35±0.04*	0.37±0.04*
EDL			
Control	0.014±0.001	0.93±0.02	0.94±0.02
+BCAA	0.012±0.001	0.31±0.05*	0.32±0.05*
Soleus			
Control	0.017±0.001	0.87±0.04	0.89±0.05
+BCAA	0.014±0.001	0.26±0.03*	0.27±0.03*
Chick			
EDC			
Control	ND	1.72±0.18	1.72±0.18
+BCAA	ND	0.20±0.05**	0.20±0.05**

Table 5.2. Transamination of methionine and leucine in intact rat and chick skeletal muscles.

Intact rat and chick skeletal muscles were incubated with 0.5 mM L-[1-^{14}C]methionine (196 dpm/nmol) or 0.5 mM L-[1-^{14}C]leucine (214 dpm/nmol) in the absence of other amino acids as described in the text. Results are given as means ± SEM for 6 observations per treatment. ND: not detectable.

Muscle	Methionine		Leucine	
	Intracellular specific radioactivity (dpm/nmol)	Transamination (nmol/mg tissue/2h)	Intracellular specific radioactivity (dpm/nmol)	Transamination (nmol/mg tissue/2h)
Rat				
Diaphragm	202.3±7.8	0.036±0.003	158.8±6.5	3.87±0.28
EDL	193.6±9.5	ND	165.7±9.1	2.57±0.19
Soleus	200.2±8.5	ND	168.5±7.3	2.02±0.31
Chick				
EDC	167.5±7.4	1.15±0.08	153.1±6.4	4.55±0.43

Table 5.3. Methionine transamination in intact chick EDC muscle.

Contralateral EDC muscles held at resting length were incubated in media containing either 0.2 or 0.5 mM L-[1-^{14}C]methionine in the absence of other amino acids (Control), or in the presence of either leucine, isoleucine plus valine (BCAA) or all other amino acids (All AA) at normal plasma concentrations. Results, given as means ± SEM for 6 observations per treatment, were statistically analysed by the paired t-test. Treatment means marked with ** are significantly different (P<0.001) from control means obtained from the contralateral muscles. ND: not detectable.

[Methionine]	Addition	Methionine decarboxy-lation	Oxomethionine production	Net methionine transamination	Intracellular concentrations	
					Methionine	BCAA
		(nmol/mg tissue/2 h)			(nmol/mg tissue)	
0.2 mM	Control	0.048±0.003	0.37±0.04	0.42±0.05	0.93±0.06	0.19±0.02
	+BCAA	0.028±0.003**	ND	0.028±0.003**	0.42±0.04**	1.29±0.06**
	Control	0.054±0.003	0.40±0.04	0.45±0.05	1.13±0.08	0.21±0.02
	+All AA	0.029±0.001**	ND	0.029±0.001**	0.18±0.01**	1.17±0.09**
0.5 mM	Control	0.052±0.005	0.99±0.04	1.04±0.04	1.57±0.13	0.20±0.01
	+BCAA	0.034±0.003**	ND	0.034±0.003**	0.71±0.06**	1.35±0.12**
	Control	0.063±0.005	1.05±0.05	1.11±0.05	1.64±0.15	0.21±0.02
	+All AA	0.025±0.002**	ND	0.025±0.002**	0.39±0.07**	1.13±0.15**

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VI. The Effect Of Glutamine On Protein Turnover In Chick Skeletal Muscle

A. Introduction

Glutamine is the most abundant free amino acid in human and animal blood plasma and skeletal muscles [1,2]. It plays an important role as a regulator of cellular metabolism, such as inhibition of lipolysis [3], ketogenesis [3,4] and protein degradation [5], and stimulation of both glycogenesis and lipogenesis [4] in the liver. Glutamine has also been shown to inhibit intracellular protein degradation in cultured rat skeletal muscle cells [6]. Recently, Rennie and his associates have shown that increased intracellular glutamine concentrations increase the rate of protein synthesis in perfused rat skeletal muscles [7]. These workers also demonstrated by a ^{15}N dilution technique that 15 mM glutamine inhibited non-myofibrillar protein degradation in perfused rat skeletal muscles [8]. A positive relationship between intracellular glutamine concentration and the rate of protein synthesis has been reported in skeletal muscles from fasted, injured, and endotoxin-treated rats in vivo [9]. A role for glutamine in improving whole body nitrogen balance during catabolic states has recently been demonstrated in patients following major sugery [10].

Garlick and Grant [11], however, have recently reported that intravenous infusion of glutamine has no effect on protein synthesis in skeletal muscles from postabsorptive rats in vivo. This lack of an effect [11] may not neccesarily negate a role for glutamine in

regulating muscle protein synthesis in vivo, since a large amount of infused glutamine is actively taken up by postabsorptive rat intestine where it is the preferred oxidative substrate [12] and thus glutamine may not be directed to skeletal muscle at concentrations sufficient to increase intramuscular glutamine concentrations. It is unfortunate that intramuscular glutamine concentrations were not provided in the study of Garlick and Grant [11].

Preliminary studies from our laboratory have shown that 0.5-2.0 mM glutamine increases tyrosine release from incubated chick skeletal muscle [13], which has been widely interpreted to reflect the net rate of protein degradation in rat skeletal muscles [eg. 14-17]. The increased release of tyrosine in these conditions may not necessarily indicate an increased rate of muscle protein degradation, but rather may result from transamination of glutamine with hydroxy-phenylpyruvate catalysed by glutamine transaminases which are widely distributed in animal tissues [18]. Also, the absence of tyrosine from the incubation medium [13] may limit muscle protein synthesis, thereby making the interpretation of these results somewhat ambiguous. The role of glutamine in regulating skeletal muscle protein metabolism in avian species has not been reported in the literature. This study was therefore designed to investigate whether physiological concentrations of glutamine regulate protein synthesis and degradation in chick skeletal muscle in the presence of all amino acids. A new technique for estimating total protein degradation in skeletal muscles incubated in the presence of all amino acids was developed, in which the release of ^{3}H-phenylalanine

from prelabelled protein was measured. Protein synthesis was estimated by measuring the incorporation of ^{3}H-phenylalanine into muscle protein.

B. Materials and Methods

Chemicals.

L-[2,6-^{3}H]phenylalanine and L-[U-^{14}C]serine were obtained from ICN Radiochemicals, Montreal, Que. Amino acids, D-glucose, bovine insulin, p-hydroxyphenylpyruvate and all other chemicals were obtained from Sigma Chemical Co., St. Louis, U.S.A.

Animals.

Day-old male broiler chicks were obtained from a local hatchery and were provided free access to a broiler starter ration and water. Extensor digitorum communis (EDC) muscles (19.7 ± 0.3 mg, n=20) were removed when the chicks were 10-days old and 120-130 g in weight.

Muscle preparation and incubation.

Chicks were anaesthetized with halothane and the EDC muscles were carefully dissected and inserted into stainless supports by means of their tendons [19] to prevent contraction thereby improving nitrogen balance and energy status of the tissue [20]. Muscles were preincubated for 30 min, and then transferred to fresh media and incubated for 2 h at 37°C in Krebs-Ringer bicarbonate buffer (pH 7.4) [19], with continuous gassing with O_2:CO_2 (95%:5%) throughout the incubation period. The buffer also contained insulin (0.01 U/ml), 12 mM glucose, 2 mM Hepes, 1.0 mM phenylalanine and plasma concentrations of other amino acids (Appendix 1) except glutamine and tyrosine in some experiments. Glutamine was added to

the incubation media as indicated in the tables.

Measurement of intramuscular amino acids and protein synthesis.

Protein synthesis in EDC muscles was estimated by incubating the muscles in the presence of 1.0 mM L-[2,6-^{3}H]phenylalanine (0.64 mCi/mmol) as previously described [14,20]. At the end of the 2-h incubation period, muscles were rinsed in nonradioactive medium, blotted and homogenized in 2 ml ice-cold 2% (w/v) trichloroacetic acid. The rate of muscle protein synthesis was estimated as previously described [14,20]. Intramuscular concentrations of amino acids were measured by h.p.l.c [19], while tyrosine was measured by a fluorometric method [21]. Intracellular concentrations of amino acids were calculated following correction for the extracellular space [22].

Measurement of protein degradation.

Individual nine-day-old chicks were injected with 0.45 ml physiological saline containing 0.4 mM L-[2,6-^{3}H]phenylalanine (0.83 Ci/mmol) via the intraperitoneal route. Twenty four hours after injection, the chicks were anaesthetized with halothane and the EDC muscles were dissected, preincubated twice for 15 min in fresh media and then transferred to fresh media and incubated for 2 h as described above. At the end of the incubation, muscles were removed and homogenized in 2 ml 2% (w/v) trichloroacetic acid. The radioactivity in ^{3}H-phenylalanine in the intramuscular free pool and in the media was measured by liquid scintillation spectrometry [22]. EDC muscle proteins were hydrolysed with 6 N HCl at 110^{0} for 24 h. The hydrolysates were evaporated to dryness and the residue was dissolved in 10 ml water. One ml of the solution was counted for

^{3}H-radioactivity while 0.2 ml was analyzed for phenylalanine by h.p.l.c. [19,20]. The rate of protein degradation was calculated by dividing the amount of ^{3}H-phenylalanine radioactivity released to the incubation medium during the final 2-h incubation period by the specific radioactivity of protein-bound ^{3}H-phenylalanine.

To measure the percentage of ^{3}H-phenylalanine in protein-bound radioactivity, protein hydrolysate was run on silica gel thin layer chromatography using phenol:water (75:25, w/w) as the solvent [23]. ^{3}H-phenylalanine and ^{3}H-tyrosine accounted for 96.3 ± 0.2% and 2.2 ± 0.3% (means ± SEM, n=3), respectively, of the total protein-bound radioactivity.

Measurement of tyrosine release in muscles incubated in the absence of tyrosine.

EDC muscles were incubated in the presence of all amino acids except glutamine and tyrosine. Glutamine was added to incubation media as shown in Table 6.5. Tyrosine released to the medium and present in the tissue at the end of the 2-h incubation period was measured [21] as an index of the net rate of protein degradation [14-17].

Measurement of serine uptake by EDC muscles.

Serine uptake was measured by incubating EDC muscles in 0.7 mM L-[U-^{14}C]serine (0.16 mCi/mmol) and plasma concentrations of all amino acids except glutamine. Glutamine was added to the incubation medium as shown in Table 3. At the end of the 2-h incubation period, muscles were thoroughly rinsed with nonradioactive medium, blotted and placed in 0.5 ml Soluene. Solubilised tissues were counted for ^{14}C-radioactivity [22]. Since no $^{14}CO_2$ was produced from

L-[U-^{14}C]serine in chick EDC muscles (results not shown), the accumulation of ^{14}C-serine in the tissue can be used to estimate uptake of serine. Intracellular L-[U-^{14}C]serine radioactivity was calculated following correction for ^{14}C-serine in the extracellular space [22]. Uptake of serine was calculated by dividing the intracellular ^{14}C radioactivity by the specific radioactivity of L-[U-^{14}C]serine in the incubation medium.

Measurement of glutamine-p-hydroxyphenylpyruvate transamination in EDC muscle mitochondrial and cytosolic fractions

The isolation of chick EDC muscle mitochondria was carried out as described by Lee and Martens [24]. The 14000 X g supernatant was used as the cytosolic fraction. Glutamine-p-hydroxyphenylpyruvate transamination in the mitochondrial and cytosolic fractions (0.1 ml) was measured at 37^{0}C as described by Cooper and Meister [25], except that the transamination product, tyrosine, rather than the formation of α-keto[^{14}C]-glutaramate from ^{14}C-glutamine [25] was measured by a fluorometric method [21].

Statistical analysis.

Results, given as means ± S.E.M., were analyzed by one-way analysis of variance, paired t-test or simple correlation analysis [26].

C. Results and Discussion

Effect of glutamine on intracellular amino acid concentrations and serine uptake by muscles.

Glutamine is the most abundant free amino acid in chick EDC muscles incubated in the presence of plasma concentrations of amino

acids (Table 6.1) as previously shown for mammalian skeletal muscle [1]. The addition of 0.5 to 15 mM glutamine increased ($P<0.01$) intracellular glutamine concentrations by 31 to 670% over that measured in the absence of glutamine (Table 6.1). Intracellular glutamine concentrations approached a plateau as medium glutamine concentration increased above 10 mM. These results suggest that glutamine is rapidly taken up and accumulated by chick skeletal muscle as previously reported for rat skeletal muscle [7,27].

Glutamine (1-15 mM) increased ($P<0.05$) the intracellular concentrations of glutamate, aspartate and asparagine (Table 6.1), probably owing to formation of these amino acids from glutamine as we have recently found that glutamine is extensively metabolised in chick skeletal muscle (Chapter 4). On the other hand, the addition of 2-15 mM glutamine decreased ($P<0.05$) intracellular concentrations of alanine and the non-metabolisable amino acids including serine, glycine, threonine, arginine, tyrosine, methionine and lysine (Table 6.1), probably resulting from inhibition of their uptake by glutamine [27] and/or from the effect of glutamine on protein turnover in this tissue. In this regard, we found that 2-15 mM glutamine decreases ($P<0.05$) the uptake of serine by chick EDC muscles (Table 6.2), as previously shown in perfused rat skeletal muscles by Hundal et al [27]. However, inhibition of serine uptake (eg. 39% at 15 mM Gln) by chick EDC muscles cannot fully account for the decrease in intracellular concentration of serine (eg. 52% at 15 mM Gln) in the presence of elevated concentrations of glutamine, suggesting that glutamine may influence the rate of protein turnover in chick skeletal muscle. The addition of 10-15 mM glutamine slightly

decreased ($P<0.05$) intracellular concentrations of leucine, isoleucine, valine and phenylalanine (Table 6.1).

Effect of glutamine on protein synthesis in the presence of all amino acids

Increasing the incubation medium concentration of glutamine from 1.0 mM to 15 mM increased ($P<0.05$) the rate of protein synthesis by 19-58% over that obtained in the absence of glutamine in EDC muscles (Table 6.3). There is a positive relationship between intracellular glutamine concentration and the rate of protein synthesis in chick skeletal muscle ($r=0.975$, $P<0.01$). The stimulating effect of glutamine on the rate of protein synthesis in chick skeletal muscle is consistent with results obtained with perfused rat skeletal muscle preparations [7].

The mechanism by which elevated intracellular concentrations of glutamine increase the rate of protein synthesis in skeletal muscle remains unknown. It is unlikely that the rate of protein synthesis is increased simply because of an increased availability of glutamine as a precursor for protein synthesis [7]. Jepson et al. [9] suggest that the stimulating effect of glutamine on the rate of protein synthesis in skeletal muscle is not related to glutamine oxidation since these authors believe that glutamine oxidation is negligible in this tissue. However, studies presented in Chapter 4 of this thesis clearly show that mitochondrial phosphate-activated glutaminase activity is high in chick EDC muscle and that glutamine is extensively oxidised in this tissue even to a greater extent than leucine (Chapter 4). Thus, it may be possible that the stimulating effect of glutamine on protein synthesis is related to its extensive

oxidation in skeletal muscle.

Effect of glutamine on protein degradation in the presence of all amino acids.

The intramuscular free ^{3}H-phenylalanine radioactivity in prelabelled chick muscles before preincubation was 370.0±4.4 dpm/mg tissue (n=6) and was decreased by 94% after the first 15 min preincubation period. Following the second 15 min preincubation period, it was decreased ($P<0.001$) to 12.5±0.6 dpm/mg tissue (n=6) and remained fairly constant throughout the final 2-h incubation period. These results indicate a rapid exchange between intracellular and extracellular phenylalanine. Since the phenylalanine pool in the incubation medium (3500 nmol Phe) is much larger than the muscle intracellular pool (about 20 nmol Phe), only a negligible amount of ^{3}H-phenylalanine arising from muscle protein degradation would be expected to be reincorporated into muscle protein. Thus, the release of ^{3}H-phenylalanine into the incubation medium should reflect total protein degradation in skeletal muscle.

Table 6.4 shows the effect of glutamine on protein degradation in chick EDC muscles incubated in the presence of all amino acids including tyrosine. It is interesting to note that glutamine has a biphasic effect on protein degradation in chick skeletal muscle. The addition of 1 mM glutamine to the incubation medium increases ($P<0.05$) the rate of protein degradation by 12 % while the addition of 0.5, 2.0, 4.0 and 6.0 mM glutamine had no significant effect ($P>0.05$) on protein degradation as compared to 0 mM glutamine. The addition of 10.0-15.0 mM glutamine significantly inhibited protein degradation by 20-23% as compared to the value obtained at 0 mM

glutamine. The addition of 4-15 mM glutamine decreased ($P<0.05$) the rate of muscle protein degradation by 15-30% as compared to the value obtained at 0.5-1.0 mM glutamine (its normal plasma concentration range).

The inhibition of the rate of protein degradation by elevated concentrations of glutamine in chick EDC muscle incubated in the presence of all amino acids is consistent with the results of Rennie et al. [8] who reported that the infusion of 15 mM glutamine inhibits protein degradation in perfused rat skeletal muscle. However, the 20% inhibition of the rate of protein degradation in chick EDC muscles by 15 mM glutamine (Table 6.4) is much less than the 64% inhibition of protein degradation reported for rat skeletal muscles perfused with 15 mM glutamine [8]. This quantitative discrepancy may be due to the different species studied, or the different experimental approaches used for estimating protein degradation. The mechanism by which elevated intracellular glutamine concentrations influence protein degradation in skeletal muscle of rats and chicks remains unknown. Since glutamine is extensively oxidised in skeletal muscle of chicks (Chapter 4), the inhibiting effect of glutamine on protein degradation may be mediated by the production of ammonia which is a potent inhibitor of lysosomal proteases [28].

Effect of glutamine on protein synthesis and tyrosine release from EDC muscles incubated in the absence of tyrosine.

The rate of protein degradation in, in vitro preparations of rat skeletal muscles is often estimated by measuring the rate of tyrosine release from muscles incubated in the absence of tyrosine [eg. 14-17, 20]. In some studies, the rates of skeletal muscle protein

degradation and synthesis have been simultaneously estimated in muscles incubated in the absence of tyrosine [eg. 16]. From the present study it is clear that the presence of plasma concentrations of tyrosine is required to maximize the effect of elevated glutamine concentrations on the rate of protein synthesis in chick skeletal muscles incubated in vitro. This observation is supported by the following experimental findings. (1) The basal rate of protein synthesis in EDC muscles incubated in the absence of tyrosine (0.248 nmol Phe/2 h/ mg tissue, Table 6.5) is lower ($P<0.01$) than that in muscles incubated in the presence of tyrosine (0.385 nmol Phe/2 h/mg tissue, Table 6.3). (2) The addition of p-hydroxyphenylpyruvate, the α-ketoacid analogue of tyrosine, increased ($P<0.01$) tyrosine formation and the rate of protein synthesis in muscles incubated in the absence of tyrosine (Table 6.7).

The addition of 15 mM glutamine to the tyrosine-free incubation media decreased ($P<0.05$) the release and tissue concentrations of tyrosine, which is in contrast to the addition of 0.5 or 1.0 mM glutamine (Table 6.5). This decrease in tyrosine concentration is probably accounted for by the ability of 15 mM glutamine to simultaneously increase protein synthesis and decrease protein degradation. The ability of 0.5 or 1.0 mM glutamine to increase ($P<0.01$) the tissue concentration ($P<0.01$) and release ($P<0.05$) of tyrosine (Table 6.5) confirms our early observations [13], in which we suggested that glutamine may increase the rate of protein degradation to a greater extent than protein synthesis in chick skeletal muscle. These results, however, are in contrast to our measurements of ^{3}H-phenylalanine release from protein and its

incorporation into protein which indicated that the addition of 0.5 or 1.0 mM glutamine did not increase the rate of muscle protein degradation more than the rate of muscle protein synthesis in the presence of all amino acids (Tables 6.3 and 6.4). Possibly the rate of protein degradation exceeds the rate of protein synthesis in skeletal muscles incubated in the absence of tyrosine, whereas in the presence of tyrosine this is not the case. These observations suggest that the validity of measuring tyrosine release as an index of protein degradation in skeletal muscles incubated in the absence of tyrosine should be reevaluated. An additional complication associated with results obtained in the absence of tyrosine may be that skeletal muscle can use glutamine as an amino donor for providing small amounts of tyrosine.

Is tyrosine synthesised from glutamine in skeletal muscle?

Although it is assumed that tyrosine is not synthesised in skeletal muscle [14], there is no direct information in the literature to confirm this assumption. In the presence of 0.1 mM or less p-hydroxyphenylpyruvate, there was no detectable glutamine-p-hydroxyphenylpyruvate transamination in the mitochondrial or cytosolic fractions of chick EDC muscles even though glutamine concentrations ranged from 2.5 to 50 mM (Table 6.6). However, as the concentration of p-hydroxyphenylpyruvate increased above 0.5 mM in the presence of 10 mM glutamine, tyrosine formation was demonstrated (Table 6.6).

In the presence of 1.0 mM p-hydroxyphenylpyruvate, both tyrosine formation and the rate of protein synthesis in intact chick EDC muscles were increased ($P<0.01$)(Table 6.7). It has been reported that

plasma concentrations of p-hydroxyphenylpyruvate in chicks under normal feeding conditions are much lower than 3 μM [29]. Thus, although chick skeletal muscle has the potential for tyrosine synthesis from glutamine, it would not normally produce adequate amounts of tyrosine to complicate data obtained in the present experiments.

Possible role for glutamine in improving nitrogen balance

Although physiological concentrations of glutamine (0.5-1.0 mM) increased the net rate of protein degradation in chick skeletal muscles incubated in the absence of tyrosine (Table 6.5), elevated concentrations of glutamine appear to have an anabolic effect on protein metabolism in chick skeletal muscles incubated in the presence of plasma amino acids by increasing protein synthesis and/or decreasing protein degradation (Tables 6.3 and 6.4). In this regard, glutamine has a similar effect on skeletal muscle protein turnover as the branched-chain amino acids, particularly leucine [15,16]. However, unlike leucine, glutamine is rapidly taken up by skeletal muscle and accumulated to a large extent in this tissue (Table 6.1) [27]. It is of interest to note that Stehle et al. [10] have recently shown that the administration of 54 mg L-alanyl-L-glutamine-N/kg per day to patients following major injury prevented the postoperative loss of intramuscular glutamine and dramatically decreased urinary nitrogen release by more than 50%. Thus, it appears that glutamine may be useful to reduce muscle wasting associated with sepsis, injury and other catabolic conditions which are characterized by a dramatic decrease in intracellular free glutamine concentrations in skeletal muscle [1,9].

Table 6.1. Effect of glutamine on intracellular amino acid concentrations in chick skeletal muscle

Chick EDC muscles were incubated in the presence of all amino acids except glutamine as indicated. Results (nmol/mg tissue, mean ± SEM) were analysed by the one-way variance analysis. *,** Different from the 0 mM glutamine group at $P<0.05$ and $P<0.01$, respectively. †, ‡, § Different from 0.5-2.0 mM, 0.5-4.0 mM, and 6 mM glutamine at $P<0.05$, respectively. n=13.

Amino acid	Medium glutamine concentration (mM)							
	0.0	0.5	1.0	2.0	4.0	6.0	10.0	15.0
Asp	0.66 ±0.05	0.67 ±0.02	0.75* ±0.08	0.79* ±0.03	0.95**† ±0.06	0.92**† ±0.05	1.53**‡§ ±0.11	1.31**‡§ ±0.08
Glu	1.74 ±0.09	1.75 ±0.09	2.10* ±0.13	1.98* ±0.11	2.36**† ±0.07	2.54**† ±0.08	2.61**‡ ±0.09	2.71**‡ ±0.12
Asn	0.09 ±0.01	0.10 ±0.01	0.12* ±0.01	0.12* ±0.02	0.16**† ±0.03	0.15**† ±0.01	0.26**‡§ ±0.05	0.24**‡§ ±0.02
Gln	3.32 ±0.21	4.23** ±0.40	6.65** ±0.32	9.95** ±0.52	14.18**† ±0.51	17.11**† ±0.74	22.74**‡§ ±0.90	25.34**‡§ ±0.71
Ser	1.98 ±0.12	1.82 ±0.11	1.74 ±0.13	1.65* ±0.10	1.41*† ±0.05	1.23*† ±0.03	1.16**‡ ±0.07	0.95**‡§ ±0.05
Gly	2.89 ±0.34	2.78 ±0.38	2.72 ±0.22	2.36* ±0.21	2.49* ±0.28	2.48* ±0.25	2.47* ±0.21	2.38* ±0.17
Thr	1.18 ±0.10	1.23 ±0.10	1.21 ±0.12	1.01* ±0.06	1.02* ±0.06	1.00* ±0.06	1.04* ±0.06	0.82* ±0.04
Arg	0.52 ±0.07	0.48 ±0.08	0.53 ±0.07	0.44* ±0.07	0.37*† ±0.07	0.34**† ±0.04	0.38*† ±0.07	0.34**† ±0.06
Ala	4.05 ±0.20	4.10 ±0.25	3.95 ±0.33	3.53* ±0.31	3.52* ±0.23	3.42* ±0.16	3.21** ±0.18	2.99**‡ ±0.13
Tyr	0.20 ±0.02	0.19 ±0.02	0.20 ±0.01	0.17* ±0.01	0.17* ±0.01	0.16** ±0.01	0.16** ±0.01	0.14**‡ ±0.01
Trp	0.18 ±0.02	0.21 ±0.02	0.19 ±0.02	0.18 ±0.01	0.19 ±0.01	0.15*‡ ±0.05	0.11**‡ ±0.01	0.12**‡§ ±0.02
Met	0.12 ±0.01	0.11 ±0.01	0.11 ±0.01	0.09* ±0.01	0.09* ±0.02	0.09* ±0.01	0.08** ±0.01	0.08** ±0.01
Val	0.43 ±0.02	0.41 ±0.01	0.42 ±0.03	0.42 ±0.03	0.38 ±0.03	0.40 ±0.02	0.34*† ±0.02	0.35*† ±0.02
Phe	1.23 ±0.03	1.20 ±0.02	1.18 ±0.05	1.19 ±0.06	1.20 ±0.03	1.18 ±0.05	1.13* ±0.03	1.12* ±0.04
Ile	0.21 ±0.03	0.22 ±0.02	0.20 ±0.03	0.21 ±0.03	0.20 ±0.03	0.19 ±0.02	0.17* ±0.02	0.17* ±0.01
Leu	0.28 ±0.02	0.27 ±0.01	0.26 ±0.03	0.26 ±0.03	0.25 ±0.02	0.27 ±0.02	0.24* ±0.02	0.24* ±0.01
Lys	0.54 ±0.10	0.49 ±0.05	0.55 ±0.11	0.43* ±0.08	0.44* ±0.06	0.35* ±0.06	0.28**‡ ±0.07	0.30**‡ ±0.06

Table 6.2. Effect of glutamine on serine uptake by chick EDC muscles

Chick EDC muscles were incubated in the presence of plasma concentrations of all amino acids including 0.7 mM L-[U-^{14}C]serine except glutamine which was added as indicated. Results (mean ± SEM) were analysed by one-way analysis of variance. *,** different from 0-1.0 mM glutamine at $P<0.05$ and $P<0.01$, respectively. †,‡ different from 2.0 mM glutamine at $P<0.05$ and $P<0.01$, respectively. n=8.

Incubation medium glutamine (mM)	Serine uptake (nmol/2 h/ mg tissue)
0	1.86±0.05
1.0	1.79±0.03
2.0	1.67±0.04*
6.0	1.30±0.04**†
15.0	1.13±0.02**‡

Table 6.3. Effect of glutamine on protein synthesis in chick EDC muscles in the presence of all amino acids.

Data were from the same experiment as described in Table 1. Results (mean ± SEM) were analysed by one-way analysis of variance. *,** different from 0 mM glutamine at P<0.05 and P<0.01, respectively. † different from 0.5 mM glutamine at P<0.05; ‡ different from 1 mM glutamine at P<0.05; § different from 0.5-2.0 mM glutamine at P<0.01. n=13.

Incubation medium glutamine (mM)	Intracellular Specific radioactivity of ^{3}H-Phe (dpm/nmol)	Protein synthesis (nmol Phe/ 2 h/mg tissue)
0	1481±42	0.385±0.013
0.5	1408±16	0.428±0.018
1.0	1451±26	0.459±0.031*
2.0	1413±21	0.493±0.034**
4.0	1432±15	0.536±0.021**†
6.0	1343±32	0.578±0.027**‡
10.0	1423±17	0.606±0.028**§
15.0	1382±20	0.610±0.017**§

Table 6.4. Effect of glutamine on protein degradation in chick EDC muscles in the presence of all amino acids

EDC muscles prelabelled in vivo with ^{3}H-phenylalanine were preincubated twice for 15 min and then incubated in fresh media for 2 h in the presence of all amino acids except glutamine unless indicated otherwise. Results (mean ± SEM) were analysed by one-way analysis of variance. All mean values for intracellular glutamine concentrations were significantly different ($P<0.01$) from each other. * different from 0 mM glutamine at $P<0.05$; † different from 0 mM glutamine at $P<0.05$; ‡ different from 0-6.0 mM glutamine at $P<0.01$. n=8.

Incubation medium glutamine (mM)	Intracellular glutamine (nmol/mg tissue)	Intracellular free ^{3}H-Phe (dpm/mg tissue)	Protein degradation (nmol Phe/2 h /mg tissue)
0	3.15±0.22	13.7±0.3	0.381±0.012
0.5	4.27±0.43	12.4±0.3	0.407±0.010
1.0	6.26±0.79	11.5±0.5	0.423±0.018*
2.0	9.81±0.66	12.6±0.2	0.398±0.016
4.0	13.77±0.41	12.4±0.7	0.366±0.011†
6.0	17.40±0.49	13.0±0.2	0.373±0.025†
10.0	21.42±0.62	13.6±0.9	0.[illegible]5±0.015‡
15.0	24.64±1.46	12.3±0.5	0.303±0.025‡

Table 6.5. Effect of glutamine on protein turnover in chick EDC muscles incubated in the absence of tyrosine.

Chick EDC muscles were incubated for 2 h following 30 min preincubation in the presence of chick plasma concentrations of amino acids except tyrosine and glutamine as indicated. Results (mean ± SEM) were analysed by the paired t-test. *, ** different from the control (0 mM gln) at $P<0.05$ and $P<0.01$, respectively. n=10.

Incubation medium glutamine (mM)	Intracellular glutamine concentration (nmol/mg tissue)	Protein synthesis (nmol Phe/2h mg tissue)	Free tyrosine in	
			Tissue (pmol/mg tissue)	Medium (pmol/mg/ tissue)
0	3.29±0.26	0.245±0.017	49.6±7.7	29.1±5.1
0.5	4.43±0.32**	0.252±0.016	66.5±9.2**	37.4±3.3*
0	3.54±0.17	0.248±0.012	55.8±8.0	27.7±5.9
1.0	6.68±0.41**	0.278±0.014*	78.9±6.9**	35.8±3.8*
0	3.31±0.34	0.233±0.008	50.8±7.9	31.3±4.3
15.0	24.83±1.36**	0.277±0.009**	36.7±8.4**	26.4±3.2*

Table 6.6. Glutamine p-hydroxyphenylpyruvate transamination in chick EDC muscles.

Glutamine-p-hydroxyphenylpyruvate transamination was assayed in the presence of 0.025-1.0 mM p-hydroxyphenylpyruvate and 10 mM glutamine, or in the presence of 2.5-50 mM glutamine and 0.1 mM p-hydroxy-phenylpyruvate as described in the text. Results are expressed as means ± SEM. ND, not detectable. n=5.

p-Hydroxy-phenylpyruvate (mM)	Tyrosine formation (nmol Tyr formed /h /mg tissue) Mitochondria	Cytosol	Gln (mM)	Tyrosine formation (nmol Tyr formed/h/ mg tissue) Mitochondria	Cytosol
0.025	ND	ND	2.5	ND	ND
0.05	ND	ND	5.0	ND	ND
0.10	ND	ND	10.0	ND	ND
0.50	ND	0.253±0.041	20.0	ND	ND
1.0	0.079±0.005	0.778±0.022	50.0	ND	

Table 6.7. Effect of p-hydroxyphenylpyruvate on tyrosine release and protein synthesis in chick EDC muscles.

Chick EDC muscles were incubated for 2 h following 30 min preincubation in the presence of plasma concentrations of glutamine (1.0 mM) and other amino acids except that tyrosine was absent. p-Hydroxyphenylpyruvate was added to the incubation medium at 0.1 or 1.0 mM. Results (mean ± SEM) were analysed by the paired t-test. *, ** different from the control (0 mM p-hydroxyphenylpyruvate) at $P<0.05$ and $P<0.01$, respectively. n=6.

Incubation medium p-hydroxy-phenylpyruvate (mM)	Protein synthesis (nmol Phe/2h mg tissue)	Free tyrosine in Tissue (pmol/mg tissue)	Free tyrosine in Medium (pmol/2 h/ mg tissue)
0	0.220±0.010	62.1±4.3	[illegible]±4.2
0.1	0.261±0.016*	68.5±8.1	36.2±5.3
0	0.231±0.012	57.8±9.2	34.1±4.8
1.0	0.382±0.017**	128.3±7.7**	974.2±49.5**

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VII. The Effect Of Ketone Bodies On Protein Turnover In Chick Skeletal Muscle

A. Introduction

Ketone bodies have been implicated to play a role in sparing skeletal muscle protein in humans and animals under catabolic conditions such as prolonged fasting [1,2]. Increased plasma ketone body concentrations have been shown to improve nitrogen balance in traumatized man [3] and in postoperative patients [4]. In additon, the urinary release of 3-methylhistidine, a valid estimator of skeletal muscle protein degradation in humans [5-7], has been shown to be reduced in injured patients with elevated ketone body concentrations [8]. Moreover, the infusion of DL-β-hydroxybutyrate decreases the urinary release of nitrogen and 3-methylhistidine from obese subjects on low energy diets [9]. These findings have been taken to suggest that ketone bodies inhibit skeletal muscle protein degradation [10]. However, whether ketone bodies influence protein turnover by acting directly or indirectly on skeletal muscle has not been established.

There are conflicting reports in the literature concerning the direct effect of ketone bodies on skeletal muscle protein degradation. Ketone bodies have been shown either to decrease [11] or to have no effect [12] on tyrosine release from fasted-rat diaphragms incubated in the absence of amino acids. Similarly, 0.5 mM β-hydroxybutyrate has been reported to have no effect on tyrosine release in fed-rat diaphragms incubated in the absence of amino acids but in the presence of 0.5 mM cycloheximide, an inhibitor of protein synthesis [13]. The interpretation of these results [11-12] is problematic since tyrosine

release from muscle represents only the net rate of protein degradation, which depends upon the balance between the rate of protein synthesis and the rate of protein degradation. In addition, the presence of cycloheximide [13] may disturb muscle metabolism as it has been shown to inhibit protein degradation in cultured muscle cells [14].

There is limited information concerning the effect of ketone bodies on skeletal muscle protein synthesis. It has been reported in one study [12] that ketone bodies have no effect on protein synthesis in fed rat diaphragms incubated in the absence of amino acids. However, the absence of amino acids from the incubation medium may decrease the sensitivity of the rate of muscle protein synthesis to ketone body concentrations. The present study was therefore designed to evaluate the effect of ketone bodies on protein synthesis and protein degradation in skeletal muscles from fed and fasted chicks incubated in the presence of plasma concentrations of all amino acids. The rate of protein synthesis was estimated from the incorporation of ^{3}H-phenylalanine into protein, while the release of ^{3}H-phenylalanine from muscle proteins prelabelled in vivo was measured as an index of protein degradation.

B. Materials and Methods

Chemicals.

Lithium acetoacetate, sodium DL-β-hydroxybutyrate, sodium acetate, sodium pyruvate, bovine insulin, glucose, amino acids, Hyamine hydroxide and other chemicals were purchased from Sigma Chemical Co., St. Louis, U.S.A. L-[2,6-^{3}H]phenylalanine was

obtained from Amersham, Ontario, Canada. L-[U-^{14}C]glutamine was obtained from American Radiolabeled Chemicals, St. Louis, U.S.A. The radiochemical purity of [U-^{14}C]glutamine was determined to be greater than 99% by descending paper chromatography [15].

Animals.

Day-old male broiler chicks (Hubbard strain) were obtained from a local hatchery and were allowed free access to water and food. Chicks were fed a broiler starter ration containing 24% crude protein. At the age of nine days, chicks weighing 110-120 were either continuously fed or fasted for 24 h.

Muscle preparation and incubation.

Individual chicks were anesthetised with halothane and the extensor digitorum communis (EDC) muscles were carefully exposed and inserted into stainless wire supports by means of their tendons [15] to prevent contraction and thus improve protein balance and energy status [16]. Each muscle was immediately dissected, preincubated for 30 min in 3.5 ml Krebs-Ringer bicarbonate buffer saturated with O_2/CO_2 (95%:5%) [15], and then transferred to fresh media of a similar composition for the final 2-h incubation period. The incubation media also contained 2 mM HEPES (pH 7.4), 12 mM glucose, insulin (0.01 U/ml), 1.0 mM phenylalanine, 1.0 mM glutamine and other amino acids at concentrations found in young chick plasma (Appendix 1). Ketone bodies or pyruvate were added to the incubation media as indicated in the Tables. In all experiments, one muscle was used for the control while the contralateral was used for the treatment.

Measurement of protein synthesis

EDC muscles were incubated in media containing 1.0 mM

[2,6-^{3}H]phenylalanine (0.64 mCi/mmol) as described above. The incubation media were continuously gassed with O_2/CO_2 (19:1) throughout the incubation periods. At the end of the 2-h final incubation period, EDC muscles were rinsed in nonradioactive medium, blotted and homogenised in 2 ml 2% trichloroacetic acid. The rate of muscle protein synthesis and the intracellular concentrations of amino acids were measured as described in Chapter 6.

Measurement of protein degradation

At nine days of age, chicks were injected via the intraperitoneal route with 0.45 ml physiological saline containing 0.4 mM [2,6-^{3}H]phenylalanine (0.83 Ci/mmol). Twenty four hours after isotope injection, EDC muscles were isolated and preincubated twice for 15 min in Krebs-Ringer bicarbonate buffer supplemented with insulin, glucose and amino acids as previously described (Chapter 6). The release of ^{3}H-phenylalanine from muscle protein and the rate of protein degradation were measured as previously described (Chapter 6).

Measurement of L-[U-^{14}C]glutamine oxidation

EDC muscles were incubated for 2 h in media containing 1.0 mM L-[U-^{14}C]glutamine (54 d.p.m./nmol carbon) as described above except that the media was not gassed during the final incubation period. At the end of the incubation period, $^{14}CO_2$ from glutamine oxidation was collected in Hyamine hydroxide [15]. The rate of CO_2 production from glutamine carbons 1-5 was calculated on the basis of the intracellular specific radioactivity of [U-^{14}C]glutamine (d.p.m./nmol carbon), which was measured as described in Chapter 4.

The results were statistically analysed by the paired t-test or analysis of variance as described by Steel and Torrie [17].

C. Results and Discussion

Effect of ketone bodies on protein turnover in chick EDC muscles

Ketone bodies at 4 mM significantly decreased ($P<0.05$) the rate of protein synthesis but had no significant effect ($P>0.05$) on the rate of protein degradation in skeletal muscles from fed chicks (Table 7.1). On the other hand, ketone bodies decreased ($P<0.05$) the rates of both protein synthesis and protein degradation in EDC muscles from 24-h fasted chicks (Table 7.1). The present findings demonstrate, for the first time, that inhibition of the rate of skeletal muscle protein synthesis and protein degradation by ketone bodies can arise from the direct action of ketone bodies on this tissue.

Effect of ketone bodies on intracellular concentrations of amino acids in chick EDC muscles

Ketone bodies have been shown to regulate amino acid metabolism and their intracellular concentrations in chick skeletal muscle [15], which is confirmed in this study (Table 7.2). Ketone bodies decreased ($P<0.01$) intracellular concentrations of aspartate, glutamine and alanine, but increased ($P<0.01$) intracellular concentrations of glutamate in EDC muscles from fed chicks (Table 7.2). The intracellular concentrations of BCAA (Table 7.2) and other amino acids were not influenced by the addition of 4 mM ketone bodies to the incubation media of EDC muscles from fed chicks (data not shown). By contrast, ketone bodies decreased ($P<0.01$) intracellular concentrations of aspartate and alanine but increased ($P<0.01$) intracellular concentrations of glutamate, glutamine and BCAA in EDC muscles from fasted chicks. Therefore, it was of interest to investigate whether the action of ketone bodies on protein turnover in

chick skeletal muscle is mediated by their effect on amino acid metabolism, since BCAA [12] and glutamine [18-20] have been shown to regulate the rate of protein synthesis and degradation in skeletal muscle.

Possible involvement of amino acids in the inhibition of protein synthesis by ketone bodies in chick EDC muscles

Increased concentrations of leucine have been shown to increase the rate of protein synthesis in rat skeletal muscle [eg. 12]. However, ketone bodies decreased the rate of protein synthesis in skeletal muscles from either fed or fasted chicks (Table 7.1) whether or not BCAA concentrations increased (Table 7.2). These results suggest that BCAA concentrations do not mediate the inhibiting effect of ketone bodies on protein synthesis in chick skeletal muscle.

It was of interest to note in EDC muscles from fed chicks that ketone bodies markedly decreased ($P<0.01$) intracellular glutamine concentrations, which have recently been shown to be positively related to the rate of protein synthesis in skeletal muscles from rats [18,19] and chicks (Chapter 6). Therefore we investigated whether glutamine mediates the effect of ketone bodies on protein synthesis in skeletal muscles from fed chicks. As shown in Table 7.3, the addition of 0.5 mM glutamine to the incubation media containing 1.0 mM glutamine prevented the ketone body-induced decrease in intracellular glutamine concentration (Table 7.3) as well as the small but significant inhibitory effect of ketone bodies on protein synthesis in skeletal muscles from fed chicks (Table 7.3). These results suggest that glutamine may be involved in the action of ketone bodies on protein synthesis in EDC muscles from fed chicks.

This suggestion is further supported by the following observations. We have recently found that glutamine is extensively oxidised in chick skeletal muscles incubated in the presence of plasma concentrations of glucose and amino acids (Chapter 4). The oxidation of glutamine is increased in EDC muscles from fed chicks incubated in the presence of 4 mM ketone bodies (Table 7.4), thereby accounting for the associated decrease in intracellular concentration of glutamine (Table 7.2). The addition of 5 mM pyruvate to the incubation medium prevented the ketone-body induced increase in glutamine oxidation (Table 7.4) and the associated decrease in intracellular glutamine concentration (Table 7.3). The rate of protein synthesis was not influenced ($P>0.05$) by the addition of pyruvate to the incubation media in the absence of ketone bodies (Table 7.3), however, the addition of 5 mM pyruvate blocked the inhibiting effect of ketone bodies on protein synthesis in skeletal muscles from fed chicks (Table 7.3). These results suggest that ketone bodies decrease protein synthesis by reducing intracellular glutamine concentrations due to an increase in glutamine oxidation in skeletal muscles from fed chicks.

The mechanism by which ketone bodies increase glutamine oxidation in skeletal muscle is not clear. Ketone body oxidation via the Krebs cycle requires oxaloacetate. Since ketone bodies inhibit glycolysis and the degradation of BCAA and alanine [15], the source of pyruvate and oxaloacetate from glucose, glycogen and most glucogenic amino acids may be limiting. Since oxaloacetate is not supplied from other sources, oxidation of ketone bodies may be inhibited. Thus, an increase in glutamine oxidation would be required to provide oxaloacetate to the Krebs cycle to help oxidise ketone bodies. This

suggestion is supported by the findings that the addition of 5 mM pyruvate, which can be converted to oxaloacetate via pyruvate carboxylase in skeletal muscle [22], prevents the ketone body-induced increase in glutamine oxidation.

It is interesting to note that fasting dramatically decreases ($P<0.01$) the intracellular concentration of glutamine in chick skeletal muscle (Table 7.2). In contrast to skeletal muscles from fed chicks, ketone bodies increase ($P<0.05$) the intracellular concentration of glutamine in skeletal muscles from fasted chicks (Table 7.2) probably due to an increase in its synthesis since there is a reduced amount of pyruvate for alanine synthesis [15]. It is unlikely that the increased concentration of glutamine is involved in the inhibition of protein synthesis by ketone bodies in skeletal muscle from the fasted chick. It remains to be determined how ketone bodies decrease protein synthesis in skeletal muscles from fasted chicks.

Is KIC involved in the inhibition of protein degradation by ketone bodies in skeletal muscle from the fasted chick?

Leucine inhibits protein degradation in skeletal muscle in vitro via the production of α-ketoisocaproate (KIC) [12,23]. Newsholme and Leech [10] have speculated that ketone bodies increase production of a metabolite of leucine, thereby inhibiting muscle protein degradation. Recently, we have shown that ketone bodies markedly increase the net production of KIC in skeletal muscles from fed chicks [24]. However, ketone bodies had no effect on protein degradation in muscles from fed chicks (Table 7.1). In contrast, DL-β-hydroxybutyrate had no effect on the net production of KIC in

skeletal muscles from fasted chicks [24], yet it decreased ($P<0.05$) the rate of protein degradation in these muscles (Table 7.1). Thus, leucine or KIC does not appear to mediate the ability of ketone bodies to inhibit protein degradation in EDC muscles from fasted chicks. The mechanism by which ketone bodies inhibit protein degradation in skeletal muscles from fasted chicks remains unknown.

Physiological significance of the inhibition of protein turnover by ketone bodies

The present finding that elevated concentrations of ketone bodies inhibit protein degradation in skeletal muscles from fasted chicks lends direct evidence to support earlier suggestions that ketone bodies may play an important role in sparing skeletal muscle protein under catabolic conditions such as prolonged fasting [1,2]. An inhibition of protein synthesis by ketone bodies in skeletal muscle may direct amino acids to other tissues such as the liver and kidneys. This may be of physiological importance in animals and humans with high ketone body concentrations due to starvation or the feeding of high-fat diet, since the liver may need more amino acids to synthesize the proteins for carrying plasma circulating lipids. Also, the kidneys may need to extract more glutamine for regulating acid/base balance during ketoacidosis.

In conclusion, ketone bodies act directly on skeletal muscle by decreasing the rate of protein synthesis in muscles from both fed and fasted chicks as well as the rate of protein degradation in muscles from fasted chicks. Our results suggest that ketone bodies decrease protein synthesis in skeletal muscles from fed chicks by decreasing intracellular concentrations of glutamine due to an increase in the

rate of its oxidation. However, it remains to be determined how ketone bodies inhibit protein turnover in skeletal muscles from fasted chicks.

Table 7.1. Effect of ketone bodies on protein turnover in EDC muscles from fed and 24 h fasted chicks.

Chick EDC muscles were incubated in the presence of plasma concentrations of glucose and amino acids as described in the text. DL-β-hydroxybutyrate (HB) (4 mM) or acetoacetate (AcAc) (4 mM) was added to incubation media as indicated. Protein synthesis was determined by measuring the incorporation of ^{3}H-phenylalanine into muscle protein. Protein degradation was determined by measuring the release of ^{3}H-phenylalanine from muscle proteins prelabelled in vivo. *,** different from the control at $P<0.05$ and $P<0.01$, respectively, as analysed by the paired t-test. † different ($P<0.01$) from the fed control, as analysed by analysis of variance. n=10.

Treatment	Protein Synthesis (nmol Phe/2 h/mg tissue)	Protein Degradation (nmol Phe/2 h/mg tissue)
Fed chicks		
Control	0.345±0.023	0.485±0.022
HB	0.307±0.021*	0.455±0.031
Control	0.331±0.016	0.472±0.043
AcAc	0.284±0.014*	0.506±0.050
Fasted chicks		
Control	0.244±0.006†	0.732±0.080†
HB	0.219±0.008*	0.673±0.074*
Control	0.269±0.010†	0.686±0.038†
AcAc	0.226±0.009**	0.592±0.024**

Table 7.2. Effect of ketone bodies on intracellular concentration of amino acids in EDC muscles from fed and 24 h fasted chicks.

Chick EDC muscles were incubated in the presence of plasma concentrations of glucose and amino acids as described in the text. DL-β-hydroxybutyrate (HB) (4 mM) or acetoacetate (AcAc) (4 mM) was added to incubation media as indicated. Results were analysed by the paired t-test. *,** different from the control at $P<0.05$ and $P<0.01$, respectively. n=10.

Treatment	Intracellular amino acids (nmol/mg tissue)						
	Asp	Glu	Gln	Ala	Val	Ile	Leu
Fed chicks							
Control	0.98 ±0.04	2.04 ±0.11	6.51 ±0.51	4.24 ±3.40	0.45 ±0.02	0.23 ±0.01	0.35 ±0.02
HB	0.81* ±0.04	2.49** ±0.10	5.43** ±0.42	3.86* ±0.18	0.45 ±0.02	0.22 ±0.01	0.38 ±0.02
Control	1.04 ±0.06	2.19 ±0.15	6.42 ±0.26	4.47 ±0.18	0.44 ±0.02	0.24 ±0.02	0.34 ±0.01
AcAc	0.64** ±0.04	3.53** ±0.28	4.95** ±0.58	3.66** ±0.28	0.42 ±0.01	0.23 ±0.01	0.35 ±0.02
Fasted chicks							
Control	1.26 ±0.11	1.62 ±0.08	3.69 ±0.23	3.19 ±0.24	0.35 ±0.02	0.15 ±0.01	0.23 ±0.01
HB	0.82** ±0.05	3.04** ±0.24	4.18** ±0.49	2.54** ±0.15	0.43** ±0.01	0.20** ±0.01	0.28** ±0.02
Control	1.11 ±0.12	1.70 ±0.06	3.52 ±0.18	2.90 ±0.07	0.36 ±0.01	0.16 ±0.01	0.23 ±0.01
AcAc	0.73 ±0.04	4.10** ±0.12	3.98* ±0.17	2.08** ±0.10	0.44** ±0.02	0.22** ±0.01	0.29** ±0.02

Table 7.3. Effect of glutamine and pyruvate on intracellular concentrations of amino acids and protein synthesis in EDC muscles from fed chicks incubated in the presence of ketone bodies.

EDC muscles from fed chicks were incubated in the presence of plasma concentrations of glucose and amino acids as described in the text. The basal incubation medium contained 1.0 mM glutamine. DL-β-hydroxybutyrate (HB) (4 mM), acetoacetate (AcAc) (4 mM), pyruvate (Pyr) (5 mM) or 0.5 mM glutamine (Gln) was added to incubation media as indicated. Results were analysed by the paired t-test. * different ($P<0.01$) from the control. n=10.

Treatment	Intracellular amino acids (nmol/mg tissue)				Protein synthesis (nmol Phe/ 2 h/ mg) tissue
	Glu	Asp	Ala	Gln	
Control	2.08±0.17	0.97±0.04	4.07±0.23	6.28±0.38	0.332±0.014
HB + 0.5 mM Gln	3.05±0.14*	0.76±0.05*	3.65±0.18*	6.73±0.43	0.314±0.016
Control	2.22±0.16	0.96±0.03	3.92±0.11	6.22±0.35	0.349±0.011
AcAc + 0.5 mM Gln	3.59±0.18*	0.72±0.04*	3.24±0.14*	6.65±0.36	0.326±0.013
Control	1.94±0.13	0.83±0.04	3.96±0.23	6.23±0.84	0.353±0.033
Pyr	1.59±0.07*	0.73±0.04*	4.97±0.16*	5.77±0.87	0.338±0.024
Control	1.98±0.10	0.92±0.07	3.90±0.26	6.18±0.40	0.318±0.021
Pyr + HB	1.82±0.09	0.88±0.05	4.73±0.34*	5.92±0.43	0.302±0.017
Control	2.12±0.14	0.90±0.03	4.47±0.38	6.47±0.76	0.325±0.007
Pyr + AcAc	1.95±0.07	0.92±0.02	5.63±0.65*	6.18±0.63	0.311±0.012

Table 7.4. Effect of ketone bodies on glutamine oxidation in EDC muscle from the fed chick.

EDC muscles from fed chicks were incubated in the presence of 1.0 mM L-[U-^{14}C]glutamine (54 d.p.m./nmol labelled carbon) and plasma concentrations of glucose and amino acids as described in the text. DL-β-hydroxybutyrate (HB) (4 mM), acetoacetate (AcAc) (4 mM), or pyruvate (Pyr) (5 mM) was added to incubation media as indicated. Results were analysed by the paired t-test. ** different ($P<0.01$) from the control. n=10.

Treatment	Intracellular specific radioactivity of [U-^{14}C]Gln (d.p.m./nmol labelled carbon)	CO_2 production from glutamine carbons 1-5 (nmol/2 h/ mg tissue
Control	18.1±0.6	12.30±0.55
HB	16.4±0.5	15.42±0.70**
Control	18.5±0.4	12.85±0.75
AcAc	17.3±4.7	18.71±1.35**
Control	19.2±0.9	13.72±0.57
Pyr	18.7±0.8	14.25±0.60
Control	19.6±0.7	12.64±0.48
Pyr + HB	18.8±0.9	13.98±0.46
Control	16.8±0.5	14.23±0.63
Pyr + AcAc	16.0±0.7	16.22±0.76

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VIII. General Discussion and Conclusions

A. Amino acid metabolism in skeletal muscle

1. *De novo* synthesis of alanine and glutamine in skeletal muscle and its regulation by ketone bodies

The early discovery that large amounts of alanine and glutamine relative to other amino acids are released from skeletal muscle puzzled scientists for some time [1-2] until Goldberg and his coworkers demonstrated that alanine and glutamine are synthesised *de novo* in mammalian skeletal muscle [3]. The results presented in this thesis show that chick skeletal muscle also extensively synthesises alanine and glutamine (Chapter 2), therefore casting doubt on an early report that chick skeletal muscle does not contain glutamine synthetase activity [4]. The coupling of BCAA transamination with *de novo* synthesis of alanine and glutamine is shown in Fig. 1.2 as originally proposed by Goldberg and his associates [3]. In this diagram, BCAA transaminate with α-ketoglutarate to form glutamate, which is then transaminated with pyruvate to produce alanine or amidated with NH_4^+ to produce glutamine.

It is well known that the release of alanine from skeletal muscle is reduced during prolonged fasting characterized by an increase in plasma ketone body concentrations [5]. In vitro studies have demonstrated that ketone bodies inhibit alanine synthesis in rat skeletal muscle [6], but the mechanism of action of ketone bodies has not been thoroughly understood. Ketone bodies markedly increase intracellular glutamate concentrations in skeletal muscles from fasted

chicks, suggesting that glutamate is not limiting for alanine synthesis (Chapter 3). On the other hand, ketone bodies decrease glycolysis and intracellular pyruvate concentrations in skeletal muscles from fasted chicks, suggesting that they decrease alanine synthesis by limiting the availability of intracellular pyruvate due to inhibition of glycolysis (Fig. 8.1). This suggestion is supported by the following findings from this thesis. (1) Glucose increases alanine synthesis in chick skeletal muscle from the fasted chick in the absence of ketone bodies, but has no effect on alanine synthesis in the presence of 4 mM ketone bodies. (2) The addition of 5 mM pyruvate to the incubation media prevents the inhibiting effect of ketone bodies on alanine synthesis. These results emphasize the importance of glycolysis in alanine synthesis in chick skeletal muscle as previously shown in rat skeletal muscle [3].

In contrast to alanine synthesis, ketone bodies increase glutamine synthesis in skeletal muscles from fasted chicks. This likely results from increased availability of glutamate due to inhibition of alanine synthesis (Fig. 8.1). The increased release of glutamine from skeletal muscle may increase the amount of glutamine for extraction by the kidneys where it plays an important role in regulation of acid/base balance [7]. Thus, in ketoacidosis, skeletal muscle itself may be able to regulate the synthesis of glutamine in response to metabolic challenges.

2. Regulation of BCAA degradation by ketone bodies

Earlier studies demonstrated that ketone bodies inhibit BCAA oxidative decarboxylation in skeletal muscle from both fed and fasted chicks [8]. In addition, ketone bodies markedly inhibit BCAA

transamination in skeletal muscle from the fasted chick (Chapter 3). Inhibition of BCAA degradation by ketone bodies can spare these essential amino acids during fasting. This makes physiological sense since ketone bodies produced in the liver are extensively oxidised as alternative energy sourses in extrahepatic tissues including skeletal muscle, thereby sparing BCAA for protein synthesis in organs such as the liver and the kidneys.

The mechanisms by which ketone bodies inhibit BCAA degradation have not previously been studied. Recent studies have suggested that the availability of amino group acceptors such as α-ketoglutarate and pyruvate can regulate BCAA transamination in skeletal muscle [9]. As shown in Chapter 3, the addition of 5 mM pyruvate prevents the inhibiting effect of ketone bodies on BCAA transamination in the fasted-chick skeletal muscle. These results suggest that ketone bodies inhibit BCAA transamination by decreasing intracellular concentration of pyruvate due to inhibition of glycolysis, which decreases the regeneration of α-ketoglutarate. (Fig. 8.1). On the other hand, ketone bodies inhibit BCKA oxidative decarboxylation by a different mechanism than decreasing pyruvate availability, since pyruvate also markedly inhibits BCAA oxidative decarboxylation [9]. The activation of ketone bodies and pyruvate for oxidation requires CoA and thus decreases its intracellular concentration. Since CoA is also required for the oxidative decarboxylation of branched-chain α-keto acids (BCKA), a decrease in CoA availability in the presence of ketone bodies or pyruvate may explain their inhibitory effect on BCKA oxidative decarboxylation in chick skeletal muscle (Fig. 8.1).

3. Oxidation of glutamine in skeletal muscle and its regulation by ketone bodies

Although the importance of glutamine synthesis in skeletal muscle is well recognized (Fig. 1.2), the role of glutamine degradation in regulating intramuscular glutamine concentration has remained largely ignored [3]. Studies presented in this thesis clearly demonstrate, for the first time, that skeletal muscles from both rats and chicks extensively oxidise glutamine (Chapter 4). Indeed, the rate of oxidative decarboxylation of physiological concentrations of glutamine is greater than that of leucine in both rat and chick skeletal muscle (Chapter 4). It follows that the release and intracellular concentration of glutamine in skeletal muscle depends not only on the rate of its synthesis but also on the rate of its degradation in this tissue.

Oxidation of glutamine in skeletal muscle produces ammonia by either the phosphate-activated glutaminase pathway or the glutamine aminotransferase pathway (Chapter 4). The studies presented in this thesis challenge the traditional concept that the purine nucleotide cycle is the only significant source of ammonia released from skeletal muscle [10]. It is interesting to hypothesize that oxidation of glutamine in skeletal muscle may be increased during exercise, as previously shown for the branched-chain amino acids [11], thereby accounting for, in part, the decreased intramuscular glutamine concentration and increased release of ammonia from exercised skeletal muscle [10].

The oxidation of glutamine in skeletal muscle supplies 4 and 5 carbon Krebs cycle intermediates. This may be of particular importance

when there is an increased demand for oxaloacetate to oxidise acetyl-CoA produced from partial oxidation of fatty acids and ketone bodies. This point is substantiated by the findings from this thesis that the rate of glutamine oxidation in chick skeletal muscle increases in the presence of elevated concentrations of ketone bodies (Chapter 7) (Fig. 8.2). In addition, the conversion of acetoacetate to acetoacetyl-CoA is stoichiometrically linked to the conversion of succinyl-CoA to succinate, thus favouring the oxidative decarboxylation of α-ketoglutarate and hence glutamine oxidation (Fig. 8.2).

The suggestion that ketone bodies increase the rate of glutamine degradation to provide oxaloacetate for oxidation of acetyl-CoA is also supported by the following evidence from this thesis. Pyruvate (5 mM), which can be converted to oxaloacetate in skeletal muscle via pyruvate carboxylase [12], prevents the ketone body-induced increase in glutamine oxidation as well as the ketone body-induced decrease in intramuscular glutamine concentrations in chick skeletal muscle (Chapter 7). These results suggest a role for oxaloacetate flux in regulating amino acid degradation in skeletal muscle. They also extend the concept proposed in this thesis that both glutamine synthesis and its degradation, rather than glutamine synthesis alone [3], are important in regulating the intracellular concentration of glutamine in skeletal muscle.

4. Metabolic fates of amino acids in skeletal muscle

In view of previous studies by Goldberg and his associates [3] and the data presented herein, it can be concluded that skeletal muscle extensively degrades glutamine, alanine, glutamate, aspartate,

leucine, isoleucine and valine, and actively synthesises glutamate, alanine and glutamine. Although methionine has recently been reported to be extensively oxidised in skeletal muscle via a transamination pathway under relatively nonphysiological conditions [eg. 13], methionine transamination in skeletal muscle is shown in this thesis to be quantitatively of little importance in the presence of plasma concentrations of amino acids (Chapter 6). This is likely due to the very low affinity of BCAA aminotransferase towards methionine in the presence of plasma concentrations of amino acids [14]. Thus, the metabolic fates of amino acids in skeletal muscle reported in the literature (Table 1.2) should be modified as shown in Table 8.1.

5. Species differences in amino acid degradation in skeletal muscle

It is well known that there are many differences in nitrogen metabolism between the ureotelic mammal and the uricotelic avian species, which secrete urea and uric acid as nitrogenous end products, respectively [15]. Glutamine is the most important intermediate in detoxification of ammonia liberated from amino acids and the purine nucleotide cycle in extrahepatic organs in mammals. On the other hand, glutamine is a direct precursor in the pathway of uric acid synthesis in birds [15]. Consistant with these concepts are the different activities of glutamine-metabolising enzymes in the liver between rats and chicks. For example, glutamine synthetase activity in chick liver is about 10-15 times higher than in rat liver, while phosphate-activated glutaminase activity in chick liver is about 8 times lower than in rat liver [16].

There are also species differences in glutamine degradation in skeletal muscle between rats and chicks in terms of the pathway for

its conversion to α-ketoglutarate (Chapter 4). For example, the mitochondrial phosphate-activated glutaminase activity in chick skeletal muscle is about 7-15 times higher than that in rat skeletal muscle, while the cytosolic activities of glutamine aminotransferases K and L in chick skeletal muscle are about 20-30 times lower than those in rat skeletal muscle. The distribution of these enzymes suggests different pathways and locations for initiating glutamine degradation in chick and rat skeletal muscle (Chapter 4). The physiological significances of these differences remain to be determined.

Another example of species difference in skeletal muscle amino acid degradation is methionine transamination. Methionine is extensively transaminated in chick skeletal muscle incubated in the absence of other amino acids (Chapter 5), However, there is essentially no methionine transamination in rat skeletal muscle incubated in the absence of other amino acids (Chapter 5). Although these results are unlikely of physiological importance, they may indicate a different affinity of BCAA aminotransferase towards methionine in chick and rat skeletal muscles.

B. Protein turnover in skeletal muscle

1. Evaluation of the measurement of protein degradation on the basis of tyrosine release from skeletal muscle.

The problems associated with using the tyrosine release technique to estimate skeletal muscle protein degradation have been discussed in Chapter 1. One must be cautious in interpreting experimental data obtained by this technique as demonstrated by the following evidence

developed from this thesis. (1) Glutamine (0.5 or 2.0 mM) significantly increases tyrosine release from chick EDC muscle (Chapter 6), however, it has no significant effect on the rate of protein degradation in chick EDC muscles incubated in the presence of all amino acids when measured by the release of ^{3}H-phenylalanine from prelabelled proteins (Chapter 6). (2) Ketone bodies decrease tyrosine release from chick EDC muscles obtained from fed chicks incubated in the absence of tyrosine [17], but have no effect on protein degradation in EDC muscles from fed chicks incubated in the presence of all amino acids when measured by the release of ^{3}H-phenylalanine from prelabelled proteins (Chapter 7). These results suggest that the validity of measurement of protein degradation on the basis of tyrosine release from skeletal muscle should be reevaluated.

2. Role of glutamine in regulating protein synthesis and degradation in skeletal muscle

Glutamine is a unique amino acid in terms of its abundance in skeletal muscle and the dynamic changes in its concentration under a variety of physiological and pathological conditions [18]. Glutamine has been shown to increase protein synthesis [19] and decrease protein degradation [20] in perfused rat skeletal muscle. A similar effect of glutamine on protein synthesis is also demonstrated in incubated rat skeletal muscle (Appendix 6). Glutamine also increases protein synthesis and decreases protein degradation in chick skeletal muscle in vitro (Chapter 6). These findings indicate that glutamine plays a role in regulating nitrogen balance in skeletal muscle, which is similar to the effect of branched-chain amino acids (BCAA) [21].

However, unlike BCAA, glutamine is extensively accumulated by skeletal muscle (Chapter 6) [19]. It is of interest to note that the intraveinous administration of L-alanyl-L-glutamine to patients following major surgery prevented the loss of intramuscular glutamine and dramatically improved nitrogen balance [22].

The mechanism by which glutamine regulates protein synthesis is not known. It is unlikely that glutamine availability is limiting for protein synthesis in skeletal muscle since glutamine is the most abundant intramuscular free amino acid [19] (Chapter 6). It is possible that the increased rate of skeletal muscle protein synthesis in the presence of elevated glutamine concentrations is closely related to its extensive oxidation in this tissue (Chapter 4). It has been reported that glutamine increases glycogen synthesis in the liver by increasing the activity of glycogen synthase, which, like the eIF-2 protein in skeletal muscle [23], is inactivated by phosphorylation and activated by dephosphorylation [24]. Therefore, it is interesting to hypothesize that glutamine increases the rate of protein synthesis in skeletal muscle by regulating phosphorylation of polypeptide initiating factors such as eIF-2. On the other hand, glutamine may decrease the rate of protein degradation by increasing the production of ammonia, which is a potent inhibitor of muscle lysosomal proteases [25].

Since glutamine is able to regulate the rate of protein synthesis in skeletal muscle, it is important to determine whether glutamine mediates the action of other compounds that are known to increase or decrease the rate of protein synthesis in this tissue. It appears that ketone bodies inhibit protein synthesis by decreasing intracellular

glutamine concentrations in skeletal muscles from fed chicks on the basis of evidence presented in this thesis. (1) ketone bodies decrease the intracellular concentration of glutamine and also the rate of protein synthesis in skeletal muscle from the fed chick, which can be prevented by the addition of 0.5 mM glutamine to the incubation media (Chapter 7). (2) Pyruvate (5 mM) prevented the ketone body-induced decrease in the intracellular concentration of glutamine by blocking the associated increase in glutamine oxidation, and also prevented the inhibiting effect of ketone bodies on the muscle protein synthesis (Chapter 7). These results lend additional support to the concept proposed by MacLennan et al. [19] and in this thesis that glutamine plays an important role in regulating skeletal muscle protein synthesis.

3. Inhibition of protein synthesis and degradation by ketone bodies in skeletal muscle from the fasted chick

It is well documented that protein synthesis and protein degradation in skeletal muscle are decreased during prolonged fasting [26]. These changes may be partly due to decreased plasma concentrations of insulin, glucagon, and thyroid hormones [26], but may be also partly due to increased concentrations of ketone bodies since they decrease protein synthesis and degradation in skeletal muscles from fasted chicks (Chapter 7). These results provide the first evidence that ketone bodies regulate protein metabolism in skeletal muscle by acting directly on this tissue and also lend direct evidence to support the concept that ketone bodies can spare muscle proteins [3,5].

In contrast to muscles from fed chicks, ketone bodies increase

intracellular glutamine concentrations in skeletal muscles from fasted chicks (Chapter 7) probably by increasing glutamine synthesis because of a decreased availability of pyruvate as a nitrogen acceptor (Chapter 3). It appears that the inhibitory effect of ketone bodies on protein synthesis in skeletal muscles from fasted chicks is not mediated by intracellular glutamine concentrations. The mechanism by which ketone bodies inhibit protein synthesis in skeletal muscles from fasted chicks remains to be determined. Also, the differences in the response of protein turnover (Chapter 7) and amino acid metabolism [8,9] to ketone bodies between skeletal muscles from fed and fasted chicks need to be explained.

C. Conclusions

In summary, the following conclusions can be drawn from this thesis.

(1) The transamination of BCAA with α-ketoglutarate in chick skeletal muscle provides amino groups for de novo synthesis of alanine and glutamine (Chapter 2).

(2) Ketone bodies decrease alanine synthesis and BCAA transamination in fasted-chick skeletal muscle by decreasing tissue pyruvate concentrations due to inhibition of glycolysis (Chapter 3).

(3) Glutamine is extensively oxidised in skeletal muscle in the presence of plasma concentrations of amino acids and glucose, thus challenging the traditional view that oxidation of glutamine in this tissue is negligible (Chapter 4).

(4) Methionine transamination in skeletal muscle is quantitatively of little significance in the presence of plasma concentrations of

amino acids, thus questioning the physiological role of the methionine transamination pathway recently proposed in this tissue. (Chapter 5).

(5) Elevated concentrations of glutamine increase protein synthesis and decrease protein degradation in chick skeletal muscle, suggesting a role for glutamine in improving N balance in this tissue (Chapter 6).

(6) Ketone bodies decrease protein synthesis in skeletal muscles from fed chicks by decreasing intracellular concentrations of glutamine due to an increase in its oxidation (Chapter 7).

(7) Ketone bodies inhibit both protein synthesis and protein degradation in skeletal muscles from fasted birds (Chapter 7), suggesting that decreased rates of protein synthesis observed during fasting may be partly due to increased plasma concentration of ketone bodies.

D. Suggestions for future studies

Although the objectives listed in the Chapter 1 of this thesis have been achieved, many additional interesting questions have arisen that remain to be answered. Several suggestions are therefore made for future studies.

(1) Although skeletal muscle can synthesise a large amount of glutamine, there is no information available regarding the regulation of glutamine synthetase in this tissue. Is glutamine synthetase activity in skeletal muscle regulated by phosphorylation and dephosphorylation as recently shown for the glutamine synthetase in mycobacterium (27)?

(2) Do ketone bodies inhibit BCKA decarboxylation by decreasing intracellular concentrations of CoA-SH in skeletal muscle? Does the feeding of high-fat diets to animals, which results in an increase in plasma ketone body concentrations [5], inhibit BCAA degradation in skeletal muscle? Is the degradation of BCAA decreased in skeletal muscle of diabetic patients, who have abnormally high ketone body concentrations [5]?

(3) Since glutamine oxidation in skeletal muscle is not complete, what are the metabolic fates of glutamine carbons 2-5? Also, how does skeletal muscle handle the ammonia produced from glutamine oxidation, particularly in the fed?

(4) Methionine transamination is of little quantitative importance in skeletal muscle in the presence of plasma concentrations of amino acids. Does methionine transmination occur in other tissues such as the liver and kidneys in the presence of all amino acids?

(5) Since glutamine has been shown to improve nitrogen balance in subjects under catabolic conditions [22], it is of importance to understand how intramuscular glutamine concentration is regulated. It is also important to know how glutamine regulates protein turnover in skeletal muscle. Does glutamine regulate phosphorylation of certain regulatory proteins (eg. eIF-2) involved in protein synthesis in skeletal muscle?

(6) How do ketone bodies inhibit protein synthesis and degradation in skeletal muscle from the fasted chick? Which proteolytic pathway, lysosomal or nonlysosomal, is involved in the inhibition of protein degradation by ketone bodies?

Table 8.1. Metabolic fates of amino acids in skeletal muscle under relatively physiological conditions

Amino acids	Metabolic fates
1. Leu, Ile, Val	Extensively degraded
2. Gln, Glu, Ala, Asp, Asn	Extensively synthesized and degraded
3. Met, Gly, Ser, Cys, Phe, Tyr, His, Lys, Thr, Trp, Pro, Arg.	Neither synthesized nor degraded

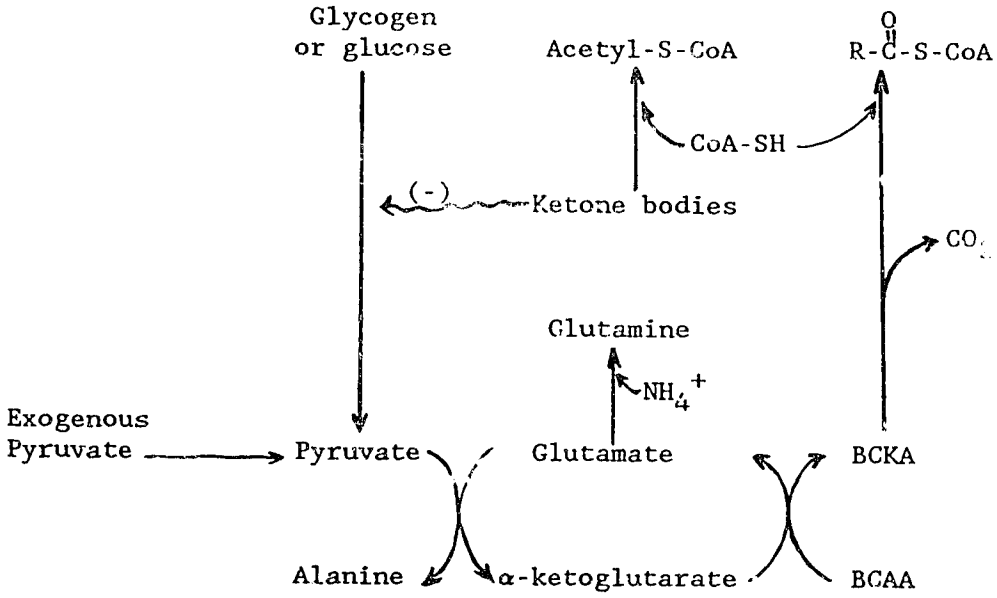

Fig. 8.1 Regulation of alanine and glutamine synthesis and BCAA transamination by ketone bodies in skeletal muscle

(-) Inhibition of glycolysis by ketone bodies;

BCAA, branched-chain amino acids; BCKA, branched-chain α-ketoacids

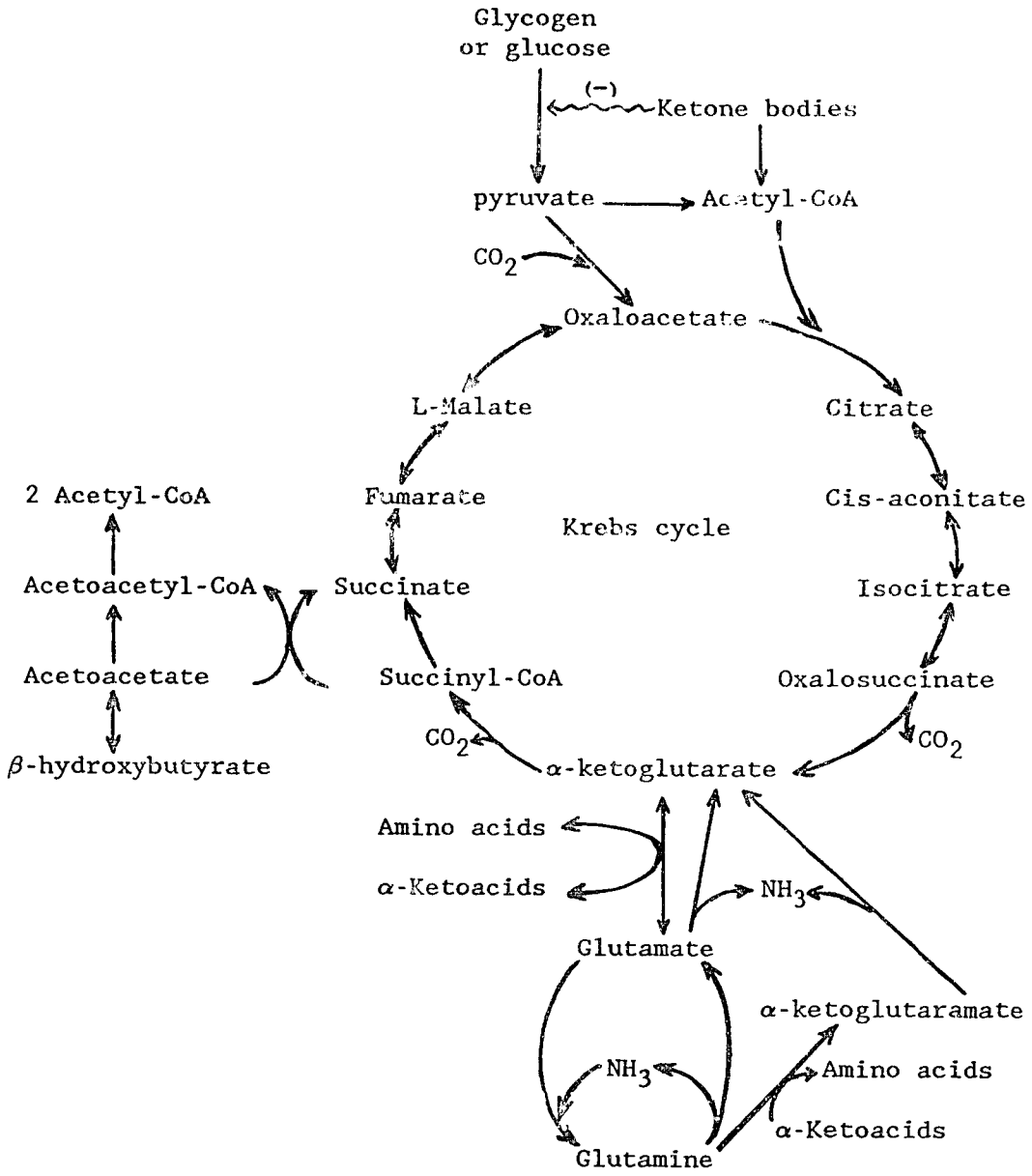

Fig. Relationships between the Krebs cycle, ketone body utilization and glutamine oxidation in skeletal muscle

(-) Inhibition of glycolysis by ketone bodies

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IX. Appendices

Appendix 1. Plasma amino acid concentrations in chicks and rats

Ten-day-old male broiler chicks of the Hubbard strain and 21-day old female Sprague Dawley rats of the Buffalo strain were fed as described in Chapter 4. Chicks and rats were anaesthetised with halothane and blood was collected by cardiac puncture of individual animals. Plasma was analysed by h.p.l.c. for amino acids as described in Chapter 2. Results are given as mean ± SEM, n=10 for chicks and n=6 for rats. * Values were adapted from Maruyama et al. (Poultry Sci. 55, 1615-1626; 1976) for chicks and from Mallette et al. (J. Biol. Chem. 244, 5713-5723; 1969) for rats.

Amino acid	Chicks (mM)	Rats (mM)
Asp	0.22 ± 0.01	0.04 ± 0.01
Glu	0.23 ± 0.01	0.16 ± 0.02
Asn	0.04 ± 0.01	0.11 ± 0.01
Ser	0.71 ± 0.04	0.39 ± 0.02
Gln	1.10 ± 0.08	0.71 ± 0.04
His	0.20 ± 0.01	0.13 ± 0.01
Gly	0.64 ± 0.03	0.51 ± 0.03
Thr	0.58 ± 0.07	0.43 ± 0.02
Cit	0.06 ± 0.01	0.07 ± 0.01
Arg	0.38 ± 0.02	0.23 ± 0.02
Tau	0.16 ± 0.02	0.12 ± 0.02
Ala	0.91 ± 0.04	0.88 ± 0.07
Tyr	0.11 ± 0.01	0.12 ± 0.01
Trp	0.08 ± 0.01	0.14 ± 0.02
Met	0.09 ± 0.01	0.11 ± 0.01
Val	0.39 ± 0.02	0.32 ± 0.01
Phe	0.40 ± 0.03	0.11 ± 0.01
Ile	0.19 ± 0.03	0.16 ± 0.01
Leu	0.27 ± 0.01	0.26 ± 0.01
Lys	0.34 ± 0.05	0.48 ± 0.0[illegible]
Cys*	0.03	0.07
Pro*	0.33	0.18

Appendix 2. Proportion of glutamate, glutamine, alanine and tyrosine in the incubation medium, the intracellular free amino acid pool and the protein in EDC muscles from 24-h fasted chicks after 2 h incubation period

EDC muscles from 24-h fasted chicks were incubated for 2 h following 30 min preincubation in Krebs Ringer bicarbonate buffer in the absence of all amino acids as described in Chapter 3. Results are given as mean ± SEM for 10 observations. The glutamate value for muscle protein includes glutamine. Values marked with ** ($P<0.01$) are significantly different from those for EDC muscle protein. The proportion of glutamate in the incubation medium is significantly lower ($P<0.01$) than that in the intracellular free amino acid pool.

	Incubation medium	Intracellular free amino acid pool	EDC muscle protein
Glutamate (%)	2.3 ± 0.1	10.7 ± 0.6	12.8 ± 0.4
Glutamine (%)	22.5 ± 1.2	21.4 ± 0.9	
Alanine (%)	20.6 ± 1.1**	18.8 ± 0.7**	10.7 ± 0.3
Tyrosine (%)	2.0 ± 0.1	1.4 ± 0.1**	2.1 ± 0.1
(Glu+Gln):Tyr	12.2 ± 0.8**	22.7 ± 1.0**	6.1 ± 0.3
Ala:Tyr	10.3 ± 0.4**	13.3 ± 0.7**	5.0 ± 0.2

Appendix 3. Release and intracellular concentrations of glutamate, glutamine and alanine in EDC muscles from 24-h fasted chicks after 2 h incubation

EDC muscles from 24-h fasted chicks were incubated in the Krebs Ringer bicarbonate buffer containing plasma concentrations of amino acids except glutamate, glutamine, alanine, tyrosine and histidine, which were absent. Following 30 min preincubation, one EDC muscle was placed in 2% trichloroacetic acid while the contralateral one from the same bird was transferred to fresh media and incubated for 2 h. Results are given as mean ± SEM for 10 observations. Values marked with ** ($P<0.01$) are significantly different from the corresponding 0 time values as analyzed by the paired t-test.

	Final incubation period (h)	Release to incubation medium	Intracellular concentration	Release plus intracellular concentration
		(nmol/mg tissue)		
Glutamate	0	--	1.28 ± 0.06	1.28 ± 0.06
	2	0.39 ± 0.04	1.20 ± 0.05	1.59 ± 0.04**
Glutamine	0	--	2.18 ± 0.12	2.18 ± 0.12
	2	2.06 ± 0.22	1.94 ± 0.10	4.10 ± 0.19**
Alanine	0	--	2.13 ± 0.05	2.13 ± 0.05
	2	2.12 ± 0.07	2.20 ± 0.14	4.32 ± 0.18**

Appendix 4. Effect of 1 mM acetoacetate or DL-β-hydroxybutyrate on the release and intracellular concentrations of glutamate, glutamine and alanine in EDC muscles from 24-h fasted chicks

EDC muscles from 24-h fasted chicks were incubated for 2 h following 30 min preincubation in the Krebs Ringer bicarbonate buffer containing 12 mM glucose and plasma concentrations amino acids except glutamate, glutamine, alanine, tyrosine and histidine, which were absent. Acetoacetate (AcAc) or DL-β-hydroxybutyrate (HB) (1 mM) was added to incubation media as indicated. Results are given as mean ± SEM for 10 observations per treatment. Values marked with * ($P<0.05$) and ** ($P<0.01$) are significantly different from the corresponding control value as analyzed by the paired t-test.

Treatment	Release			Intracellular concentrations		
	Glutamate	Glutamine	Alanine	Glutamate	Glutamine	Alanine
	(nmol/mg wet tissue/2 h)			(nmol/mg wet tissue)		
Control	0.38±0.03	1.91±0.09	2.16±0.10	1.31±0.04	2.10±0.12	2.29±0.14
1 mM AcAc	0.49±0.03**	2.25±0.10**	1.60±0.06**	2.20±0.08**	2.33±0.14*	1.78±0.05**
Control	0.35±0.02	2.13±0.12	2.01±0.12	1.28±0.04	2.24±0.23	2.11±0.10
1 mM HB	0.46±0.03**	2.34±0.17*	2.33±0.15*	1.45±0.06*	2.53±0.32**	2.38±0.09*

Appendix 5. Activities of mitochondrial glutamine aminotransferases K and L in chick EDC and rat diaphragm muscles

Mitochondrial glutamine aminotransferase K was assayed in the presence of 1-50 mM glutamine and 50 μM phenylpyruvate as described in Chapter 4. Mitochondrial glutamine aminotransferase L activity was assayed in the presence of 1-50 mM L-albizziin and 50 μM α-keto-γ-methiolbutyrate as described in Chapter 4. Results are given as mean ± SEM, n=3. Means within a column followed by different superscripts are significantly different ($P<0.05$) as determined by analysis of variance.

Glutamine aminotransferase K (nmol Phe/h/mg tissue)			Glutamine aminotransferase L (nmol Met/h/mg tissue)		
[Gln] (mM)	Chick EDC	Rat diaphragm	[Albizziin] (mM)	Chick EDC	Rat diaphragm
1.0	0.16 ± 0.01^{a}	0.29 ± 0.02^{a}	1.0	0.08 ± 0.01^{a}	0.15 ± 0.01^{a}
2.5	0.18 ± 0.01^{ab}	0.33 ± 0.01^{b}	2.5	0.10 ± 0.01^{a}	0.20 ± 0.01^{b}
10.0	0.22 ± 0.01^{bc}	0.35 ± 0.01^{ab}	10.0	0.11 ± 0.01^{ab}	0.23 ± 0.01^{b}
20.0	0.24 ± 0.01^{c}	0.36 ± 0.01^{b}	20.0	0.11 ± 0.01^{ab}	0.24 ± 0.01^{b}
50.0	0.24 ± 0.01^{c}	0.38 ± 0.02^{b}	50.0	0.13 ± 0.01^{b}	0.30 ± 0.01^{c}

Appendix 6. Effects of glutamine on protein turnover in incubated EDL muscles from fed rats.

Rat EDL muscles were incubated for 3 h following 30 min preincubation in Krebs-Ringer bicarbonate buffer containing insulin (0.1 U/ml), 5 mM glucose, 1.0 mM phenylalanine and plasma concentrations of other amino acids (Appendix 1) except glutamine, unless otherwise indicated. One muscle was used for control while the contralateral one was used for treatment. The rate of protein synthesis was estimated by measuring the incorporation of ^{3}H-phenylalanine into muscle protein as described in Chapter 6. The rate of protein degradation was estimated by measuring the release of ^{3}H-phenylalanine from muscle protein prelabelled in vivo as described in Chapter 6. The percentage of protein degradation was calculated by dividing the amount of ^{3}H-phenylalanine radioactivity (d.p.m.) released to incubation media by the the total amount of ^{3}H-phenylalanine radioactivity (d.p.m.) in muscle protein. Results are given as mean ± SEM with the numbers of observations in the parentheses. Values significantly different from the control value are indicated (* $P<0.05$) as analysed by the paired t-test.

Medium [Gln] (mM)	Intracellular [Gln] (nmol/mg tissue	Protein synthesis (pmol Phe/3 h/ mg tissue	Protein degradation (% Phe degraded from protein)
0	4.8 ± 0.22 (46)	432 ± 18 (46)	3.71 ± 0.13 (36)
0.7	10.1 ± 0.78 * (11)	503 ± 33 * (11)	3.44 ± 0.20 (10)
2.0	14.0 ± 1.23 * (11)	550 ± 29 * (11)	3.31 ± 0.23 (8)
4.0	20.5 ± 1.93 * (11)	576 ± 35 * (11)	3.43 ± 0.18 (11)