UTILIZATION OF ENERGY SUBSTRATES BY CHICKEN AND PIG ENTEROCYTES

A Thesis

by

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ABSTRACT

Enterocytes, the major absorptive cells in small intestine, have high rates of growth and turnover at the early stage. These properties make the cells have a high demand for energy. An integrated knowledge on metabolic fates of diverse nutrients and their contributions to ATP production by enterocytes helps to improve the strategy for formulating balanced diets. Glutamate and glutamine are known to be major energy substrates for mammalian enterocytes, but little is known about metabolic fuels for post-hatching chicken enterocytes. In addition, there is a paucity of information about the metabolism of other amino acids and fatty acids in chicken or pig enterocytes.

In the present study, enterocytes isolated from 0- to 42-day-old chickens or 0- to 21-day-old piglets were used to determine the metabolic fates of amino acids, fatty acids and glucose. Enterocytes isolated from the jejunum of chickens or pigs were incubated at 40°C or 37°C, respectively, for 30 min in Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 5 mM D-glucose and one of the following: 0.5-5 mM L-[U-^{14}C]glutamate, 0.5-5 mM L-[U-^{14}C]glutamine, 0.5-5 mM L-[U-^{14}C]aspartate, 0.5-5 mM L-[U-^{14}C]alanine, 0.5-2 mM L-[U-^{14}C]palmitate, D-[U-^{14}C]glucose, 0.5-5 mM [U-^{14}C]propionate, or 0.5-5 mM [1-^{14}C]butyrate. At the end of the incubation, $^{14}CO_2$ produced from each ^{14}C-labeled substrate was collected for counting.

Rates of oxidation of each substrate by enterocytes from all age groups of chickens and pigs increased ($P < 0.01$) progressively with increasing its extracellular concentrations. The rates of oxidation of glutamate by enterocytes from 0- to 41-day-old chickens were much greater ($P < 0.01$) than those for the same concentrations of other substrates. Oxidation of glutamine, propionate, butyrate and palmitate by enterocytes of chickens was limited during their post-

hatching growth. In contrast, the oxidation of glutamate, glutamine, aspartate, and glucose contributed to much more ATP production by enterocytes from 0- to 21-day-old piglets than any other substrates. Compared with 0-day-old pigs, the rates of oxidation of glutamate, aspartate, glutamine, alanine, and glucose by enterocytes from 21-day-old pigs decreased ($P < 0.01$) markedly, without changes the rate of palmitate oxidation. Oxidation of alanine, propionate, and butyrate by enterocytes of piglets was limited during their postnatal growth.

Our results indicate that glutamate is the major metabolic fuel in enterocytes of 0- to 42-day-old chickens. However, amino acids (glutamate plus glutamine plus aspartate) are the major metabolic fuels in enterocytes of 0- to 21-day-old pigs. Because of limited uptake of arterial glutamate by enterocytes of chickens and pigs, dietary glutamate is essential to the integrity and function of their small intestines.

DEDICATION

This thesis is dedicated to my family. Their solicitude and patience help me overcome all the struggles associated with earning a Master of Science degree. I would not have been able to complete the project without their support.

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Contributors

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NOMENCLATURE

AA	Amino acid
Ala	Alanine
AMEn	Apparent metabolizable energy corrected by nitrogen
Asp	Aspartate
ATP	Adenosine triphosphate
CPT-I	Carnitine palmitoyltransferase I
DD	Distilled and deionized
EAA	Essential Amino Acid
GDH	Glutamate dehydrogenase
Glu	Glutamate
Gln	Glutamine
GOT	Glutamate-oxaloacetate transaminase
GPT	Glutamate-pyruvate transaminase
GS	Glutamine synthetase
HPLC	High-performance liquid chromatography
NEAA	Non-essential amino acid
PAG	phosphate-activated glutaminase
PFK	Phosphofructose kinase

TABLE OF CONTENTS

	Page
ABSTRACT	ii
DEDICATION	iii
ACKNOWLEDGEMENTS	iv
CONTRIBUTORS AND FUNDING SOURCES	v
NOMENCLATURE	vii
TABLE OF CONTENTS	viii
LIST OF FIGURES	x
LIST OF TABLES	xi
CHAPTER I INTRODUCTION	1
CHAPTER II LITERATURE REVIEW	3
Developmental changes in the small intestine of young animals	3
Amino acid catabolism in poultry	4
Amino acid nutrition in poultry	7
Amino acid catabolism in mammals	8
Amino acid nutrition in mammals	10
Physiological roles of glucose in animal tissues	11
Fatty acid metabolism in animals	12
Summary	13
CHAPTER III METABOLISM OF AMINO ACIDS, GLUCOSE, AND FATTY ACIDS IN CHICKEN ENTEROCYTES	15
Synopsis	15
Introduction	16
Materials and Methods	17
Chemicals	17
Animals and the isolation of the jejunum	17
Preparation of enterocytes	18
Determination of substrate oxidation	18
ATP production	19

Enzymatic activities in chicken enterocytes 20
Measurement of metabolites 21
Statistical analysis 21
Results 22
Oxidation of amino acids, glucose and fatty acids in chicken enterocytes 22
ATP production from the oxidation of energy substrates to CO_2 in chicken enterocytes 25
Production of nitrogenous metabolites from amino acids 27
Production of lactate and pyruvate from glucose 30
Utilization of amino acids 33
Utilization of glucose 35
Enzyme activities 37
Discussion 39
Metabolic fates of amino acids, glucose, and fatty acids in chicken enterocytes ... 39
Developmental changes of the metabolism of amino acids, glucose, and fatty acids in chicken enterocytes 42
Energetic roles of amino acids, glucose, and fatty acids in chicken enterocytes.... 43
Physiological significance of amino acids in chicken enterocytes 44

CHAPTER IV METABOLISM OF AMINO ACIDS, GLUCOSE, AND FATTY ACIDS IN PIG ENTEROCYTES 46

Synopsis 46
Introduction 48
Materials and Methods 49
Chemicals 49
Animals and the isolation of the jejunum 49
Preparation of enterocytes 50
Determination of substrate oxidation 50
ATP production 51
Enzymatic activities in pig enterocytes 52
Measurement of metabolites 53
Statistical analysis 53
Results 54
Oxidation of amino acids, glucose and fatty acids in pig enterocytes 54
ATP production from the oxidation of energy substrates to CO_2 in pig enterocytes 57
Production of nitrogenous metabolites from amino acids 59
Production of lactate and pyruvate from glucose 62
Utilization of amino acids 65
Utilization of glucose 67
Enzyme activities 69
Discussion 71
Metabolic fates of amino acids, glucose, and fatty acids in pig enterocytes 71
Developmental changes of the metabolism of amino acids, glucose, and fatty acids in pig enterocytes 74

Energetic roles of amino acids, glucose, and fatty acids in pig enterocytes 75
Physiological significance of amino acids in pig enterocytes 76

CHAPTER V SUMMARY AND CONCLUSIONS 78

Summary 78
Conclusions 83

REFERENCES 86

APPENDIX A 96

APPENDIX B 97

APPENDIX C 98

LIST OF FIGURES

FIGURE Page

3.1. Utilization of glucose by chicken enterocytes in the presence of 5 mM glucose...... 36

4.1. Utilization of glucose by pig enterocytes in the presence of 5 mM glucose 68

5.1 Oxidation of amino acids, glucose and fatty acids in chicken and pig enterocytes.... 85

LIST OF TABLES

TABLE Page

3.1. Oxidation of energy substrates to CO_2 by chicken enterocytes 24

3.2. ATP production from the oxidation of energy substrates to CO_2 in chicken enterocytes 26

3.3. Production of nitrogenous metabolites from amino acids in chicken enterocytes..... 29

3.4. Production of lactate by chicken enterocytes in the presence of 5 mM glucose 31

3.5. Production of pyruvate by chicken enterocytes in the presence of 5 mM glucose.... 32

3.6. Utilization of amino acid substrates by chicken enterocytes 34

3.7. Maximal activities of some key enzymes in carbohydrate and amino acid metabolism as well as the transport of long-chain fatty acids in chicken enterocytes 38

4.1. Oxidation of energy substrates to CO_2 by pig enterocytes 56

4.2. ATP production from the oxidation of energy substrates to CO_2 in pig enterocytes 58

4.3. Production of nitrogenous metabolites from amino acids in pig enterocytes 61

4.4. Production of lactate by pig enterocytes in the presence of 5 mM glucose............... 63

4.5. Production of pyruvate by pig enterocytes in the presence of 5 mM glucose 64

4.6. Utilization of amino acid substrates by pig enterocytes 66

4.7. Maximal activities of some key enzymes in carbohydrate and amino acid metabolism as well as the transport of long-chain fatty acids in pig enterocytes............... 70

CHAPTER I

INTRODUCTION

Amino acids play many physiological roles in animals during their postnatal growth. The functions of amino acids include serving as building blocks for protein; metabolic fuels; and regulators of cell signaling pathways, immune response, intracellular protein turnover, and reproduction. Amino acids exert their specific functions to support the growth and health of organs. In most cases, amino acids are metabolized within a tissue- in a species-specific manner. For example, in mammals, catabolism of glutamine via phosphate-activated glutaminase is extensive in the small intestine and kidneys, but is absent from the placenta and mammary tissue (Wu 2013). In these species, glutamine carries peripheral ammonia into the liver for urea synthesis as a primary route for ammonia removal. In contrast, the liver of chickens lacks phosphate-activated glutaminase but synthesizes a large amount of glutamine and converts glutamine into uric acid as a major mechanism for the detoxification of ammonia. Although many cell types can initiate the degradation of amino acids, the complete amino acid oxidation to CO_2, urea, and water in the whole body depends on the cooperation of multiple organs, which is known as the compartmentation or inter-organ metabolism of amino acids. Furthermore, the utilization of amino acids by tissues vary with animal species. Perhaps with the exception of fish (Jia et al. 2017), dietary amino acids contribute much less energy to animals than fatty acids and digestible carbohydrates. However, studies with pigs and rats have shown that the major sources of energy for their small intestine in the fed state are amino acids rather than fatty acids and digestible carbohydrates (Wu 2018).

In addition to amino acids, glucose (the product of terminal hydrolysis of starch and glycogen in the small intestine) and fatty acids are also important nutrients for animals. Glucose is known as a major energy source for brain, its metabolites (pyruvate and oxaloacetate) are also involved in the metabolism of amino acids. Short-chain fatty acids serve as the major energy source for the large intestine, and propionate is also a substrate for gluconeogenesis in the liver of ruminants, pigs and birds.

As the major digestive and absorptive organ in animals, the small intestine has important roles in their nutrition, growth and development. Thus, the gut is vital to the health and survival of the organisms. The growth of the small intestine is characterized by the proliferation of mucosal cells, morphogenesis of intestinal mucosal cells, and differentiation of stem cells in the crypt into four major epithelial cell types. Because the small intestine is responsible for the absorption of most nutrients and for their delivery into the portal circulation, the normal growth of the gut is of great significance for animal health and production. The process of gut development is fast in newborns and has a high demand for energy. The energetic efficiencies of amino acids, glucose, and fatty acids are different in animals, with the amino acids having the lowest values (Wu 2013). Comparison of the metabolism of nutrients can help better understand the major role that these nutrients play in specific animal tissues. This review will focus on 1) developmental changes in the small intestine of young mammals and chickens; 2) amino acid catabolism in mammals and birds; 3) the functional roles of amino acids in mammalian and avian metabolism; and 4) the physiological roles of glucose in mammals and birds.

CHAPTER II

LITERATURE REVIEW

Developmental changes in the small intestine of young animals

The small intestine consists of the duodenum, jejunum and ileum, and is an important organ for digestion and absorption (Wu 2018). More than 85% of the total mucosal cell number in the small intestine is the enterocyte. Digestive enzymes are distributed on the surface of the microvillus of enterocyte and are also present in the lumen of the small intestine. These enzymes are responsible for the terminal digestion of proteins, carbohydrates, and lipids, as well as the absorption of amino acids, small peptides, and monosaccharides.

For the small intestine of newborn pigs, sheep, and cattle, macromolecules (includingmaternal colostral immunogloblins) can be absorbed to meet their physiological requirements for immune response (Kelly et al. 1992). This absorption process is mediated by specific receptors on the surface of enterocytes. By binding to these receptors, macromolecules can be absorbed intact by endocytosis. One to two days after birth, this transmission of macromolecules across enterocytes ends, and this event is known as gut closure. During the first day after birth, the weight of the small intestine of newborn piglets increases by up to 70%, which is associated with increases in its length and diameter by 20% and 15%, respectively (Widdowson et al. 1976; Xu et al. 1992). The fast growth rate of the small intestine in newborns is also characterized by increases in the villus volume and the ratio of enterocyte number to villus volume by 5-fold and 2-fold, respectively. New mucosal epithelial cells arise through the mitosis of stem cells in the crypts of Leiberkuhn, then migrate up along the villi and eventually extrude at the villous tips (Johnson 1988). The high turnover rate of mucosal epithelial cells is also associated with an increase in DNA synthesis (Widdowson et al. 1976). An increase in cell

proliferation rate in the crypts, a decrease in cell extrusion rate at the villous tip, or both results in a net increase in the epithelial cell population (growth).

Amino acid catabolism in poultry

Although the rates of uptake of di- and tri-peptides by enterocytes are greater than those for free amino acids (Matthews 2000), luminal amino acids can be rapidly absorbed by the apical membrane of the enterocyte (Yen et al. 2004). There are many amino acid transporters distributed in the apical membrane of enterocytes. The international Nomenclature Committee named amino acid transporters according to their solute carrier (SLC) families based on their gene sequence similarities. Na^+-dependent amino acid transporters and Na^+-independent amino acid transporters account for the uptake of 60% and 40% of free amino acids from the lumen of the small intestine into enterocytes, respectively. Of particular note, before binding to an amino acid, the Na^+-dependent amino acid transporter binds to Na^+ first, which will increase its affinity for the amino acid. As a result, both Na^+ and the amino acid will be transported into the cytoplasm of the enterocyte. To maintain the balance of electrolytes within the enterocyte, the Na^+/K^+-ATPase in its basolateral membrane is responsible for pumping Na^+ out of the cell and getting K^+ into the cell at the expenditure of ATP. Among all the amino acids in the arterial blood, only glutamine can be absorbed by the basolateral membrane of the enterocyte in the post-absorptive state (Wu 2013). This is because only glutamine transporters are highly expressed in the basolateral membrane of the enterocyte. For adult rats and young pigs, the small intestine takes up 30% of glutamine from the arterial blood in the post-absorptive state (Wu et al. 1994).

Previous studies have demonstrated that amino acids can be oxidized by enterocytes as metabolic fuels. In poultry, glutamate and glutamine can be utilized by skeletal muscle (Wu et al. 1991), as well as the liver, small intestine, brain, and kidney (Smith and Campbell 1983; Tinker

et al. 1986; Watford et al. 1981; Watford and Wu 2005). The rate of oxidation of glutamate and glutamine in skeletal muscle differs among its fiber types. In chickens, the rates of oxidation of glutamate and glutamine are greater in the breast muscle (mainly glycolytic fibers) than in the leg muscle (mainly oxidative fibers) (Wu et al. 1991, 1998). In addition to oxidation to CO_2 and water, glutamate may be used as a precursor for glutamine synthesis by glutamine synthetase in a cell-specific manner. This conversion is of physiological significance due to its function in scavenging free ammonia in the blood and other tissues. In addition, glutamine can be converted into glutamate by the mitochondrial phosphate-activated glutaminase. This conversion can be used to alleviate glutamine accumulation in plasma, which can inhibit the synthesis of nitric oxide by vascular endothelial cells (Wu and Meininger 2002). Other research indicated that the rate of oxidation of glutamate in the breast muscle of chickens is lower than the rate of synthesis of glutamine from glutamate (Wu and Thompson 1987).

In the avian small intestine, glutamine and fructose-6-phosphate are known as substrates for the synthesis of glucosamine-6-phosphate and, thus, glycoproteins (including mucins and membrane receptors) (Wu 2013). In addition, glutamine is capable of activating the mTOR signaling pathway to stimulate tissue protein synthesis and animal growth. Because of limited glutaminase activity and abundant glutamine content in common feedstuffs for poultry diets, the concentration of glutamine in the plasma of chickens is about 1 mM, which doubles the concentration of glutamine in the plasma of mammals (Wu 2018). In contrast, the concentration of glutamate in plasma is relatively low (<100 μM) in poultry, although glutamate is abundant in common feedstuffs for poultry diets. It is likely that glutamate is utilized as a substrate for intestinal glutathione synthesis by poultry (Porteous 1980). Previous studies indicated that the rate of glutamine consumption by chicken enterocytes was higher than that for proline, serine,

glutamate, aspartate, asparagine, and glucose at 2.5 mM for each amino acid and 5 mM for glucose (Porteaus 1980). The author also showed that the rate of glutamate consumption was only 20% of that for glucose. However, Wu et al. (1995) reported that the enterocytes of growing chickens had a low activity of glutaminase and a limited ability to utilize this amino acid. Unfortunately, the rates of glutamate, aspartate and glucose metabolism in chicken enterocytes were not determined in the study of Wu et al. (1995). Thus, whether amino acids are major metabolic sources for these cells remain unknown. This issue should be addressed, because energy metabolism is the basis of life (Wu 2018).

The liver of chickens takes up glutamine from the arterial blood at a higher rate in the fasting state than in the fed state (Tinker et al. 1986). In contrast, the liver of chickens in the fed state actively takes up glutamate, and the hepatic uptake of glutamate is the highest among all the amino acids measured, including glutamine, arginine, alanine and aspartate (Tinker et al. 1986). Due to the lack of glutaminase in the liver of chickens, glutamine is mainly used to synthesize purine and pyrimidine nucleotides. The purine can be further converted into uric acid, which is an important antioxidant in birds (Fang et al. 2002). In contrast, the liver of chickens can readily degrade glutamate by either glutamate dehydrogenase or glutamate transaminases (e.g., glutamate-pyruvate transaminase and glutamate-oxaloacetate transaminase), with the carbon skeletons of glutamate being mainly converted into CO_2 and water. This is because, in avian hepatocytes, phosphoenolpyruvate carboxykinase (PEPCK) is localized exclusively in mitochondria and, therefore, glutamate is not converted into glucose in these cells under fed or fasting conditions (Watford et al. 1981). This aspect of hepatic amino acid metabolism in birds is distinct from that in mammals.

In contrast to pigs (Hou and Wu 2018), the kidneys of chickens in the fed state take up glutamine from the arterial blood, but not glutamate (Tinker et al. 1986). In the long-term (6-day) fasting state, there is no uptake of both glutamine and glutamate by chicken kidney (Tinker et al. 1986). PEPCK is present in both the cytosol and mitochondria, which allows for the production of glucose from Glu under both fed and fasting conditions (Watford et al. 1981). This is significant for the regulation of glucose homeostasis in birds (Wu 2018). When renal Glu dehydrogenase activity is enhanced under acidotic conditions, the Glu-derived ammonia contributes to the maintenance of acid-base balance in the whole body.

Amino acid nutrition in poultry

Chickens grow fast and respond sensitively to dietary amino acids (Baker 2009). Because of the differences in genetic selection, environment, and dietary composition, chickens nowadays have different requirements for amino acids. Although previous studies reported that adult roosters did not need dietary glutamate or glutamine to maintain their body at a zero or positive nitrogen balance when fed a purified diet during the 3-day experimental period (Leveille and Fisher 1959), dietary glutamate and glutamine are still vital for chickens to maintain a zero or positive nitrogen balance for long-term survival (Maruyama et al. 1976). Note that nitrogen balance is not highly sensitive to assess dietary requirements for amino acids in animals within a short period time (Wu 2014). One longer-term study (> a 14-day period) demonstrated that the absence of glutamate from diets decreased the body weight gain of 1- to 14-day-old chickens fed a purified diet, while 10% of glutamate supplementation to a glutamate-free basal diet increased the body weight gain of young chickens by 4-fold (Maruyama et al. 1976). Due to the limited enzymic activities of arginase (Klain and Johnson 1962) and proline oxidase (Furukawa et al. 2018) in chickens for glutamine synthesis from arginine and proline, dietary glutamine is of great

significance for the growth of birds. These findings establish that growing chickens cannot synthesize enough glutamine to meet their growth requirements.

Amino acid catabolism in mammals

Amino acids play an important role in the growth of mammals. “Nutritionally essential amino acids” are defined as the amino acids that animals cannot synthesize or the rates of their synthesis are insufficient to meet maximal growth. Modern breeds of pigs grow faster and gain more lean tissues than the previous breeds and, therefore, have greater requirements for dietary amino acids (Strathe et al. 2017; Weber et al. 2015). As a result, glutamate and glutamine have recently been considered as nutritionally essential amino acids (Hou and Wu 2017; Rezaei et al. 2013a). To date, the major metabolic fate of glutamine is its hydrolysis into glutamate by mitochondrial phosphate-activated glutaminase. In addition, glutamine and fructose-6-phosphate are converted into glutamate and glucosamine-6-phosphate by glutamine:fructose-6-phosphate transaminase (GFAT). Glutamine also participates in the synthesis of nucleic acids in pigs, with glutamate being a by-product (Wu 2018). Furthermore, glutamine transaminase is capable of initiating glutamine catabolism to yield α-ketoglutaramate, which is further deaminated to α-KG by ω-amidase (Wu et al. 1991). Glutamine-derived glutamate is catabolized by multiple metabolic pathways in a cell- and tissue-specific manner (Wu 2013). In mammals and birds, transamination serves as the major metabolic pathway for glutamate degradation to yield α-KG, which is either oxidized to CO_2, and H_2O or converted into glucose, depending on their physiological and nutritional status. Glutamate-pyruvate transaminase and glutamate-oxaloacetate transaminase are abundant in both mitochondria and the cytosol of most mitochondria-containing cells. In addition to transamination, glutamate can be dehydrogenated by glutamate dehydrogenase in the liver, pancreas, and kidney (Wu 2003). Furthermore,

glutamate decarboxylase is abundant in the brain and pancreas, where glutamine-derived glutamate can be decarboxylated to GABA (Wu 2013).

The small intestine of pigs has high requirements for glutamate and glutamine. Previous studies have shown that 95-97% and 70% of the orally administered glutamate and glutamine, respectively, at a regular intake level (0.5 g Glu/kg BW/day and 0.5 g Gln/kg BW per day) are utilized by the small intestine of pigs during the single pass, with only 3-5% of enteral glutamate and 30% of enteral glutamine entering the portal vein (Reeds et al. 1996; Wu et al. 2010). Of note, in pigs, glutamate can only be taken up by the small intestine from the luminal side, but glutamine can be taken up from both the luminal side and arterial blood (Wu et al. 1994). Only a moderate increase in the circulating level of glutamine was detected 1 hour after oral administration of glutamine at 2 times its normal dietary intake (Haynes et al. 2009; Wang et al. 2008). In young pigs, a transient 75% increase in the circulating level of glutamate was detected 1 hour after oral administration of glutamate at 4 times its normal dietary intake (Janeczko et al. 2007) or receiving dietary supplementation with 4% monosodium glutamate (MSG, 2 g/kg BW/day per day) (Rezaei et al. 2013a). Because of the extensive utilization of dietary glutamate by pig enterocytes, de novo synthesis of glutamate in insulin-sensitive tissues (skeletal muscle, heart, and adipose tissue) and the brain is responsible for maintaining glutamate level in body (Wu 2013; Curthoys and Watford 1995). These findings are consistent with previous work that indicated that dietary glutamate, aspartate, glutamine, and arterial glutamine contributed 80% of ATP to porcine intestinal mucosal cells (Reeds et al. 2000). No difference was detected in the rates of oxidation of glutamate and glutamine by enterocytes of 60-kg pigs (Blachier et al. 1999). This finding can be explained by a low activity of glutamate dehydrogenase in pig enterocytes,

where the production of ammonia from glutamate or monosodium glutamate was limited (Blachier et al. 1999).

Amino acid nutrition in mammals

Growing evidence shows that beyond their use for protein synthesis, amino acids are extensively degraded by the small intestine and liver of mammals as metabolic fuels. A major cell type for the oxidation of glutamate and glutamine is the enterocyte, as reported for pigs, rats, and cattle (Windmueller 1980; Wu et al. 1994, Wu et al. 2005). The rates of utilization of glutamate and glutamine by the liver, and kidney of mammals are also high to support renal ammoniagenesis as a mechanism for the regulation of whole-body acid-base balance (Ytrebø et al. 2006, Wu 2018). In addition, amino acids also play regulatory roles in mammalian metabolism. For example, amino acids can regulate gene expression in animal cells at the levels of transcription, translation, and post-translational protein modification. The general control non-derepressible protein 2 (GCN2) kinase can be activated by a deficiency of an amino acid, which decreases global protein synthesis (Kilberg et al. 2005; Palii et al. 2008). Furthermore, existing studies indicated that an excess of an amino acid may down- or up-regulate expression of genes, depending on its side chains and target proteins (Flynn et al. 2008; Stipanuk et al. 2008). The complexity of the regulatory mechanisms whereby amino acids modulate gene expression is further confirmed by the finding that glutamine can stimulate the expression of argininosuccinate synthetase in Caco-2 cells at the transcriptional level (Brasse-Lagnel et al. 2003) but reduce the abundance of the glutamine synthetase protein in mouse skeletal muscle (Huang et al. 2007). Of interest, glutamine, arginine, and leucine can regulate intracellular protein turnover by stimulating the phosphorylation of mTOR1 in a cell-specific manner (Escobar et al. 2005, 2006; Meijer and Dubbelhuis 2004; Yao et al. 2008).

Physiological roles of glucose in animal tissues

The uptake of glucose by skeletal muscle can be stimulated by increasing extracellular insulin concentrations, which helps to transport glucose across the plasma membrane (Chrousos and Gold, 1992; McGrowder and Brown, 2007; Szabo et al., 2012). Subsequent studies further indicated that the certain level of insulin can accelerate the uptake of glucose by tissues through specific glucose transporter (Duclos et al., 1993; Kono et al., 2005; McGrowder and Brown, 2007). In addition, AMP-activated protein kinase can enhance glucose metabolism and control energy homeostasis in the whole body (Hardie et al., 1998, 1999, 2003; Hardie, 2007; Leclerc and Rutter, 2004; Xue and Kahn, 2006; Bungo et al., 2011; Doustar et al., 2012). The regulatory role of AMP-activated protein kinase in glucose metabolism was further confirmed by studies with cultured cells, such as glucose uptake by glucose transporter-4 (GLUT4) in mice C2C12 cells (Kim et al., 2013).

Insulin treatment can increase glucose transport by the skeletal muscle tissue of chickens in the absence of the GLUT4 homolog (Ewart et al., 1998; Tokushima et al., 2005; Zhao et al., 2009). The low rate of glucose utilization by the avian muscle contributes to a high concentration of glucose (e.g., > 12 mM) in the plasma of chickens (Wu 2018) that would be considered to be a diabetic state in mammals. In addition, diverse signaling molecules, such as PI3K, serine/threonine kinase Akt2 were demonstrated to play regulatory roles in insulin signaling and glucose uptake in mammals (Sweazea and Braun, 2005, 2006; Buren et al., 2008; Zhao et al., 2009). It is possible that the rate of metabolism of glucose in the enterocytes of birds is limited, therefore promoting the entry of diet-derived glucose into the portal circulation.

Fatty acid metabolism in animals

Although the complete digestion of lipids relies on the inter-organ cooperation, the digestion and absorption of these nutrients occur mainly in the jejunum of mammals and chickens (Iqbal and Hussain 2009; Wu 2018). The mitochondrial fatty acid β oxidation includes the activation of long-chain fatty acid to acyl-CoA, the transfer of acyl-CoA from the cytosol to the mitochondrial matrix via carnitine palmitoyltransferase-I (CPT-I), and the β oxidation of acyl-CoA to produce acetyl-CoA (Bartlett and Eaton 2004). Peroxisomal pathways I and II are responsible for shortening very long-chain fatty acids ($\geq C_{20}$) and very long branched-chain fatty acids ($\geq C_{20}$) into shorter-chain fatty acids in the peroxisome (Van Veldhoven 2010). The latter enter the mitochondria via CPT-I for oxidation to CO_2 and water.

CPT-I serves as a rate-controlling factor for the mitochondrial β-oxidation of long-chain fatty acids which is required for the transport of long-chain acyl-CoA from the cytosol into the mitochondrial matrix (McGarry and Brown 1997). The activity of CPT-I is inhibited by malonyl-CoA, the product of acetyl-CoA carboxylation (Jobgen et al. 2007). In insulin-sensitive tissues, the β-oxidation of long-chain fatty acids is mainly controlled by CPT-I under normal conditions (Bartlett and Eaton 2004). Previous studies have shown that fatty acid oxidation is enhanced under fasting conditions due to the decreased concentration of malonyl-CoA in the liver, skeletal muscle, and heart (Saha et al. 1995). Results of subsequent research indicate that cGMP-dependent protein kinase G can stimulate fatty acid oxidation by activating AMP-activated protein kinase and inhibit acetyl-CoA carboxylase through protein phosphorylation in the liver and muscle (Jobgen et al. 2006).

Although the oxidation of long-chain fatty acids in porcine enterocytes is known to be limited (Wu 2018), there is a paucity of information regarding the oxidation of short- and long-

chain fatty acids in the enterocytes of birds. As noted previously, oxidation of butyrate to CO_2 accounts for about 80% of the total oxygen consumption by colonocytes (Roediger 1982). Of note, the rate of oxidation of butyrate by colonocytes is 10 and 70 times that of glutamine and glucose, respectively (Clausen et al. 1995). In germ-free animals, the oxidation of butyrate by colonocytes is further enhanced possibly due to the absence of colonic microflora (Cherbuy 1995). Given the use of butyrate products as additives to swine and poultry diets (Hou et al., 2014), it is imperative to understand the role of butyrate in enterocyte metabolism in both mammals and birds.

Summary

The metabolism of amino acids, glucose, and fatty acids plays an important role in animal growth. The rapid growth of enterocytes at the early stage and their rates of high turnover have stimulated interest in sources of metabolic fuels for the cells. The trophic effects of nutrients on diverse tissues are well established for swine and poultry. Because enterocytes are the major absorptive cells in the small intestine, integrated knowledge of nutrient metabolism in the cells have pivotal implications for better understanding of their requirements for amino acids and fatty acids and for formulating balanced diets. However, limited information is available regarding the metabolic fates or energetic roles of these nutrients in enterocytes of post-hatching chickens or postnatal pigs. The contributions of amino acids, glucose, and fatty acids to ATP production by the cells remains obscure. The present study was conducted to: 1) define contributions of different substrates to energy metabolism in chicken and pig enterocytes, 2) uncover the metabolic fates of different substrates in the cells, and 3) identify species differences in substrate metabolism by enterocytes between chickens and pigs. Therefore, the results of this study will help to identify the major energy sources for the small intestines of both chickens and pigs;

define developmental changes in the metabolism of energy substrates by enterocytes of post-hatching chickens and postnatal pigs; and to provide a biochemical basis for dietary supplementation with functional amino acids and fatty acids to chickens and pigs for improving their intestinal growth and health as well as their whole-body growth and production performance.

CHAPTER III

METABOLISM OF AMINO ACIDS, GLUCOSE, AND FATTY ACIDS IN CHICKEN ENTEROCYTES

Synopsis

Glutamate, glutamine, and aspartate are known to be major energy substrates for mammalian enterocytes. However, little is known about oxidation of these amino acids in avian cells. In the present study, enterocytes from 0-, 7-, 21- and 42-day-old chickens were used to determine the oxidation of amino acids, fatty acids and glucose. Cells were incubated at 40°C for 30 min in Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 5 mM D-glucose and one of the following: 0.5-5 mM L-[U-^{14}C]glutamate, 0.5-5 mM L-[U-^{14}C]glutamine, 0.5-5 mM L-[U-^{14}C]aspartate, 0.5-5 mM L-[U-^{14}C]alanine, 0.5-2 mM L-[U-^{14}C]palmitate, D-[U-^{14}C]glucose, 0.5-5 mM [U-^{14}C]propionate, and 0.5-5 mM [1-^{14}C]butyrate. $^{14}CO_2$ produced from each ^{14}C-labeled substrate was collected for determination of radioactivity. Among all the substrates studied, glutamate had the greatest rate of oxidation in enterocytes from 0- to 42-day-old chickens and its degradation was initiated primarily by transaminases. Rates of oxidation of each amino acid and fatty acid by cells increased ($P < 0.05$) progressively with increasing extracellular concentrations from 0.5 to 5 mM. The rates of oxidation of glutamate and glucose in enterocytes decreased progressively ($P < 0.05$) with increasing age, and those for glutamine, aspartate, propionate and butyrate were lower ($P < 0.05$) in 42-day-old chickens than in 0-day-old chickens. In contrast, rates of oxidation of alanine and palmitate increased ($P < 0.05$) by 15% and 220%, respectively, in cells from 42-day-old chickens, compared with 0-day-old chickens. Compared with glutamate, oxidation of glutamine, aspartate, alanine, propionate, butyrate and

palmitate was limited in cells from all age groups of chickens. Collectively, these results indicate that glutamate is the major metabolic fuel in enterocytes of 0- to 42-day-old chickens.

Introduction

Enterocytes account for 85% of total cell numbers in the small intestine and are responsible for terminal digestion and absorption of nutrients in chickens (Wu 2018). The small intestine of neonatal chicken develops fast at the early stage of growth. Specifically, the volume of the small intestinal villus volume increases 5-fold while the ratio of enterocyte number to villus area increases 2-fold between days 7 and 0 after the hatching of chicks (Uni et al., 1995). This rapid development of enterocytes is associated with a high rate of cell turnover. Previous studies have shown that glutamate and glutamine are extensively degraded in the enterocytes of sheep, rats, pigs, and calves (Wu et al., 2005). However, no research has been conducted to determine the roles of amino acids as energy sources for avian enterocytes, in comparison with glucose as well as short- and long-chain fatty acids.

Metabolism of amino acids differs between birds and mammals (Wu 2013). For example, in chickens, glutamine is used for the hepatic synthesis of uric acid and there is no glutaminase activity for glutamine hydrolysis in their livers. In contrast, the livers of mammals have a high glutaminase activity and synthesize glutamine from glutamate and ammonia. In addition to the liver, the intestinal metabolism of amino acids may also differ between avian and mammalian species. In support of this view, Wu et al. (1995a) have shown that, in contrast to pig enterocytes, chicken cells lack the ability to synthesize ornithine, citrulline, arginine, and proline from glutamine. Therefore, we surmise that glutamine is not a significant source of ATP in these cells. At present, little is known about metabolic fuels for avian enterocytes.

In the present study, we compared the rates of oxidation and contributions of amino acids and glucose as well as short- and long-chain fatty acids to ATP production by the enterocytes of post-hatching chickens. We also assessed the rates of utilization of these substrates by the cells. To provide a biochemical basis for the metabolic results, we determined the activities of key enzymes involved in the metabolic pathways. Our results indicated that glutamate was extensively oxidized in enterocytes of 0- to 42-say-old chickens and provided most ATP to the cells.

Materials and Methods

Chemicals. The following radiolabeled chemicals were purchased from American Radiolabeled Chemicals (St. Louis, MO): D-[U-^{14}C]glucose, L-[U-^{14}C]glutamine, L-[U-^{14}C]glutamate, L-[U-^{14}C]aspartate, L-[U-^{14}C]alanine, [U-^{14}C]propionate, [1-^{14}C]butyrate, and [U-^{14}C]palmitic acid. Before use, ^{14}C-labeled glutamine was purified by using the Dowex AG1-X8 resin (acetate form, 200-400 mesh) (Self et al. 2004). ^{14}C-Labeled glutamate was purified by adding an equal volume of 1.5 M $HClO_4$ and then neutralized by a half volume of 2 M K_2CO_3. Soluene was procured from Perkin-Elmer. The liquid scintillation cocktail for $^{14}CO_2$ was prepared by dissolving 5 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis(5-phenyloxazol-2-yl) benzene into 500 of toluene and 500 mL of 2-methoxyethanol (Wu and Thompson. 1987). The sources of other chemicals were the same as described previously (Li et al. 2016; Self et al. 2004).

Animals and the isolation of the jejunum. Male chickens (Cobb 500) at days 0, 7, 21, and 42 post hatching were obtained from the Poultry Science Center of Texas A&M University. Before isolating enterocytes from 0-day-old chicks, the animals received no feed. The chickens that remained in the study had free access to corn- and soybean meal-based diets (Appendix #A

and #B) and drinking water. There were 8 birds per age group. All experimental procedures were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University. On the day of euthanasia, chickens were asphyxiated in a sealed barrel with 100% CO_2. Thereafter, the abdomen was opened, and a mid-jejunal segment was obtained.

Preparation of enterocytes. Enterocytes were prepared from the jejunum as described by Wu et al. (1995a). Briefly, the jejunum was thoroughly rinsed with saline to remove its luminal contents and then rinsed three times with 50 ml of oxygenated (95% O_2-5% CO_2) Ca^+ -free Krebs-Henseleit bicarbonate (KHB) buffer (119 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 25 mM $NaHCO_3$, pH 7.4) containing 20 mM HEPES (pH 7.4) and 5 mM EDTA (disodium). The jejunum was filled with 40 ml of the same Ca_2^+-free KHB buffer and placed in 200 ml of this buffer. The intestine was incubated at 40°C (the normal body temperature of chickens) in a shaking water bath (70 oscillations/min) for 20 min. At the end of the incubation, the jejunum was gently patted with fingertips for 1 min, and the lumen fluid was drained into 40-ml polystyrene tubes. The cells were centrifuged at 400 g for 3 min, washed three times with oxygenated (95% O_2-5% CO_2) KHB buffer (pH 7.4) containing 2.5 mM $CaCl_2$ and 20 mM HEPES (pH 7.4) but no EDTA, and then resuspended in this buffer. The viability of the isolated enterocytes was greater than 95%, as assessed by trypan blue exclusion (Wu et al. 1995a).

Determination of substrate oxidation. Metabolic studies were conducted as previously described (Wu 1995b) except that the temperature of the incubation bath was 40°C rather than 37°C. Briefly, 2×10^6 enterocytes were incubated at 40°C for 30 min in 1 ml of oxygenated (95% O_2/5% CO_2) KHB buffer (pH 7.4) containing 5 mM D-glucose, 1 nM insulin, and one of the following substrates: 5 mM D-[U-^{14}C]glucose, 0.5 mM L-[U-^{14}C]glutamate, 2 mM L-[U-

^{14}C]glutamate, 5 mM L-[U-^{14}C]glutamate, 0.5 mM L-[U-^{14}C]glutamine, 2 mM L-[U-^{14}C]glutamine, 5 mM L-[U-^{14}C]glutamine, 0.5 mM L-[U-^{14}C]aspartate, 2 mM L-[U-^{14}C]aspartate, 5 mM L-[U-^{14}C]aspartate, 0.5 mM L-[U-^{14}C]alanine, 2 mM L-[U-^{14}C]alanine, 5 mM L-[U-^{14}C]alanine, 0.5 mM [U-^{14}C]palmitate, 2 mM [U-^{14}C] palmitate, 0.5 mM [U-^{14}C]propionate, 2 mM [U-^{14}C]propionate, 5 mM [U-^{14}C]propionate, 0.5 mM [U-^{14}C]butyrate, 2 mM [U-^{14}C]butyrate, and 5 mM [U-^{14}C]butyrate. The specific radioactivity of each tracer in the incubation medium was approximately 200 dpm/nmol. In all experiments, media containing the same components, as well as 0.2 ml 1.5 M $HClO_4$ (added before the addition of cells) were run as blanks. Incubation was initiated by the addition of a cell suspension. After a 30-min incubation period, the reaction was terminated by the addition, through the rubber stopper, of 0.2 ml Soluene into a center-well suspended within the tube for CO_2 collection, followed by the addition, through the rubber stopper, of 0.2 ml of 1.5 M $HClO_4$ into the incubation medium. After a further 1-h incubation, $^{14}CO_2$ radioactivity was measured by a liquid scintillation counter (Self et al. 2004).

ATP production. The rate of oxidation of a substrate in enterocytes ($CO_2/10^6$ cell per 30 min) was calculated as the amount of $^{14}CO_2$ (dpm) produced by the enterocytes divided by the specific radioactivity of the substrate in incubation medium. The rate of ATP production was calculated from the rate of CO_2 production by multiplying by the coefficient (ATP/CO_2, mol/mol) of the substrate according to the following equations:

Glutamate: $C_5H_9NO_4 + 4.5O_2 \rightarrow NH_3 + 5CO_2 + 3H_2O$;

22.5 mol ATP/mol Glu, 4.5 mol ATP/mol CO_2

Glutamine: $C_5H_{10}N_2O_3 + 4.5O_2 \rightarrow 2NH_3 + 5CO_2 + 2H_2O$;

22.5 mol ATP/mol Gln, 4.5 mol ATP/mol CO_2

Aspartate: $C_4H_7NO_4 + 3.75O_2 \rightarrow NH_3 + 4CO_2 + 3.5H_2O$;

15 mol ATP/mol Asp, 3.75 mol ATP/mol CO_2

Alanine: $C_3H_7NO_2 + 3.75O_2 \rightarrow NH_3 + 3CO_2 + 3.5H_2O$;

15 mol ATP/mol Asp, 5 mol ATP/mol CO_2

Palmitate: $C_{16}H_{32}O_2 + 23O_2 \rightarrow 16CO_2 + 16H_2O$;

106 mol ATP/mol PA, 6.625, mol ATP/mol CO_2

Glucose: $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$;

30 mol ATP/mol Glucose, 5 mol ATP/mol CO_2

Propionate: $C_3H_6O_2 + 3.5O_2 \rightarrow 3CO_2 + 3H_2O$;

15 mol ATP/mol Propionate, 5 mol ATP/mol CO_2

Butyrate: $C_4H_8O_2 + 5O_2 \rightarrow 4CO_2 + 4H_2O$;

22 mol ATP/mol Butyrate, 5.5 mol ATP/mol CO_2

The rates of ATP production from the oxidation of substrates (expressed as mol ATP/mol substrate) are as follows: glutamate, 22.5; glutamine, aspartate, 15; alanine, 15; palmitate, 106; and glucose, 30; propionate, 15; butyrate, 22 (Wu 2013, 2018). The coefficients of ATP production per mole of CO_2 produced from the oxidation of substrates (expressed as mol ATP/mol CO_2) are as follows: glutamate, 4.5; glutamine, 4.5; aspartate, 3.75; alanine, 5; palmitate, 6,625; and glucose, 5; propionate, 5; butyrate, 5.5 (Wu 2013, 2018).

Enzymatic activities in chicken enterocytes. Mitochondria were isolated from enterocytes as described previously (Wu (1997). The supernatant fluid from the 10,000 × g centrifugation was used as the cytosolic fraction. Both mitochondria and the cytosol were used to measure enzyme activities. The activities of glutaminase, glutamine synthetase, glutamate dehydrogenase,

glutamate-pyruvate transaminase, and glutamate-oxaloacetate transaminase were determined as described by Wu et al. (2000) and Self et al. (2004). The activities of hexokinase, phosphofructokinase, and pyruvate kinase were determined using kits from Nipro Enzymes (3-9-3 HONJO-NISHI KITA-KU OSAKA 531-8510 JAPAN). Carnitine palmitoyltransferase-1 activity was determined with a kit (Carnitine Palmitoyltransferase I activity assay) by measuring the formation of ^{14}C-labeled palmitoyl-carnitine from palmitoyl-CoA and ^{14}C-labeled carnitine (McGarry et al. 1983). The details of the enzyme assays are provided in Appendix #C.

Measurement of metabolites. Amino acids (including glutamate, glutamine, aspartate and alanine) in neutralized cell extracts were quantified by our established HPLC method (Wu et al., 1994). Ammonia (NH_4^+ plus NH_3; or simply referred to as NH_3 in text) was determined using an enzymatic method involving glutamate dehydrogenase and α-ketoglutaric acid (Wu 1995). L-Lactate and pyruvate were determined using enzymatic methods involving L-lactate dehydrogenase (Wu et al. 1991). Glucose was determined using an enzymic method involving glucose-6-P dehydrogenase and hexokinase (Wu et al. 1995). Based on the production of metabolites, the rates of utilization of glutamate, glutamine, alanine, aspartate and glucose were calculated as follows:

Glutamate utilization (nmol/10^6 cells per 30 min) = (CO_2/5 + Ala + Asp mol)/cell number;

Glutamine utilization (nmol/10^6 cells per 30 min) = (CO_2/5 + Glu + Ala + Asp)/cell number;

Alanine utilization (nmol/10^6 cells per 30 min) = (CO_2/3 + Asp + Glu)/cell number;

Aspartate utilization (nmol/10^6 cells per 30 min) = (CO_2/4 + Glu + Ala)/cell number;

Glucose utilization (nmol/10^6 cells per 30 min) = (CO_2/6 + Lactate/2 + Pyruvate/2)/cell number

Statistical analysis. Data were analyzed by one-way ANOVA and the Student-Newman-Keuls multiple comparison test (Assaad et al. 2014). Probability values < 0.05 were taken to indicate statistical significance.

Results

Oxidation of amino acids, glucose and fatty acids in chicken enterocytes. Data on the rates of CO_2 production from the oxidation of different substrates in chicken enterocytes are summarized in Table 3.1. Rates of oxidation of glutamate, glutamine, aspartate and alanine in enterocytes from all age groups of chickens increased ($P < 0.05$) progressively with increasing their extracellular concentrations from 0.5 mM to 5 mM. In cells from 0- to 42-day-old chickens, the rate of CO_2 production from [U-^{14}C]glutamate was the highest among all the tested substrates. Particularly, in cells from 0-day-old chickens, the rate of CO_2 production from [U-^{14}C]glutamate was 4-fold greater than that from the sum of all the other tested substrates and was 15-fold greater than that from glutamine or aspartate. Compared with glutamate, the oxidation of glutamine, alanine and aspartate was limited in enterocytes from 0- to 42-day-old chickens.

There were developmental changes in the oxidation of amino acids by chicken enterocytes. The rate of oxidation of glutamate in the cells decreased progressively ($P < 0.05$) by 84% with increasing age from 0 to 42 days. The rate of oxidation of aspartate decreased ($P < 0.05$) in 7-day-old versus 0-day-old chickens, remained relatively constant between 7- and 21-day-old chickens, and decreased ($P < 0.05$) thereafter in 42-day-old chickens by 84% compared with 0-day-old chickens. In contrast, the rate of oxidation of glutamine increased ($P < 0.05$) in 7-day-old versus 0-day-old chickens, did not differ ($P > 0.05$) between 7- and 21-day-old chickens, and decreased ($P < 0.05$) thereafter in 42-day-old chickens by 51% compared with 0-day-old chickens. The rate of oxidation of alanine increased ($P < 0.05$) progressively with increasing the

age of chickens from 0 to 21 days, and decreased ($P < 0.05$) thereafter in 42-day-old chickens by 45% compared with 21-day-old chickens.

The rate of CO_2 production from 5 mM glucose by enterocytes of chickens at each post-hatching age was the second highest among all the tested substrates. The rate of oxidation of glucose decreased progressively ($P < 0.05$) by 76% with increasing the age of chickens from 0 to 42 days. The largest decline (62%) occurred between 0 and 7 days of age.

Compared with glutamate, oxidation of propionate, butyrate and palmitate was limited in enterocytes from 0- and 42-day-old chickens. However, there were developmental changes in the oxidation of short- and long-chain fatty acids by the cells. The rate of oxidation of palmitate increased ($P < 0.05$) by 78% in 7-day-old versus 0-day-old chickens, did not differ ($P > 0.05$) between 7- and 21-day-old chickens, and increased ($P < 0.05$) thereafter in 42-day-old chickens by 20% compared with 21-day-old chickens. In contrast, the rate of oxidation of propionate decreased progressively ($P < 0.05$) by 76% with increasing age from 0 to 42 days. The rate of oxidation of butyrate increased ($P < 0.05$) by 33% in 7-day-old versus 0-day-old chickens, did not differ ($P > 0.05$) between 7- and 21-day-old chickens, and decreased ($P < 0.05$) thereafter in 42-day-old chickens by 20% compared with 21-day-old chickens.

Table 3.1. Oxidation of energy substrates to CO_2 by chicken enterocytes[1].

Substrate in medium	Concentration (mM)	Age of chickens (days)			
		0	7	21	42
Glutamate	0.5	70.1 ± 3.1[a]	20.1 ± 1.0[b]	12.8 ± 0.45[c]	13.7 ± 1.8[c]
	2.0	155 ± 7.9[a]	71.1 ± 5.1[b]	41.0 ± 3.3[c]	23.4 ± 1.7[d]
	5.0	227 ± 5.3[a]	150 ± 9.2[b]	83.0 ± 2.8[c]	36.1 ± 0.96[d]
Glutamine	0.5	3.44 ± 0.33[b]	6.46 ± 0.27[a]	7.20 ± 0.22[a]	3.15 ± 0.77[b]
	2.0	10.6 ± 1.4[b]	12.7 ± 0.38[a]	12.1 ± 0.82[ab]	5.55 ± 0.89[c]
	5.0	16.9 ± 1.8[b]	22.0 ± 1.7[a]	19.5 ± 0.92[a]	8.36 ± 1.2[c]
Aspartate	0.5	4.87 ± 0.40[b]	5.24 ± 0.75[b]	8.75 ± 0.44[a]	4.24 ± 0.34[b]
	2.0	11.8 ± 0.76[b]	11.1 ± 1.3[b]	14.6 ± 1.0[a]	6.34 ± 0.25[c]
	5.0	27.9 ± 2.0[a]	19.9 ± 0.73[b]	18.7 ± 1.3[b]	9.55 ± 0.57[c]
Alanine	0.5	2.06 ± 0.08[b]	1.17 ± 0.12[c]	5.73 ± 0.41[a]	1.86 ± 0.12[b]
	2.0	4.61 ± 0.22[b]	3.31 ± 0.16[c]	10.7 ± 0.76[a]	4.48 ± 0.36[b]
	5.0	6.54 ± 0.14[c]	7.08 ± 0.18[b]	13.6 ± 0.65[a]	7.54 ± 0.88[b]
Palmitate	0.5	2.32 ± 0.18[c]	3.65 ± 0.17[d]	3.61 ± 0.51[b]	4.47 ± 0.20[a]
	2.0	3.97 ± 0.07[c]	7.12 ± 0.28[a]	7.31 ± 0.56[b]	8.65 ± 0.74[a]
Glucose	5.0	74.0 ± 2.2[a]	28.4 ± 1.2[b]	22.1 ± 1.3[c]	17.7 ± 3.2[d]
Propionate	0.5	2.53 ± 0.22[a]	1.31 ± 0.08[c]	1.40 ± 0.17[b]	0.97 ± 0.18[d]
	2.0	3.66 ± 0.27[a]	2.14 ± 0.13[b]	1.95 ± 0.22[b]	1.56 ± 0.26[c]
	5.0	4.72 ± 0.29[a]	3.12 ± 0.19[b]	2.76 ± 0.23[bc]	2.34 ± 0.24[c]
Butyrate	0.5	0.84 ± 0.02[b]	1.12 ± 0.08[a]	1.19 ± 0.13[a]	0.91 ± 0.16[ab]
	2.0	1.53 ± 0.12[bc]	2.15 ± 0.11[a]	1.81 ± 0.21[a]	1.28 ± 0.19[b]
	5.0	2.15 ± 0.16[b]	2.51 ± 0.12[a]	2.48 ± 0.28[b]	1.61 ± 0.20[c]

[1]Data, expressed as nmol $CO_2/10^6$ cell per 30 min, are means ± SEM, n = 8 chickens/age group. Enterocytes were incubated at 40°C for 30 min in the KHB buffer, as described in text. The medium contained 5 mM D-glucose and one of the following: 0.5-5.0 mM [U-^{14}C]glutamate, 0.5-5.0 mM [U-^{14}C]glutamine, 0.5-5.0 mM [U-^{14}C]aspartate, 0.5-5.0 mM [U-^{14}C]alanine, 0.5-2.0 mM [U-^{14}C]palmitate, 0.5-5.0 mM [U-^{14}C]propionate, and 0.5-5.0 mM [1-^{14}C]butyrate. The rate of oxidation of each substrate increased ($P < 0.05$) with increasing extracellular concentration from 0.5 to 5 mM.

a-d: Within a row, means not sharing the same superscript letters differ ($P < 0.05$).

ATP production from the oxidation of energy substrates to CO_2 in chicken enterocytes. Data on ATP production from the oxidation of substrates in chicken enterocytes are summarized in Tables 3.2. Results on the rates of ATP production from the oxidation of energy substrates in enterocytes were generally similar to the rates of substrate oxidation noted previously. Overall, the rates of ATP production from all substrates in enterocytes from all age groups of chickens increased ($P < 0.01$) progressively with increasing extracellular concentrations from 0.5 mM to 5 mM. In cells from 0- to 42-day-old chickens, the rate of ATP production from glutamate was the highest among all the tested substrates. Compared with glutamate, ATP production from glutamine, alanine and aspartate was limited in enterocytes from all age groups of chickens.

The rate of ATP production from 5 mM glucose by enterocytes of chickens at each post-hatching age was the second highest among all the tested substrates. The rate of ATP production from glucose decreased progressively ($P < 0.05$) with increasing age from 0 to 42 days. The largest decline occurred between 0 and 7 days of age.

Compared with glutamate, ATP production from propionate, butyrate and palmitate was limited in enterocytes from 0- and 42-day-old chickens. As for CO_2 production, there were developmental changes in ATP production from short- and long-chain fatty acids by the cells. The rate of ATP production from palmitate increased ($P < 0.05$) in 7-day-old versus 0-day-old chickens, did not differ ($P > 0.05$) between 7- and 21-day-old chickens, and increased ($P < 0.05$) thereafter in 42-day-old chickens compared with 21-day-old chickens. In contrast, the rate of ATP production from propionate decreased progressively ($P < 0.05$) with increasing the age of chickens from 0 to 42 days. The rate of ATP production from butyrate increased ($P < 0.05$) in 7-day-old versus 0-day-old chickens, did not differ ($P > 0.05$) between 7- and 21-day-old chickens, and decreased ($P < 0.05$) thereafter in 42-day-old chickens compared with 21-day-old chickens.

Table 3.2. ATP production from the oxidation of energy substrates to CO_2 in chicken enterocytes[1]

Substrate in medium	Concentration (mM)	Age of chickens (days)			
		0	7	21	42
Glutamate	0.5	316 ± 14.0[a]	90.5 ± 6.2[b]	57.8 ± 2.0[c]	61.8 ± 8.0[c]
	2.0	698 ± 35.5[a]	323 ± 31.3[b]	184 ± 15.0[c]	105 ± 7.7[d]
	5.0	1023 ± 23.6[a]	675 ± 48.9[b]	373 ± 12.5[c]	162 ± 4.3[d]
Glutamine	0.5	15.5 ± 1.5[c]	28.9 ± 1.6[b]	32.4 ± 1.0[a]	14.2 ± 3.5[c]
	2.0	47.8 ± 6.3[b]	57.8 ± 2.1[a]	54.4 ± 3.7[a]	25.0 ± 4.0[c]
	5.0	76.1 ± 8.2[b]	102 ± 10.1[a]	87.8 ± 4.1[b]	37.6 ± 5.2[c]
Aspartate	0.5	18.3 ± 1.5[b]	19.8 ± 3.8[b]	32.8 ± 1.6[a]	15.9 ± 1.3[c]
	2.0	44.3 ± 2.8[b]	41.9 ± 6.4[b]	54.6 ± 3.8[a]	23.8 ± 0.94[c]
	5.0	105 ± 7.5[a]	75.0 ± 3.7[b]	70.2 ± 4.8[b]	35.8 ± 2.1[c]
Alanine	0.5	1.03 ± 0.39[d]	5.70 ± 0.78[c]	28.6 ± 2.0[a]	9.30 ± 0.61[b]
	2.0	23.1 ± 1.1[b]	16.6 ± 1.1[c]	53.5 ± 3.8[a]	22.4 ± 1.8[b]
	5.0	32.7 ± 0.72[b]	35.4 ± 1.2[b]	68.2 ± 3.2[a]	37.7 ± 4.4[b]
Palmitate	0.5	15.4 ± 1.2[d]	24.2 ± 1.1[a]	23.9 ± 3.4[c]	29.7 ± 1.3[b]
	2.0	26.3 ± 0.45[c]	47.2 ± 1.9[a]	48.4 ± 3.7[b]	57.3 ± 4.9[a]
Glucose	5.0	370 ± 11.1[a]	143 ± 7.8[b]	110 ± 6.4[c]	88.6 ± 15.8[d]
Propionate	0.5	12.7 ± 1.1[a]	6.41 ± 0.54[b]	6.98 ± 0.87[b]	4.84 ± 0.88[c]
	2.0	18.3 ± 1.3[a]	10.6 ± 0.88[b]	9.73 ± 1.1[b]	7.81 ± 1.3[c]
	5.0	23.6 ± 1.4[a]	15.6 ± 1.3[b]	13.8 ± 1.1[bc]	11.7 ± 1.2[c]
Butyrate	0.5	4.61 ± 0.11[c]	6.15 ± 0.44[b]	6.57 ± 0.70[a]	4.98 ± 0.93[bc]
	2.0	8.43 ± 0.65[bc]	11.8 ± 0.60[a]	9.93 ± 1.2[b]	7.01 ± 1.0[c]
	5.0	11.8 ± 0.88[b]	13.9 ± 0.66[a]	13.7 ± 1.5[a]	8.87 ± 1.1[c]

[1]Data, expressed as nmol ATP/10^6 cell per 30 min, are means ± SEM, n = 8 chickens/age group. Enterocytes were incubated at 40°C for 30 min in the KHB buffer, as described in text. The medium contained 5 mM D-glucose and one of the following: 0.5-5.0 mM [U-^{14}C]glutamate, 0.5-5.0 mM [U-^{14}C]glutamine, 0.5-5.0 mM [U-^{14}C]aspartate, 0.5-5.0 mM [U-^{14}C]alanine, 0.5-2.0 mM [U-^{14}C]palmitate, 0.5-5.0 mM [U-^{14}C]propionate, and 0.5-5.0 mM [1-^{14}C]butyrate. The rate of oxidation of each substrate increased ($P < 0.05$) with increasing extracellular concentration from 0.5 to 5 mM.

a-d: Within a row, means not sharing the same superscript letters differ ($P < 0.05$).

Production of nitrogenous metabolites from amino acids. Data on the production of nitrogenous metabolites from amino acids are summarized in Table 3.3. Alanine and aspartate were two amino acids produced from glutamate in enterocytes of 0- to 42-day-old chickens. No other amino acids (including glutamine, asparagine, ornithine, citrulline, arginine, and proline) were produced from glutamate in the cells. Rates of production of alanine and aspartate from glutamate by enterocytes from all age groups of chickens increased ($P < 0.05$) progressively with increasing the extracellular concentration of glutamate from 0.5 mM to 5 mM. Compared with 0-day-old chickens, the rates of production of both alanine and aspartate from 0.5 to 5 mM glutamate by enterocytes decreased ($P < 0.05$) in 42-day-old chickens.

In enterocytes from 0- to 42-day-old chickens, NH_3 was produced from glutamine but not from other substrates. The major amino acid formed from glutamine was glutamate, followed by alanine and then aspartate. No other amino acids (including asparagine, ornithine, citrulline, arginine, and proline) were produced from glutamine in the cells. Rates of production of NH_3, glutamate, and alanine from glutamine in enterocytes from all age groups of chickens increased ($P < 0.01$) progressively with increasing the extracellular concentration of glutamine from 0.5 mM to 5 mM. The rates of aspartate production from 0.5 and 2 mM glutamine were greater ($P < 0.05$) in enterocytes from 0-day-old chickens, compared with 7- to 42-day-old chickens. The rates of ammonia production from glutamine did not differ among the four age groups of chickens. The rate of production of glutamate from 0.5 mM glutamine by enterocytes from 42-day-old chickens decreased ($P < 0.05$) in comparison with 0-day-old chickens. At 2 and 5 mM glutamine, the rates of aspartate production from glutamate did not differ among the four age groups of chickens.

Catabolism of aspartate in enterocytes from 0- to-42-day-old chickens resulted in the production of primarily alanine and, to a lesser extent, glutamate. No other amino acids were produced from aspartate in the cells. Rates of production of alanine and glutamate from aspartate in enterocytes from all age groups of chickens increased ($P < 0.05$) progressively with increasing the extracellular concentration of aspartate from 0.5 mM to 5 mM. Compared with 0-day-old chickens, the rates of production of both alanine and glutamate from aspartate by enterocytes decreased ($P < 0.05$) in 42-day-old chickens.

Glutamate and aspartate were the only amino acids produced from the catabolism of alanine in enterocytes from 0- to 42-day-old chickens. Rates of production of glutamate and aspartate from alanine in enterocytes from all age groups of chickens increased ($P < 0.05$) progressively with increasing the extracellular concentration of alanine from 0.5 mM to 5 mM. The rates of formation of glutamate from 0.5, 2 and 5 mM alanine were greater ($P < 0.05$) in cells from 21- and 42-day-old chickens, compared with 0- and 7-day-old chickens. Similar results were obtained for the formation of aspartate from 5 mM alanine.

Table 3.3. Production of nitrogenous metabolites from amino acids in chicken enterocytes[1]

Substrate in medium	Concentration (mM)	Metabolites	Age of chickens (days)			
			0	7	21	42
Glutamate	0.5	Aspartate	4.06 ± 0.22[a]	3.06 ± 0.21[b]	2.96 ± 0.33[b]	2.74 ± 0.24[b]
	2.0		5.66 ± 0.31[a]	4.67 ± 0.43[b]	4.23 ± 0.32[bc]	3.40 ± 0.12[c]
	5.0		6.38 ± 0.44[a]	5.56 ± 0.40[ab]	4.86 ± 0.34[b]	4.35 ± 0.37[b]
	0.5	Alanine	18.3 ± 0.88[a]	17.6 ± 1.1[a]	16.6 ± 0.43[ab]	14.4 ± 1.0[b]
	2.0		24.1 ± 1.2[a]	22.2 ± 2.2[ab]	19.9 ± 1.1[ab]	18.3 ± 1.1[b]
	5.0		30.6 ± 0.77[a]	28.0 ± 1.2[ab]	24.9 ± 1.6[b]	21.6 ± 0.37[c]
Glutamine	0.5	Ammonia	12.3 ± 0.58	14.0 ± 0.94	11.9 ± 0.75	12.1 ± 1.4
	2.0		23.1 ± 0.89	25.1 ± 0.81	24.5 ± 1.4	23.0 ± 1.7
	5.0		32.6 ± 1.8	33.0 ± 1.7	31.8 ± 0.56	31.2 ± 2.3
	0.5	Glutamate	5.66 ± 0.44[b]	5.91 ± 0.56[ab]	6.80 ± 0.33[a]	7.48 ± 0.30[a]
	2.0		11.2 ± 0.79	12.4 ± 0.75	13.1 ± 0.85	14.4 ± 0.88
	5.0		14.9 ± 0.83	16.6 ± 0.20	17.0 ± 1.1	17.8 ± 1.4
	0.5	Aspartate	2.02 ± 0.15	1.85 ± 0.15	1.62 ± 0.15	1.54 ± 0.11
	2.0		2.23 ± 0.18	2.10 ± 0.26	1.82 ± 0.12	1.74 ± 0.17
	5.0		2.30 ± 0.33	2.20 ± 0.16	2.10 ± 0.14	2.07 ± 0.13
	0.5	Alanine	5.69 ± 0.30[a]	5.29 ± 0.32[ab]	5.05 ± 0.36[ab]	4.39 ± 0.26[b]
	2.0		9.44 ± 0.32[a]	9.00 ± 0.50[a]	8.52 ± 0.57[ab]	7.06 ± 0.31[b]
	5.0		14.6 ± 0.69[a]	13.6 ± 0.13[a]	12.1 ± 0.29[b]	10.3 ± 0.54[c]
Aspartate	0.5	Glutamate	7.34 ± 0.83	7.17 ± 0.60	6.17 ± 0.75	5.95 ± 0.52
	2.0		11.6 ± 1.2[a]	9.78 ± 0.46[ab]	8.95 ± 0.25[b]	8.65 ± 0.53[b]
	5.0		18.0 ± 1.5[a]	15.2 ± 1.2[ab]	13.8 ± 0.37[b]	12.4 ± 0.68[b]
	0.5	Alanine	11.7 ± 0.79	11.1 ± 0.83	10.2 ± 0.19	9.31 ± 0.62
	2.0		18.5 ± 1.3[a]	16.0 ± 0.27[b]	14.5 ± 0.34[bc]	12.7 ± 0.44[c]
	5.0		25.0 ± 1.7[a]	22.5 ± 0.98[ab]	19.8 ± 1.4[bc]	18.0 ± 0.92[c]
Alanine	0.5	Glutamate	7.53 ± 0.58[c]	10.5 ± 0.6[b]	14.6 ± 0.8[a]	13.1 ± 0.5[a]
	2.0		10.7 ± 1.0[b]	12.6 ± 0.6[b]	17.9 ± 1.0[a]	16.3 ± 0.5[a]
	5.0		12.6 ± 1.0[b]	14.9 ± 0.9[b]	21.3 ± 0.9[a]	18.9 ± 0.7[a]
	0.5	Aspartate	5.69 ± 0.47	5.74 ± 0.38	6.72 ± 0.29	6.46 ± 0.27
	2.0		6.63 ± 0.57	7.76 ± 0.42	8.39 ± 0.47	7.92 ± 0.26
	5.0		7.26 ± 0.49[b]	8.01 ± 0.21[b]	11.6 ± 0.70[a]	10.4 ± 0.40[a]

[1]Data, expressed as nmol product/10^6 cell per 30 min, are means ± SEM, n = 8 chickens/age group. Enterocytes were incubated at 40°C for 30 min in the KHB buffer, as described in text. The medium contained 5 mM D-glucose and one of the following: 0.5-5.0 mM [U-^{14}C]glutamate, 0.5-5.0 mM [U-^{14}C]glutamine, 0.5-5.0 mM [U-^{14}C]aspartate, 0.5-5.0 mM [U-^{14}C]alanine, 0.5-2.0 mM [U-^{14}C]palmitate, 0.5-5.0 mM [U-^{14}C]propionate, and 0.5-5.0 mM [1-^{14}C]butyrate. There was no production of ammonia from glutamate, alanine or aspartate from chicken enterocytes. The rate of production of metabolites from each substrate increased ($P < 0.05$) with increasing extracellular concentration from 0.5 to 5 mM.

a-d: Within a row, means not sharing the same superscript letters differ ($P < 0.05$).

Production of lactate and pyruvate from glucose. The rate of production of lactate from 5 mM glucose (Table 3.4) was approximately 10 times that for pyruvate (Table 3.5) in enterocytes from 0- to 42-day-old chickens. In cells from 0- to 42-day-old chickens, 0.5-5 mM glutamate, glutamine, aspartate or alanine reduced ($P < 0.05$) the rates of production of lactate from glucose. In enterocytes from 0- to 21-day-old chickens, 2-5 mM glutamate, 5 mM glutamine, 5 mM aspartate, and 0.5-5 mM alanine decreased ($P < 0.05$) the rates of production of pyruvate from glucose. Propionate and butyrate at 0.5-5 mM or 0.5-2 mM palmitate had no effect ($P > 0.05$) on lactate or pyruvate production from glucose. The rates of production of lactate or pyruvate did not differ ($P > 0.05$) by enterocytes from 0-, 7- and 21-day-old chickens, but their values were all greater ($P > 0.05$) than those from 42-day-old chickens.

Table 3.4. Production of lactate by chicken enterocytes in the presence of 5 mM glucose[1,2]

Substrate in medium	Concen-tration (mM)	Age of chickens (days)			
		0	7	21	42
Glutamate	0.5	32.5 ± 1.7*	27.0 ± 2.5*	25.3 ± 1.5*	19.2 ± 1.6
	2.0	30.7 ± 2.6*	26.0 ± 2.7*	26.5 ± 2.5*	20.5 ± 0.84
	5.0	29.8 ± 1.7*	24.6 ± 2.1*	25.2 ± 1.8*	20.2 ± 0.86
Glutamine	0.5	31.3 ± 2.5*	27.5 ± 3.0*	26.2 ± 1.9*	21.6 ± 2.0
	2.0	31.9 ± 1.8*	27.9 ± 2.6*	26.0 ± 2.1*	21.4 ± 1.8
	5.0	31.4 ± 0.97*	20.8 ± 1.5*	25.1 ± 1.9*	23.9 ± 0.98
Aspartate	0.5	29.0 ± 3.3*	27.3 ± 2.9*	24.9 ± 2.0*	21.5 ± 1.8
	2.0	31.8 ± 1.8*	26.4 ± 3.0*	25.1 ± 1.5*	21.6 ± 0.58
	5.0	31.5 ± 2.9*	27.5 ± 2.3*	27.5 ± 2.1*	22.2 ± 1.7
Alanine	0.5	30.4 ± 3.9*	27.4 ± 2.9*	23.5 ± 2.2*	22.3 ± 2.2
	2.0	31.8 ± 2.5*	27.3 ± 2.9*	22.7 ± 0.5*	19.1 ± 1.4
	5.0	30.9 ± 2.6*	23.2 ± 1.8*	24.6 ± 2.1*	22.2 ± 1.5
Palmitate	0.5	38.2 ± 2.8	29.9 ± 1.9	29.3 ± 0.99	23.4 ± 2.0
	2.0	38.7 ± 1.9	31.7 ± 0.72	30.7 ± 0.70	20.7 ± 1.1
Glucose	5.0	37.4 ± 2.0	31.9 ± 2.3	31.2 ± 2.9	22.6 ± 1.4
Propionate	0.5	39.5 ± 3.3	32.8 ± 1.3	29.4 ± 1.2	24.6 ± 1.7
	2.0	37.6 ± 2.6	31.7 ± 2.9	29.1 ± 2.2	24.1 ± 1.2
	5.0	36.6 ± 2.9	31.6 ± 2.0	29.8 ± 1.9	21.6 ± 1.5
Butyrate	0.5	39.3 ± 3.3	32.1 ± 2.0	30.3 ± 1.9	21.9 ± 0.76
	2.0	37.5 ± 1.2	30.0 ± 2.3	30.4 ± 1.1	22.6 ± 1.0
	5.0	36.1 ± 3.0	32.1 ± 2.5	29.6 ± 1.3	21.6 ± 1.5

[1]Data, expressed as nmol lactate/10^6 cell per 30 min, are means ± SEM, n = 8 chickens/age group. Enterocytes were incubated at 40°C for 30 min in the KHB buffer, as described in text. The medium contained 5 mM D-glucose and one of the following: 0.5-5.0 mM [U-^{14}C]glutamate, 0.5-5.0 mM [U-^{14}C]glutamine, 0.5-5.0 mM [U-^{14}C]aspartate, 0.5-5.0 mM [U-^{14}C]alanine, 0.5-2.0 mM [U-^{14}C]palmitate, 0.5-5.0 mM [U-^{14}C]propionate, and 0.5-5.0 mM [1-^{14}C]butyrate.
[2]The rates of lactate production by enterocytes from 0- to 21-day-old chickens were all greater ($P < 0.05$) than those for 42-day-old chickens.
*$P < 0.05$ vs the glucose substrate, as analyzed by the paired t-test.

Table 3.5. Production of pyruvate by chicken enterocytes in the presence of 5 mM glucose[1,2]

Substrate in medium	Concen-tration (mM)	Age of chickens (days)			
		0	7	21	42
Glutamate	0.5	4.17 ± 0.40	3.35 ± 0.28	3.07 ± 0.25	2.18 ± 0.17
	2.0	3.57 ± 0.05*	2.54 ± 0.17*	2.88 ± 0.23	2.16 ± 0.20
	5.0	3.12 ± 0.30*	2.46 ± 0.28*	2.19 ± 0.21*	2.10 ± 0.19
Glutamine	0.5	3.78 ± 0.31	3.87 ± 0.22	3.04 ± 0.18	2.09 ± 0.11
	2.0	3.99 ± 0.30	3.82 ± 0.26	2.01 ± 0.24*	2.04 ± 0.27
	5.0	3.04 ± 0.32*	2.23 ± 0.29*	2.50 ± 0.19*	2.13 ± 0.19
Aspartate	0.5	3.94 ± 0.10	3.60 ± 0.13	3.07 ± 0.21	2.21 ± 0.22
	2.0	3.97 ± 0.33	3.40 ± 0.03	3.10 ± 0.18	2.11 ± 0.21
	5.0	3.01 ± 0.04*	2.57 ± 0.24*	2.93 ± 0.23	2.15 ± 0.23
Alanine	0.5	2.40 ± 0.15*	2.51 ± 0.27*	2.23 ± 0.23*	2.16 ± 0.11
	2.0	3.00 ± 0.28*	2.65 ± 0.03*	2.54 ± 0.05*	2.02 ± 0.09
	5.0	3.01 ± 0.31*	2.67 ± 0.17*	2.43 ± 0.13*	2.20 ± 0.24
Palmitate	0.5	4.04 ± 0.20	3.81 ± 0.15	3.14 ± 0.32	2.18 ± 0.19
	2.0	3.80 ± 0.28	3.76 ± 0.26	3.07 ± 0.18	2.24 ± 0.19
Glucose	5.0	4.00 ± 0.32	3.77 ± 0.28	3.10 ± 0.23	2.27 ± 0.19
Propionate	0.5	3.94 ± 0.13	3.85 ± 0.35	3.10 ± 0.18	2.12 ± 0.15
	2.0	3.90 ± 0.25	3.78 ± 0.23	3.12 ± 0.32	2.26 ± 0.11
	5.0	3.89 ± 0.24	3.70 ± 0.17	3.04 ± 0.19	2.32 ± 0.09
Butyrate	0.5	4.05 ± 0.55	3.92 ± 0.26	3.08 ± 0.07	2.19 ± 0.24
	2.0	4.13 ± 0.16	3.67 ± 0.19	3.06 ± 0.27	2.11 ± 0.23
	5.0	3.81 ± 0.24	3.75 ± 0.14	3.12 ± 0.09	2.25 ± 0.15

[1]Data, expressed as nmol pyruvate/10^6 cell per 30 min, are means ± SEM, n = 8 chickens/age group. Enterocytes were incubated at 40°C for 30 min in the KHB buffer, as described in text. The medium contained 5 mM D-glucose and one of the following: 0.5-5.0 mM [U-^{14}C]glutamate, 0.5-5.0 mM [U-^{14}C]glutamine, 0.5-5.0 mM [U-^{14}C]aspartate, 0.5-5.0 mM [U-^{14}C]alanine, 0.5-2.0 mM [U-^{14}C]palmitate, 0.5-5.0 mM [U-^{14}C]propionate, and 0.5-5.0 mM [1-^{14}C]butyrate.

[2]The rates of lactate production by enterocytes from 0- to 21-day-old chickens were all greater ($P < 0.05$) than those for 42-day-old chickens.

*$P < 0.05$ vs the glucose substrate, as analyzed by the paired t-test.

Utilization of amino acids. Rates of utilization of glutamate, glutamine, aspartate and alanine by enterocytes from all age groups of chickens increased ($P < 0.05$) progressively with increasing their extracellular concentrations from 0.5 mM to 5 mM (Table 3.6). In cells from 0- to 42-day-old chickens, the rate of utilization of glutamate was the highest among all the tested amino acid substrates, followed by aspartate, glutamine and alanine. The rate of utilization of 5 mM glutamate by enterocytes decreased ($P < 0.05$) progressively with increasing the age of chickens from 0 to 42 days. In contrast, the rate of utilization of glutamine by enterocytes did not differ ($P > 0.05$) among 0-, 7-, 21- and 42-day-old chickens. The rate of utilization of 5 mM aspartate was greater ($P < 0.05$) in 0-day-old chickens than that for 21- to 42-day-old chickens.

The rates of utilization of 0.5 and 2 mM alanine by enterocytes increased ($P < 0.05$) progressively with increasing the age of chickens from 0 to 21 days and decreased ($P < 0.05$) thereafter in 42-day-old chickens compared with 21-day-old chickens. Interestingly, in the presence of 5 mM alanine, the rates of its utilization did not differ ($P > 0.05$) between 0- and 7-day-old chickens, and increased ($P < 0.05$) thereafter in 21- and 42-day-old chickens.

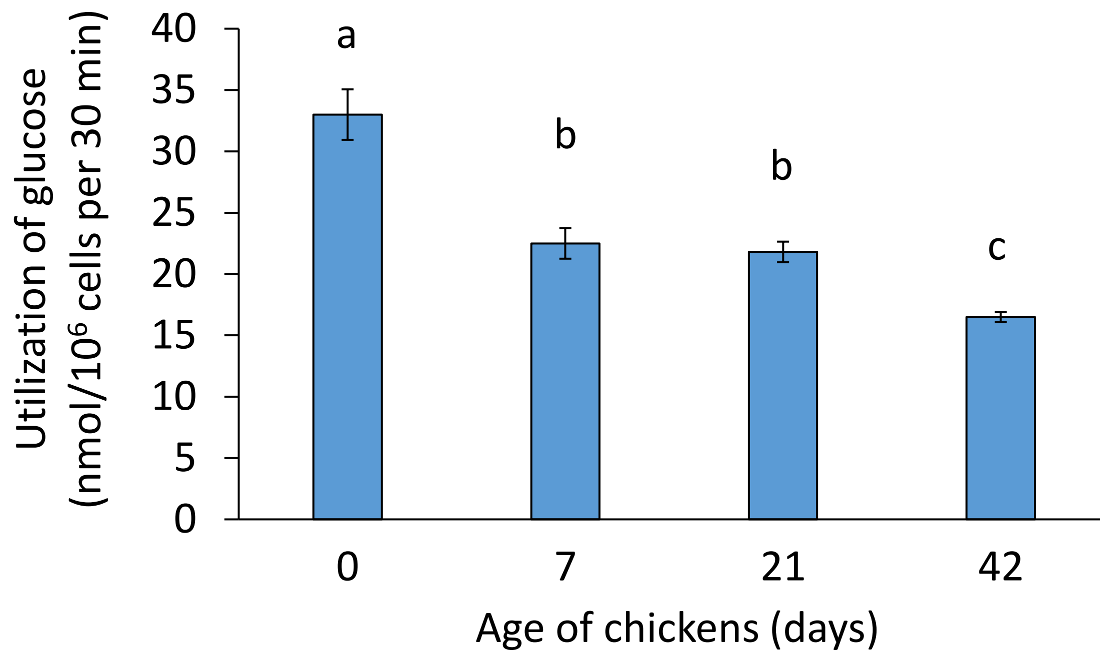
Table 3.6. Utilization of amino acid substrates by chicken enterocytes[1]

Substrate in medium	Concentration (mM)	Age of chickens (days)			
		0	7	21	42
Glutamate	0.5	40.3 ± 4.2[a]	29.7 ± 1.7[b]	30.9 ± 2.8[b]	22.5 ± 2.4[b]
	2.0	65.7 ± 4.3[a]	46.8 ± 3.2[b]	40.9 ± 3.0[bc]	32.1 ± 2.5[c]
	5.0	88.7 ± 5.6[a]	74.5 ± 5.3[b]	62.0 ± 3.7[c]	45.0 ± 2.3[d]
Glutamine	0.5	23.3 ± 1.6	22.7 ± 2.0	20.0 ± 1.3	19.9 ± 1.7
	2.0	31.9 ± 1.2	31.5 ± 1.2	31.5 ± 2.8	31.7 ± 1.5
	5.0	47.6 ± 2.7	44.8 ± 3.6	39.2 ± 2.1	40.5 ± 3.1
Aspartate	0.5	27.2 ± 2.3	24.9 ± 2.2	24.8 ± 1.4	22.1 ± 0.68
	2.0	39.2 ± 3.2	36.5 ± 4.4	34.6 ± 1.6	30.5 ± 2.0
	5.0	58.6 ± 3.6[a]	51.3 ± 3.4[ab]	46.0 ± 3.7[b]	40.9 ± 1.4[b]
Alanine	0.5	16.1 ± 1.2[c]	19.7 ± 1.1[b]	24.6 ± 1.2[a]	20.2 ± 0.2[b]
	2.0	21.2 ± 1.2[c]	25.3 ± 1.2[b]	31.9 ± 1.3[a]	27.2 ± 1.3[b]
	5.0	24.9 ± 1.3[b]	28.0 ± 1.3[b]	38.2 ± 2.4[a]	34.9 ± 1.4[a]

[1]Data, expressed as nmol/10^6 cell per 30 min, are means ± SEM, n = 8 chickens/age group. Enterocytes were incubated at 40°C for 30 min in the KHB buffer, as described in text. The medium contained 5 mM D-glucose and one of the following: 0.5-5.0 mM [U-^{14}C]glutamate, 0.5-5.0 mM [U-^{14}C]glutamine, 0.5-5.0 mM [U-^{14}C]aspartate, and 0.5-5.0 mM [U-^{14}C]alanine. The rate of glucose utilization was not affected ($P > 0.05$) with increasing extracellular concentration from 0.5 to 5 mM.
a-d: Within a row, means not sharing the same superscript letters differ ($P < 0.05$).

Utilization of glucose. The rates of glucose utilization by enterocytes from 0-, 7-, 21-, and 42-day-old chickens are shown in Figure 3.1. The rate of utilization of glucose by enterocytes decreased ($P < 0.05$) by 30% in 7-day-old versus 0-day-old chickens, did not differ ($P > 0.05$) between 7- and 21-day-old chickens, and decreased ($P < 0.05$) thereafter in 42-day-old chickens by 50%, compared with 0-day-old chickens.

Figure 3.1. Utilization of glucose by chicken enterocytes in the presence of 5 mM glucose[1]



[1]Data, expressed as nmol glucose/10^6 cell per 30 min, are means ± SEM, n = 8 chickens/age group. Enterocytes were incubated at 40°C for 30 min in the KHB buffer containing 5 mM D-glucose, as described in text.
a-c: Means not sharing the same superscript letters differ ($P < 0.05$).

Enzyme activities. Activities of key enzymes involved in the metabolism of glutamate, glutamine, aspartate, alanine, glucose and fatty acids in chicken enterocytes are summarized in Table 3.7. Glutamate dehydrogenase activity was very low in the cells from 0- to 42-day-old chickens. There were high GOT and GPT activities but a low glutaminase activity in chicken enterocytes. The activity of glutamine synthetase was low in enterocytes from 0-day-old chickens but high in cells from 7- to 42-day-old chickens. The activities of GOT and GPT in the cells decreased ($P < 0.05$) in 7-day-old versus 0-day-old chickens, did not differ between 7- and 21-day-old chickens, and decreased ($P < 0.05$) thereafter in 42-day-old chickens. In contrast, glutaminase increased ($P < 0.05$) progressively between 0 and 21 days of age and remained at the elevated level in 42-day-old chickens.

The activities of hexokinase, PFK and pyruvate kinase were high in enterocytes from 0- to 42-day-old chickens. The activities of both hexokinase and PFK decreased ($P < 0.05$) progressively with increasing the age of chickens from 0 to 21 days. Interestingly, the activity of pyruvate kinase did not differ ($P > 0.05$) among 0-, 7-, 21-, and 42-day-old chickens. The activity of CPT-I in enterocytes decreased ($P < 0.05$) progressively with increasing the age of chickens from 0 to 21 days and increased ($P < 0.05$) thereafter by 67% in 42-day-old chickens compared with 21-day-old chickens.

Table 3.7. Maximal activities of some key enzymes in carbohydrate and amino acid metabolism as well as the transport of long-chain fatty acids in chicken enterocytes[1]

Enzyme	Age of chickens (days)			
	0	7	21	42
	nmol/min/mg protein			
GDH	0.61 ± 0.09	0.48 ± 0.02	0.59 ± 0.11	0.57 ± 0.05
GOT	11.5 ± 0.98[a]	9.67 ± 0.49[b]	9.80 ± 0.65[b]	6.30 ± 0.42[c]
GPT	9.76 ± 0.91[a]	8.13 ± 0.73[ab]	7.18 ± 0.48[b]	6.18 ± 0.60[c]
PAG	0.78 ± 0.09[c]	1.57 ± 0.09[b]	1.79 ± 0.07[a]	1.91 ± 0.08[a]
GS	1.15 ± 0.09[c]	47.9 ± 0.53[a]	48.9 ± 0.65[a]	33.5 ± 0.64[b]
Hexokinase	11.5 ± 0.91[a]	8.26 ± 0.32[b]	6.62 ± 0.42[c]	5.14 ± 0.38[d]
PFK	19.8 ± 1.7[a]	16.0 ± 0.20[b]	8.15 ± 0.77[c]	6.96 ± 0.56[d]
Pyruvate kinase	9.93 ± 0.71	9.60 ± 1.0	9.02 ± 0.60	8.80 ± 0.42
CPT-I	2.94 ± 0.08[a]	0.97 ± 0.02[b]	0.45 ± 0.01[d]	0.75 ± 0.03[c]

[1]Data, expressed as nmol/min/mg protein, are means ± SEM, n = 8 chickens/age group.
CPT-I = carnitine palmitoyltransfrease I; GDH = glutamate dehydrogenase; GOT = glutamate-oxaloacetate transaminase; GPT = glutamate-pyruvate transaminase; GS = glutamine synthetase; PAG = phosphate-activated glutaminase; PFK = phosphofructose kinase.
a-c: Within a row, means not sharing the same superscript letters differ ($P < 0.05$).

Discussion

Metabolic fates of amino acids, glucose, and fatty acids in chicken enterocytes. This research quantified, for the first time, the metabolic fates of amino acids, glucose, and fatty acids in enterocytes of developing chickens. Our results indicated that glutamate was extensively oxidized in enterocytes from 0- to 42-day-old chickens and that the rate of oxidation of glutamate was much greater than that of glutamine, aspartate and alanine (Table 3.1). Because of the low activity of GDH, glutamate catabolism in these cells was initiated by GOT and GPT to form α-ketoglutarate, alanine and aspartate (Table 3.7). While the oxidation of glutamate-derived alanine and aspartate was limited, α-ketoglutarate was further oxidized to CO_2 in the cells via the Krebs cycle. In enterocytes from 0-, 7-, 21- and 42-day-old chickens, oxidation of glutamate to CO_2 accounted for 51%, 40%, 27%, and 16% of the total glutamate utilized, respectively. This indicated an important role of glutamate as a major metabolic fuel for intestinal cells, particularly during the early days post-hatching. The extensive catabolism of glutamate by enterocytes reduces the entry of dietary glutamate into the portal circulation. Thus, although glutamate is one of the most abundant amino acids in poultry diets (Wu 2014), it is among the least abundant amino acids in the blood of chickens (Wu 2018). Our finding is important because it provides a biochemical basis for designing new diets supplemented with glutamate to improve intestinal function and health in poultry, particularly under stressful conditions (e.g., heat stress and long-distance transportation).

In contrast to glutamate, the oxidation of glutamine, aspartate and alanine was limited in enterocytes from all age groups of chickens (Table 3.1). This can be explained by a low glutaminase activity in the cells (Table 3.7) as demonstrated by Wu et al. (1995). Although GOT and GPT activities were high in chicken enterocytes (Table 3.7), only a small amount of alanine

and aspartate underwent transamination to form pyruvate and oxaloacetate, respectively, in the intact cells, possibly due to a low concentration of α-ketoglutarate as a substrate for glutamate transaminase in the cytosol and mitochondria. This illustrates a major species difference in the intestinal metabolism of amino acids between birds and mammals. The low rate of catabolism of glutamine by enterocytes allows most of dietary glutamine to enter the portal circulation. Thus, the concentration of glutamine in the plasma of chickens is usually about two times that in mammals, including pigs and rats (Wu 2018). In contrast, among all the substrates studied (i.e., D-glucose, glutamate, glutamine, aspartate, alanine, palmitate, propionate, and butyrate), allowing dietary glutamine to enter the portal vein effectively in birds. In chickens, the concentration of glutamine in plasma is the highest (~1 mM) among all amino acids (Watford and Wu 2005), which doubles the concentration of glutamine in the plasma of pigs (Wu 2018). A high circulating level of glutamine in broilers stimulate protein synthesis and inhibits proteolysis in skeletal muscle (Wu and Thompson 1990), contributing to their fast growth rate. In addition to the small intestine, glutamate and glutamine are degraded in the skeletal muscle, liver, kidney, and brain (Smith and Campbell 1983; Tinker et al. 1986; Watford et al. 1981; Watford and Wu 2005). As the precursor of γ-aminobutyrate (neurotransmitters), glutamate and glutamine also play an important role in the behavior and feed intake of chickens. Collectively, results of this study further show the crucial role of glutamate and glutamine in the growth, development and homeostasis of chickens.

In chicken enterocytes, glutaminase is present in mitochondria to convert glutamine into glutamate. However, only 4-7% of the utilized glutamine was oxidized to CO_2 in enterocytes of 0- to 42-day-old chickens (Tables 3.1 and 3.6). These results suggest the compartmentation of intracellular glutamate degradation in the cells. Extracellular glutamate may be preferentially

degraded, whereas mitochondrially-derived glutamate (i.e., the glutamate formed from glutamine by glutaminase) may not be coupled efficiently with glutamate transaminases in the mitochondria. Further studies are warranted to test this hypothesis.

Glucose has long been assumed to be actively utilized by all cell types of chickens. Consistent with the presence of high activities of hexokinase, PFK and pyruvate kinase (Table 3.7) in enterocytes from 0- to 42-day-old chickens, glucose was readily metabolized to lactate and pyruvate in the cells (Tables 3.4 and 3.5). Similar results have been reported by Watford et al. (1979) for 5- to 7-week-old male chickens. The ratio of lactate to pyruvate in the incubation medium was about 10:1, which is similar to the value in the blood of animals. About 40% and 18% of the utilized glucose were oxidized to CO_2 in enterocytes from 0- and 7-day-old chickens, respectively (Table 3.1 and Figure 3.1), indicating that glucose is a significant source of energy for the cells. As chickens grew, the rate of glucose utilization by enterocytes decreased (Figure 3.1), therefore allowing more dietary glucose to enter the portal circulation. Furthermore, we found that the rate of glucose oxidation in enterocytes from 0- to 42-day-old chickens was much lower than that of glutamate (Table 3.1).

Another novel finding of this study is that among all the potential energy substrates, the oxidation of palmitate (a long-chain fatty acid) in enterocytes of 0- to 42-day-old chickens was very limited (Table 3.1). This is consistent with a low activity of CPT-I in these cells (Table 3.7). Long-chain fatty acids are activated by the cytosolic acyl-CoA synthase to form acyl-CoAs, which enter the mitochondria via the CPT transport system. The low rate of oxidation of fatty acids in enterocytes allows efficient absorption of dietary oils into the lymphatic vessel. Interestingly, oxidation of short-chain fatty acids (propionate and butyrate) was also limited in chicken enterocytes (Table 3.1). It is possible that acyl-CoA synthase activity for short-chain

fatty acids is low in chicken enterocytes, thereby limiting the activation of short-chain fatty acids into acyl-CoAs for oxidation in the mitochondria. Taken together, fatty acids are not major metabolic fuels for those cells.

Developmental changes of the metabolism of amino acids, glucose, and fatty acids in chicken enterocytes. Results of the present study revealed developmental changes in the metabolism of amino acids, glucose, and fatty acids in chicken enterocytes. Glutamate was highly oxidized by enterocytes of chickens at each post-hatching age. As chickens grew from 0 to 42 days of age, glutamate oxidation by enterocytes decreased by 80 to 85% depending on the extracellular concentration of glutamate (Table 3.1), which is consistent with the decreases in the rate of glutamate utilization (Table 3.6) and the rates of production of aspartate and alanine by the cells (Table 3.3). In contrast to glutamate, there were only small changes in the rates of oxidations of glutamine, aspartate, and alanine by enterocytes between 0- to 42-day-old chickens (Table 3.1). This is consistent with the observation that there were only moderate changes in the activities of GOT and GPT in the cells between 0- and 42-day-old chickens (Table 3.7).

The rate of oxidation of glucose by chicken enterocytes was the second highest among all the potential energy substrates. Compared with 0-day-old chickens, the rate of oxidation of glucose by enterocytes of 42-day-old chickens decreased by 76% (Table 3.1). The marked decrease in glucose oxidation was associated with decreases in the utilization of glucose as well as the production of lactate and pyruvate by the cells. Similar results on age-dependent declines in substrate oxidation were also observed for short-chain fatty acids. In contrast, the rate of oxidation of palmitate increased as chickens grew from 0 to 42 days of age (Table 3.1), but the long-chain fatty acid still contributed only a small amount of ATP to the cells, when compared with glutamate.

Energetic roles of amino acids, glucose, and fatty acids in chicken enterocytes. A salient observation of the present study is that glutamate was quantitatively the most important metabolic fuel for enterocytes in all age groups of chicken. In all age groups of chickens, glutamate oxidation accounted for 80% of total ATP production by all the tested substrates (Table 3.2). Consistent with an age-dependent decrease in metabolic rate, the contribution of glutamate to ATP production by enterocytes also decreased with age (e.g., by more than 80% in 42-day-old chickens, compared with 0-day-old chickens) (Table 3.2). The oxidation of glutamine and aspartate contributed equally only a small amount of ATP produced by chicken enterocytes at each post-hatching age. Particularly, the contribution of glutamate to ATP production by enterocytes from 0- and 42-day-old chickens was about 10 and 4 times that of glutamine, respectively. Similar results were observed for aspartate and alanine. Thus, as chickens grow, glutamine, aspartate and alanine contribute more ATP to their enterocytes.

Glucose is a significant energy substrate for enterocytes from 0- to 42-day-old chickens. The rate of ATP production from the oxidation of glucose to CO_2 by the cells was the second highest among all the tested substrates and amounted to one third of ATP production from glutamate. In contrast, propionate and butyrate were only minor energy substrates for the cells (Table 3.2). The oxidation of palmitate in chicken enterocytes produced more ATP than the short-chain fatty acids at any age post hatching. Compared with glutamate, palmitate was a minor source of energy in chicken enterocytes. Interestingly, at 0.5 and 2.0 mM, the contribution of palmitate to ATP production by enterocytes of chickens increased with age, such as that at 42 days of age, this long-chain fatty acid became a significant metabolic fuel for the cells. It is possible that the transport of short-chain fatty acids from the cytosol into the mitochondria is lower than that for long-chain fatty acids and that the conversion of short-chain fatty acids into

their acyl-CoAs in the mitochondria is limited. Further studies are warranted to examine these possibilities.

Physiological significance of amino acids in chicken enterocytes. Glutamate not only serves as an important energy substrate for chicken enterocytes, but also contributes carbon and nitrogen precursors for biosynthetic processes (Wu 2013). Our finding that the rate of oxidation of glutamate by the enterocytes of 0- to 42-day-old chickens was high (Table 3.1) was not consistent with the previous report that the rate of utilization of glutamate by enterocytes of 3- to 5-week-old male chickens was only 20% that for glucose (Porteaus 1980). The reason for this discrepancy is unknown at this time. Furthermore, glutamate is an important substrate for the synthesis of glutathione and alanine in the small intestine (Wu 2013). These physiological processes account for the extensive utilization of glutamate by chicken enterocytes. Because arterial glutamate is not taken up by enterocytes (Wu 2013), dietary glutamate plays an important role in maintaining the intestinal integrity and health of chickens. Glutamate is one of the most abundant amino acids in the whole-body protein of chickens (Wu 2014). Therefore, de novo synthesis of glutamate must be active to provide endogenous glutamate for protein synthesis in the extra-intestinal tissues of chickens.

In conclusion, results of this study demonstrate that 1) glutamate oxidation was the major energy source for enterocytes of post-hatching chickens, 2) the oxidation of other amino acids (glutamine, aspartate, and alanine) was limited in chicken enterocytes, 3) the contribution of glucose oxidation to ATP production by enterocytes was the second highest among all the tested substrates, and 4) chicken enterocytes oxidized more long-chain fatty acids than short-chain fatty acids. Our work provided the first line of evidence for extensive oxidation of glutamate by enterocytes of 0- to 42-day-old chickens. Because of limited uptake of arterial glutamate by

enterocytes, dietary glutamate is essential to the integrity and function of the chicken small intestine. This finding has important implications for developing new nutritional strategies to improve the small-intestinal and whole-body growth and health of post-hatching chickens.

CHAPTER IV

METABOLISM OF AMINO ACIDS, GLUCOSE, AND FATTY ACIDS IN PIG ENTEROCYTES

Synopsis

Glutamate and glutamine are known to be major energy substrates for pig enterocytes, and aspartate has also been reported to extensively catabolized by the small intestine of rats. However, little is known about the oxidation of aspartate and fatty acids in pig enterocytes or the relative role of potential nutrients in ATP production by the cells. In the present study, enterocytes isolated from 0-, 7-, 14- and 21-day-old piglets were used to determine the rates of oxidation of amino acids, fatty acids and glucose. Enterocytes were incubated at 37°C for 30 min in Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 5 mM D-glucose and one of the following: 0.5-5 mM L-[U-^{14}C]glutamate, 0.5-5 mM L-[U-^{14}C]glutamine, 0.5-5 mM L-[U-^{14}C]aspartate, 0.5-5 mM L-[U-^{14}C]alanine, 0.5-2 mM L-[U-^{14}C]palmitate, D-[U-^{14}C]glucose, 0.5-5 mM [U-^{14}C]propionate, or 0.5-5 mM [1-^{14}C]butyrate. At the end of the incubation, $^{14}CO_2$ produced from each ^{14}C-labeled substrate was collected. Rates of oxidation of each substrate by enterocytes from all age groups of piglets increased ($P < 0.01$) progressively with increasing its extracellular concentrations. The rates of oxidation of glutamate, glutamine, aspartate, glucose by enterocytes from 0- to 21-day-old pigs were much greater ($P < 0.01$) than those for the same concentrations of palmitate, propionate, and butyrate. Compared with 0-day-old pigs, the rates of oxidation of glutamate, aspartate, glutamine, alanine, and glucose by enterocytes from 21-day-old pigs decreased ($P < 0.01$) markedly, without changes in palmitate oxidation. Oxidation of alanine, propionate, butyrate and palmitate by enterocytes of pigs was limited during their

postnatal growth. At each postnatal age, the oxidation of glutamate, glutamine, aspartate, and glucose produced much more ATP than any other substrates. The degradation of glutamate was initiated primarily by transaminases. Our results indicated that amino acids (glutamate plus glutamine plus aspartate) are the major metabolic fuels in enterocytes of 0- to 21-day-old pigs. Because of limited uptake of arterial glutamate by enterocytes, dietary glutamate is essential to the integrity and function of the pig small intestine.

Introduction

The small intestine is a highly differentiated and complex organ in pigs. Enterocytes (the villus columnar absorptive cells) account for >85% of total mucosal cell numbers in the small intestine which are responsible for terminal digestion and absorption of nutrients in pigs (Wu 2018). Because the enterocytes have a high rate of turnover, they have high requirements for energy.

Previous studies have shown that neonatal pig enterocytes use glutamine and glucose as major metabolic fuels (Wu et al. 1995). However, the authors did not evaluate the potential roles of other amino acids or fatty acids as energy sources for these cells. In older (60-kg) pigs, the rate of oxidation of 2 mM glutamate to CO_2 by enterocytes is similar to that of 2 mM glutamine (Blachier et al. 1999). Consistent with a low activity of glutamate dehydrogenase in the small intestine (Wu 2013), there is limited production of ammonia from glutamate or monosodium glutamate by pig enterocytes (Blachier et al. 1999). Furthermore, in vivo work involving the use of ^{13}C-labeled tracer has demonstrated extensive oxidation of glutamate by the portal-drained viscera of young pigs (Reeds et al. 1996, 1997), although no measurement has been made on glutamine or aspartate oxidation.

Based on the foregoing, it is possible that glutamate, glutamine and aspartate are major sources of energy for pig enterocytes. However, at present, comparison of oxidation of glutamine, glutamate, and aspartate with oxidation of glucose and fatty acids by enterocytes in the same study is lacking. Such information is essential to our better understanding of intestinal substrate metabolism. The present study was designed to fill in this gap of knowledge by measuring the rates of oxidation of amino acids and glucose, as well as short- and long-chain fatty acids in enterocytes from 0 to 21-day-old pigs. We also assessed the contribution of these

substrates to ATP production by the cells. Finally, to provide a biochemical basis for the metabolic results, we determined the activities of key enzymes involved in the metabolic pathways.

Materials and Methods

Chemicals. The following radiolabeled chemicals were purchased from American Radiolabeled Chemicals (St. Louis, MO): D-[U-^{14}C]glucose, L-[U-^{14}C]glutamine, L-[U-^{14}C]glutamate, L-[U-^{14}C]aspartate, L-[U-^{14}C]alanine, [U-^{14}C]propionate, [1-^{14}C]butyrate, and [U-^{14}C]palmitic acid. Before use, ^{14}C-labeled glutamine was purified by using the Dowex AG1-X8 resin (acetate form, 200-400 mesh) (Self et al. 2004). ^{14}C-Labeled glutamate was purified by adding an equal volume of 1.5 M $HClO_4$ and then neutralized by a half volume of 2 M K_2CO_3. Soluene was procured from Perkin-Elmer. The liquid scintillation cocktail for $^{14}CO_2$ was prepared by dissolving 5 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis(5-phenyloxazol-2-yl) benzene into 500 of toluene and 500 mL of 2-methoxyethanol (Wu and Thompson. 1987). The sources of other chemicals were the same as described previously (Li et al. 2016; Self et al. 2004).

Animals and the isolation of the jejunum. Preweaning piglets (offspring of Yorkshire x Landrace sows and Duroc x Hampshire boars) at days 0, 7, 14, and 21 after birth were obtained from the Swine Center of Texas A&M University. Before isolating enterocytes from 0-day-old pigs, the animals received no colostrum or water. Suckling pigs were nursed by their mothers until 21 days of age, when they were removed from the sows. There were 6 pigs per age group. All experimental procedures were approved by the Institutional Agricultural Animal Care and

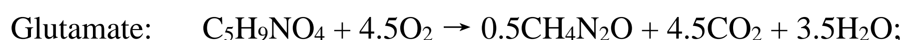
Use Committee of Texas A&M University. The jejunum was obtained from individual anaesthetized pigs.

Preparation of enterocytes. The jejunum was obtained from pigs after they were anaesthetized and used for the preparation of enterocytes,as described by Wu et al. (1995a). Briefly, the jejunum was thoroughly rinsed with saline to remove its luminal contents and then rinsed three times with 50 ml of oxygenated (95% O_2-5% CO_2) Ca^+-free Krebs-Henseleit bicarbonate (KHB) buffer (119 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 25 mM $NaHCO_3$, pH 7.4) containing 20 mM HEPES (pH 7.4) and 5 mM EDTA (disodium). The jejunum was filled with 40 ml of the same Ca_2^+-free KHB buffer and placed in 200 ml of this buffer. The intestine was incubated at 37°C (the normal body temperature of pigs) in a shaking water bath (70 oscillations/min) for 20 min. At the end of the incubation, the jejunum was gently patted with fingertips for 1 min, and the lumen fluid was drained into 40-ml polystyrene tubes. The cells were centrifuged at 400 g for 3 min, washed three times with oxygenated (95% O_2-5% CO_2) KHB buffer (pH 7.4) containing 2.5 mM $CaCl_2$ and 20 mM HEPES (pH 7.4) but no EDTA, and then resuspended in this buffer. The viability of the isolated enterocytes was greater than 95%, as assessed by trypan blue exclusion (Wu et al. 1995a).

Determination of substrate oxidation. Metabolic studies were conducted as previously described (Wu 1995b). Briefly, 3×10^6 enterocytes were incubated at 37°C for 30 min in 1 ml of oxygenated (95% O_2/5% CO_2) KHB buffer (pH 7.4) containing 5 mM D-glucose, 1 nM insulin, and one of the following substrates: 5 mM D-[U-^{14}C]glucose, 0.5 mM L-[U-^{14}C]glutamate, 2 mM L-[U-^{14}C]glutamate, 5 mM L-[U-^{14}C]glutamate, 0.5 mM L-[U-^{14}C]glutamine, 2 mM L-[U-^{14}C]glutamine, 5 mM L-[U-^{14}C]glutamine, 0.5 mM L-[U-^{14}C]aspartate, 2 mM L-[U-^{14}C]aspartate, 5 mM L-[U-^{14}C]aspartate, 0.5 mM L-[U-^{14}C]alanine, 2 mM L-[U-^{14}C]alanine, 5

mM L-[U-^{14}C]alanine, 0.5 mM [U-^{14}C]palmitate, 2 mM [U-^{14}C] palmitate, 0.5 mM [U-^{14}C]propionate, 2 mM [U-^{14}C]propionate, 5 mM [U-^{14}C]propionate, 0.5 mM [U-^{14}C]butyrate, 2 mM [U-^{14}C]butyrate, and 5 mM [U-^{14}C]butyrate. The specific radioactivity of each tracer in the incubation medium was approximately 200 dpm/nmol. In all experiments, media containing the same components, as well as 0.2 ml of 1.5 M $HClO_4$ (added before the addition of cells) were run as blanks. Incubation was initiated by the addition of a cell suspension. After a 30-min incubation period, the reaction was terminated by the addition, through the rubber stopper, of 0.2 ml Soluene into a center-well suspended within the tube for CO_2 collection, followed by the addition, through the rubber stopper, of 0.2 ml of 1.5 M $HClO_4$ into the incubation medium. After a further 1-h incubation, $^{14}CO_2$ radioactivity was measured by a liquid scintillation counter (Self et al. 2004).

ATP production. The rate of oxidation of a substrate in enterocytes ($CO_2/10^6$ cell per 30 min) was calculated as the amount of $^{14}CO_2$ (dpm) produced by the enterocytes divided by the specific radioactivity of the substrate in incubation medium. The rate of ATP production was calculated from the rate of CO_2 production by multiplying by the coefficient (ATP/CO_2, mol/mol) of the substrate according to the following equations:

Glutamate: 

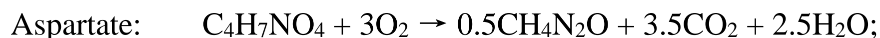
$C_5H_9NO_4 + 4.5O_2 \rightarrow 0.5CH_4N_2O + 4.5CO_2 + 3.5H_2O$;

20.5 mol ATP/mol Glu, 4.56 mol ATP/mol CO_2

Glutamine:

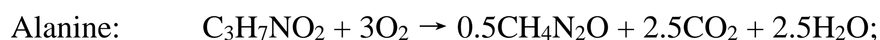
$C_5H_{10}N_2O_3 + 4.5O_2 \rightarrow CH_4N_2O + 4CO_2 + 3H_2O$;

18.5 mol ATP/mol Gln, 4.63 mol ATP/mol CO_2

Aspartate: 

$C_4H_7NO_4 + 3O_2 \rightarrow 0.5CH_4N_2O + 3.5CO_2 + 2.5H_2O$;

13 mol ATP/mol Asp, 3.71 mol ATP/mol CO_2

Alanine: 

$C_3H_7NO_2 + 3O_2 \rightarrow 0.5CH_4N_2O + 2.5CO_2 + 2.5H_2O$;

13 mol ATP/mol Ala, 5.2 mol ATP/mol CO_2

Palmitate: $C_{16}H_{32}O_2 + 23O_2 \rightarrow 16CO_2 + 16H_2O$;

106 mol ATP/mol PA, 6.625, mol ATP/mol CO_2

Glucose: $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$;

30 mol ATP/mol Glucose, 5 mol ATP/mol CO_2

Propionate: $C_3H_6O_2 + 3.5O_2 \rightarrow 3CO_2 + 3H_2O$;

15 mol ATP/mol Propionate, 5 mol ATP/mol CO_2

Butyrate: $C_4H_8O_2 + 5O_2 \rightarrow 4CO_2 + 4H_2O$;

22 mol ATP/mol Butyrate, 5.5 mol ATP/mol CO_2

The rates of ATP production from the oxidation of substrates (expressed as mol ATP/mol substrate) are as follows: glutamate, 20.5; glutamine, 18.5, aspartate, 13; alanine, 13; palmitate, 106; and glucose, 30; propionate, 15; butyrate, 22 (Wu 2013, 2018). The coefficients of ATP production per mole of CO_2 produced from the oxidation of substrates (expressed as mol ATP/mol CO_2) are as follows: glutamate, 4.56; glutamine, 4.63; aspartate, 3.71; alanine, 5.2; palmitate, 6,625; glucose, 5; propionate, 5; and butyrate, 5.5 (Wu 2013, 2018).

Enzymatic activities in pig enterocytes. Mitochondria were isolated from enterocytes as described previously (Wu 1997). The supernatant fluid from the 10,000 × g centrifugation was used as the cytosolic fraction. Both mitochondria and the cytosol were used to measure enzyme activities. The activities of glutaminase, glutamine synthetase, glutamate dehydrogenase, glutamate-pyruvate transaminase, and glutamate-oxaloacetate transaminase were determined as described by Wu et al. (2000) and Self et al. (2004). The activities of hexokinase, phosphofructokinase, and pyruvate kinase were determined using kits from Nipro Enzymes (3-9-

3 HONJO-NISHI KITA-KU, OSAKA 531-8510 JAPAN). Carnitine palmitoyltransferase-1 activity was determined with a kit (Carnitine palmitoyltransferase I activity assay) by measuring the formation of ^{14}C-labeled palmitoyl-carnitine from palmitoyl-CoA and ^{14}C-labeled carnitine (McGarry et al. 1983). The details of the enzyme assays are provided in Appendix #C.

Measurement of metabolites. Amino acids (including glutamate, glutamine, aspartate and alanine) in neutralized cell extracts were quantified by our established HPLC method (Wu et al., 1994). Ammonia (NH_4^+ plus NH_3; or simply referred to as NH_3 in text) was determined using an enzymatic method involving glutamate dehydrogenase and α-ketoglutaric acid (Wu 1995). L-Lactate and pyruvate were determined using enzymatic methods involving L-lactate dehydrogenase (Wu et al. 1991). Glucose was determined using an enzymic method involving glucose-6-P dehydrogenase and hexokinase (Wu et al. 1995). Based on the production of metabolites, the rates of utilization of glutamate, glutamine, alanine, aspartate and glucose were calculated as follows:

Glutamate utilization (nmol/10^6 cells per 30 min) = (CO_2/4 + Ala + Asp mol)/cell number;

Glutamine utilization (nmol/10^6 cells per 30 min) = (CO_2/4 + Glu + Ala + Asp)/cell number;

Alanine utilization (nmol/10^6 cells per 30 min) = (CO_2/2 + Asp + Glu)/cell number;

Aspartate utilization (nmol/10^6 cells per 30 min) = (CO_2/3 + Glu + Ala)/cell number;

Glucose utilization (nmol/10^6 cells per 30 min) = (CO_2/6 + Lactate/2 + Pyruvate/2)/cell number

Statistical analysis. Data were analyzed by one-way ANOVA and the Student-Newman-Keuls multiple comparison test (Assaad et al. 2014). Probability values < 0.05 were taken to indicate statistical significance.

Results

Oxidation of amino acids, glucose and fatty acids in pig enterocytes. Data on the rates of CO_2 production from the oxidation of different substrates in pig enterocytes are summarized in Table 4.1. Rates of oxidation of glutamate, glutamine, aspartate and alanine in enterocytes from all age groups of pigs increased ($P < 0.05$) progressively with increasing their extracellular concentrations from 0.5 mM to 5 mM. In cells from 0- to 21-day-old pigs, the rates of CO_2 production from [U-^{14}C]glutamate and [U-^{14}C]glutamine were the highest and the second highest among all the tested substrates. Particularly, in cells from 0-day-old pigs, the rate of total CO_2 production from [U-^{14}C]glutamate, [U-^{14}C]glutamine, and [U-^{14}C]aspartate was 91% of that from the sum of all the tested substrates. Compared with glutamate, glutamine, and aspartate, the oxidation of alanine was limited in enterocytes from 0- to 21-day-old pigs.

There were developmental changes in the oxidation of amino acids by pig enterocytes. The rates of oxidation of glutamate, glutamine, and aspartate in the cells decreased progressively ($P < 0.05$) by 87%, 88%, and 90%, respectively with increasing the age of piglets from 0 to 21 days. The rates of oxidation of glutamate, glutamine, aspartate, and alanine decreased ($P < 0.05$) in 14-day-old versus 0-day-old pigs, and remained relatively constant between 14- and 21-day-old pigs.

The rate of CO_2 production from 5 mM glucose by enterocytes of pigs at postnatal age was the third highest among all the tested substrates. The rate of oxidation of glucose decreased progressively ($P < 0.05$) by 87% with increasing the age of pigs from 0 to 14 days. The largest decline (74%) occurred between 7 and 14 days of age.

Compared with glutamate and glutamine, oxidation of propionate, butyrate and palmitate was limited in enterocytes from 0- and 21-day-old pigs. However, there were developmental

changes in the oxidation of short- and long-chain fatty acids by the cells. The rate of oxidation of propionate decreased progressively ($P < 0.05$) by 58% with increasing age from 0 to 21 days. The rate of oxidation of butyrate decreased ($P < 0.05$) by 40~45% in 21-day-old versus 0-day-old pigs. In contrast, the rate of oxidation of palmitate did not differ ($P > 0.05$) between 0- and 21-day-old pigs.

Table 4.1. Oxidation of energy substrates to CO_2 by pig enterocytes[1].

Substrate in medium	Concentration (mM)	Age of pigs (days)			
		0	7	14	21
Glutamate	0.5	106 ± 4.3[a]	81.6 ± 4.7[b]	15.0 ± 0.78[c]	13.4 ± 1.0[c]
	2.0	185 ± 11.9[a]	126 ± 8.2[b]	23.9 ± 1.7[c]	23.1 ± 1.0[c]
	5.0	297 ± 22.9[a]	217 ± 7.3[b]	40.6 ± 2.0[c]	39.0 ± 0.90[c]
Glutamine	0.5	83.9 ± 5.6[a]	64.7 ± 2.4[b]	11.0 ± 0.72[c]	10.6 ± 0.24[c]
	2.0	170 ± 18.7[a]	109 ± 2.3[b]	19.4 ± 1.3[c]	20.2 ± 0.82[c]
	5.0	248 ± 22.6[a]	178 ± 8.2[b]	32.0 ± 1.8[c]	29.9 ± 2.9[c]
Aspartate	0.5	67.1 ± 5.4[a]	40.7 ± 2.8[b]	6.60 ± 0.60[c]	5.00 ± 0.15[c]
	2.0	120 ± 11.3[a]	83.0 ± 2.5[b]	11.3 ± 0.80[c]	11.0 ± 1.1[c]
	5.0	166 ± 8.1[a]	136 ± 4.3[b]	19.8 ± 1.0[c]	16.3 ± 1.7[c]
Alanine	0.5	27.2 ± 1.9[a]	12.8 ± 1.0[b]	1.60 ± 0.15[c]	1.40 ± 0.13[c]
	2.0	48.4 ± 3.2[a]	28.8 ± 1.2[b]	3.50 ± 0.28[c]	3.00 ± 0.74[c]
	5.0	70.4 ± 4.8[a]	41.2 ± 0.40[b]	6.40 ± 0.63[c]	5.00 ± 1.47[c]
Palmitate	0.5	6.90 ± 0.51	7.00 ± 0.12	7.50 ± 0.49	8.00 ± 0.40
	2.0	11.5 ± 1.2	12.0 ± 0.12	12.9 ± 0.35	13.6 ± 0.20
Glucose	5.0	171 ± 6.5[a]	81.3 ± 4.1[b]	21.4 ± 1.2[c]	21.9 ± 1.4[c]
Propionate	0.5	3.10 ± 0.34[a]	2.40 ± 0.10[b]	2.00 ± 0.06[b]	1.30 ± 0.05[c]
	2.0	4.40 ± 0.11[a]	3.50 ± 0.13[b]	3.00 ± 0.07[b]	2.20 ± 0.06[c]
	5.0	7.20 ± 0.66[a]	4.40 ± 0.09[b]	3.90 ± 0.11[b]	3.10 ± 0.07[c]
Butyrate	0.5	2.00 ± 0.08[a]	1.70 ± 0.08[b]	1.50 ± 0.12[b]	1.10 ± 0.07[c]
	2.0	3.30 ± 0.18[a]	2.60 ± 0.06[b]	2.40 ± 0.14[b]	1.80 ± 0.06[c]
	5.0	4.30 ± 0.26[a]	3.50 ± 0.10[b]	3.20 ± 0.09[b]	2.60 ± 0.16[c]

[1]Data, expressed as nmol $CO_2/10^6$ cell per 30 min, are means ± SEM, n = 6 pigs/age group. Enterocytes were incubated at 37°C for 30 min in the KHB buffer, as described in text. The medium contained 5 mM D-glucose and one of the following: 0.5-5.0 mM [U-^{14}C]glutamate, 0.5-5.0 mM [U-^{14}C]glutamine, 0.5-5.0 mM [U-^{14}C]aspartate, 0.5-5.0 mM [U-^{14}C]alanine, 0.5-2.0 mM [U-^{14}C]palmitate, 0.5-5.0 mM [U-^{14}C]propionate, and 0.5-5.0 mM [1-^{14}C]butyrate. The rate of oxidation of each substrate increased ($P < 0.05$) with increasing extracellular concentration from 0.5 to 5 mM.

a-d: Within a row, means not sharing the same superscript letters differ ($P < 0.05$).

ATP production from the oxidation of energy substrates to CO_2 in pig enterocytes. Data on ATP production from the oxidation of substrates by pig enterocytes are summarized in Table 4.2. Results of the comparison of ATP production from each energy substrate oxidation by enterocytes were generally similar to those for the rates of substrate oxidation noted previously. Rates of ATP production from each substrate by enterocytes from all age groups of pigs increased ($P < 0.01$) progressively with increasing its extracellular concentrations from 0.5 mM to 5 mM. In the enterocytes of pig from 0- to 21-day-old, the contribution of amino acids oxidation to total ATP production decreased ($P < 0.01$) from 94 to 67%. Glutamate and glutamine were the largest and the second largest contributor to ATP production by enterocytes of pigs during their postnatal growth. The oxidation of glutamate, glutamine, and aspartate accounted for 90 to 95% of the total ATP production from amino acids by pig enterocytes. At each day age, the contribution of oxidation of alanine to ATP production by pig enterocytes was limited. Compared with 0-day-old pig enterocytes, the contributions of glutamate, glutamine, aspartate, and alanine to ATP production by 21-day-old pig enterocytes decreased ($P < 0.01$) by 87%, 87%, 90%, and 75%, respectively.

In the enterocytes of pig from 0- to 21-day-old, the contributions of oxidation of glucose and fatty acids to ATP production decreased ($P < 0.01$) by 87% and by 10%, respectively. Among the tested fatty acids, palmitate contributed to more ATP production than propionate and butyrate. At each day age, the contribution of propionate and butyrate to ATP production in pig enterocytes was limited.

Table 4.2. ATP production from energy substrates by pig enterocytes[1]

Substrate in medium	Concentration (mM)	Age of pigs (days)			
		0	7	14	21
Glutamate	0.5	485 ± 19.4[a]	372 ± 21.2[b]	68.6 ± 3.6[c]	60.9 ± 4.4[c]
	2.0	866 ± 54.0[a]	575 ± 34.8[b]	109 ± 7.9[c]	106 ± 4.5[c]
	5.0	1386 ± 117.9[a]	992 ± 33.1[b]	185 ± 9.1[c]	178 ± 4.1[c]
Glutamine	0.5	389 ± 25.9[a]	300 ± 11.5[b]	50.9 ± 3.3[c]	48.9 ± 1.1[c]
	2.0	827 ± 83.3[a]	504 ± 9.7[b]	89.9 ± 5.9[c]	93.7 ± 3.8[c]
	5.0	1218 ± 92.7[a]	826 ± 37.9[b]	148 ± 8.2[c]	139 ± 13.3[c]
Aspartate	0.5	249 ± 20.1[a]	151 ± 10.5[b]	24.5 ± 2.2[c]	18.6 ± 0.56[c]
	2.0	463 ± 42.4[a]	308 ± 8.4[b]	41.8 ± 3.0[c]	40.8 ± 3.9[c]
	5.0	633 ± 27.9[a]	504 ± 16.0[b]	73.4 ± 3.7[c]	60.5 ± 6.4[c]
Alanine	0.5	140 ± 10.0[a]	66.4 ± 5.6[b]	8.40 ± 0.80[c]	7.00 ± 0.66[c]
	2.0	247 ± 18.0[a]	149 ± 6.0[b]	18.1 ± 1.5[c]	15.6 ± 0.86[c]
	5.0	353 ± 25.0[a]	215 ± 3.5[b]	33.2 ± 3.3[c]	28.1 ± 2.6[c]
Palmitate	0.5	45.6 ± 3.4	46.2 ± 0.78	50.0 ± 3.2	53.0 ± 2.6
	2.0	75.8 ± 8.4	79.7 ± 0.73	85.8 ± 2.3	90.1 ± 1.3
Glucose	5.0	868 ± 34.6[a]	407 ± 20.5[b]	107 ± 5.8[c]	110 ± 6.7[c]
Propionate	0.5	15.4 ± 1.7[a]	11.9 ± 0.50[b]	9.90 ± 0.30[c]	6.70 ± 0.24[d]
	2.0	21.8 ± 0.58[a]	17.4 ± 0.60[b]	14.9 ± 0.35[c]	11.1 ± 0.31[d]
	5.0	37.4 ± 3.5[a]	22.1 ± 0.44[b]	19.6 ± 0.56[c]	15.7 ± 0.33[d]
Butyrate	0.5	10.7 ± 0.44[a]	9.10 ± 0.43[b]	8.20 ± 0.67[b]	6.20 ± 0.39[c]
	2.0	18.2 ± 1.2[a]	14.5 ± 0.33[b]	13.4 ± 0.77[b]	9.90 ± 0.34 [c]
	5.0	24.0 ± 1.5[a]	19.2 ± 0.55[b]	17.5 ± 0.50[b]	14.2 ± 0.87[c]

[1]Data, expressed as nmol ATP/10^6 cell per 30 min, are means ± SEM, n = 6 pigs/age group. Enterocytes were incubated at 37°C for 30 min in the KHB buffer, as described in text. The medium contained 5 mM D-glucose and one of the following: 0.5-5.0 mM [U-^{14}C]glutamate, 0.5-5.0 mM [U-^{14}C]glutamine, 0.5-5.0 mM [U-^{14}C]aspartate, 0.5-5.0 mM [U-^{14}C]alanine, 0.5-2.0 mM [U-^{14}C]palmitate, 0.5-5.0 mM [U-^{14}C]propionate, and 0.5-5.0 mM [1-^{14}C]butyrate. The rate of oxidation of each substrate increased ($P < 0.05$) with increasing extracellular concentration from 0.5 to 5 mM.
a-d: Within a row, means not sharing the same superscript letters differ ($P < 0.05$).

Production of nitrogenous metabolites from amino acids. Data on the production of nitrogenous metabolites from amino acids are summarized in Table 4.3. Alanine and aspartate were two amino acids produced from glutamate in enterocytes of 0- to 21-day-old pigs. No other amino acids (including glutamine and asparagine) were produced from glutamate in the cells incubated in the absence of ammonia. Rates of production of alanine and aspartate from glutamate by enterocytes from all age groups of pigs increased ($P < 0.05$) progressively with increasing the extracellular concentration of glutamate from 0.5 mM to 5 mM. Compared with 0-day-old pigs, the rates of production of both alanine and aspartate from 0.5 to 5 mM glutamate by enterocytes decreased ($P < 0.05$) in 21-day-old pigs.

In enterocytes from 0- to 21-day-old pigs, NH_3 was produced from glutamine but not from other substrates. The major amino acid formed from glutamine was glutamate, followed by alanine and then aspartate. As reported previously (Wu et al. 1994), ornithine, citrulline, proline, and arginine were produced from glutamine in pig enterocytes, and these data are not presented herein. No other amino acids were produced from glutamine in the cells. Rates of production of NH_3, glutamate, and alanine from glutamine in enterocytes from all age groups of pigs increased ($P < 0.01$) progressively with increasing the extracellular concentration of glutamine from 0.5 mM to 5 mM. The rates of production of alanine and aspartate from 0.5 and 2 mM glutamine were greater ($P < 0.05$) in enterocytes from 0-day-old pigs, compared with 7- to 21-day-old pigs. The rate of ammonia production from glutamine by enterocytes decreased ($P < 0.01$) as pigs grew. The rate of production of glutamate from 0.5 mM glutamine by enterocytes from 21-day-old pigs decreased ($P < 0.05$) in comparison with 0-day-old pigs. At 0.5, 2, and 5 mM glutamate, the rates of production of aspartate and alanine from glutamate by enterocytes decreased ($P < 0.01$) as pigs grew.

Catabolism of aspartate in enterocytes from 0- to-21-day-old pigs resulted in the production of primarily alanine and, to a lesser extent, glutamate. No other amino acids were produced from aspartate in the cells. Rates of production of alanine and glutamate from aspartate in enterocytes from all age groups of pigs increased ($P < 0.05$) progressively with increasing the extracellular concentration of aspartate from 0.5 to 5 mM. Compared with 0-day-old pigs, the rates of production of both alanine and glutamate from aspartate by enterocytes decreased ($P < 0.05$) in 21-day-old pigs.

Table 4.3. Production of nitrogenous metabolites from amino acids in pig enterocytes[1]

Substrate in medium	Concen-tration (mM)	Metabolites	Age of pigs (days)			
			0	7	14	21
Glutamate	0.5	Ammonia	0[b]	0.52 ± 0.11[a]	0.48 ± 0.08[a]	0.35 ± 0.07[a]
	2.0		0[b]	0.72 ± 0.08[a]	0.68 ± 0.10[a]	0.63 ± 0.09[a]
	5.0		0[b]	0.99 ± 0.09[a]	0.94 ± 0.11[a]	0.87 ± 0.05[a]
	0.5	Aspartate	15.8 ± 1.4[a]	8.60 ± 0.30[b]	7.71 ± 0.27[a]	6.15 ± 0.60[a]
	2.0		19.3 ± 0.55[a]	16.7 ± 1.1[a]	13.1 ± 1.2[b]	9.22 ± 0.60[c]
	5.0		25.3 ± 1.3[a]	19.8 ± 1.1[b]	15.9 ± 1.3[c]	13.0 ± 1.0[c]
	0.5	Alanine	30.3 ± 3.3[a]	20.8 ± 1.7[b]	13.0 ± 0.25[c]	12.2 ± 1.0[c]
	2.0		43.9 ± 3.5[a]	30.2 ± 1.3[b]	24.8 ± 0.49[c]	21.1 ± 1.0[c]
	5.0		51.8 ± 3.4[a]	40.0 ± 2.8[b]	32.4 ± 0.92[c]	27.3 ± 0.86[c]
Glutamine	0.5	Ammonia	98.2 ± 5.0[a]	51.7 ± 1.4[b]	40.8 ± 0.43[c]	27.2 ± 1.1[d]
	2.0		137 ± 2.8[a]	77.6 ± 2.7[b]	58.8 ± 2.5[c]	47.8 ± 1.3[d]
	5.0		200 ± 7.9[a]	125 ± 5.4[b]	87.6 ± 2.6[c]	77.7 ± 1.9[d]
	0.5	Glutamate	59.9 ± 3.5[a]	20.4 ± 1.1[b]	18.7 ± 0.44[b]	15.3 ± 0.48[b]
	2.0		78.4 ± 3.8[a]	31.0 ± 2.1[b]	29.6 ± 1.3[b]	27.3 ± 0.85[b]
	5.0		99.2 ± 3.0[a]	58.7 ± 5.5[b]	51.3 ± 1.9[b]	49.9 ± 1.6[b]
	0.5	Aspartate	11.2 ± 0.51[a]	6.24 ± 0.24[b]	5.88 ± 0.44[b]	4.35 ± 0.27[b]
	2.0		13.9 ± 0.83[a]	8.04 ± 0.48[b]	7.14 ± 0.36[b]	6.31 ± 0.19[b]
	5.0		17.0 ± 1.5[a]	10.8 ± 0.12[b]	9.32 ± 0.69[b]	8.49 ± 0.21[b]
	0.5	Alanine	24.2 ± 1.6[a]	7.11 ± 0.29[b]	6.35 ± 0.26[b]	5.38 ± 0.32[b]
	2.0		34.9 ± 1.7[a]	10.9 ± 0.38[b]	9.92 ± 0.38[b]	8.40 ± 0.61[b]
	5.0		41.2 ± 2.8[a]	17.0 ± 0.71[b]	14.7 ± 0.50[b]	12.6 ± 1.1[b]
Aspartate	0.5	Glutamate	8.11 ± 0.59[a]	7.25 ± 0.36[a]	6.64 ± 0.38[a]	6.32 ± 0.17[b]
	2.0		13.8 ± 0.95[a]	12.6 ± 0.85[a]	11.5 ± 0.67[a]	7.66 ± 0.43[b]
	5.0		19.3 ± 1.2[a]	17.5 ± 1.2[a]	15.8 ± 1.0[a]	12.9 ± 0.58[b]
	0.5	Alanine	16.5 ± 1.1[a]	13.3 ± 0.63[a]	11.6 ± 0.97[ab]	10.7 ± 0.29[b]
	2.0		22.3 ± 1.1[a]	21.0 ± 1.1[a]	17.2 ± 0.68[ab]	14.4 ± 1.0[b]
	5.0		32.7 ± 1.2[a]	28.2 ± 0.74[a]	25.2 ± 1.2a[b]	22.5 ± 0.69[b]
Alanine	0.5	Glutamate	11.5 ± 1.1[a]	7.88 ± 0.39[b]	6.54 ± 0.31[b]	5.79 ± 0.25[b]
	2.0		14.3 ± 1.2[a]	10.3 ± 0.33[b]	8.31 ± 0.51[c]	7.27 ± 0.17[c]
	5.0		17.5 ± 0.63[a]	15.6 ± 0.48[a]	12.7 ± 0.92[b]	10.7 ± 0.51[c]
	0.5	Aspartate	5.85 ± 0.24[a]	5.52 ± 0.12[a]	3.90 ± 0.34[b]	2.95 ± 0.16[c]
	2.0		6.97 ± 0.39[a]	6.03 ± 0.45[ab]	5.19 ± 0.22[bc]	4.50 ± 0.16[c]
	5.0		7.53 ± 0.44[a]	7.10 ± 0.21[ab]	6.45 ± 0.22[bc]	5.71 ± 0.11[c]

[1]Data, expressed as nmol product/10^6 cell per 30 min, are means ± SEM, n = 6 pigs/age group. Enterocytes were incubated at 37°C for 30 min in the KHB buffer, as described in text. The medium contained 5 mM D-glucose and one of the following: 0.5-5.0 mM [U-^{14}C]glutamate, 0.5-5.0 mM [U-^{14}C]glutamine, 0.5-5.0 mM [U-^{14}C]aspartate, 0.5-5.0 mM [U-^{14}C]alanine, 0.5-2.0 mM [U-^{14}C]palmitate, 0.5-5.0 mM [U-^{14}C]propionate, and 0.5-5.0 mM [1-^{14}C]butyrate. There was no production of ammonia from glutamate, alanine or aspartate from pig enterocytes. The rate of production of metabolites from each substrate increased ($P < 0.05$) with increasing extracellular concentration from 0.5 to 5 mM.
a-d: Within a row, means not sharing the same superscript letters differ ($P < 0.05$).

Production of lactate and pyruvate from glucose. The rate of production of lactate from 5 mM glucose (Table 4.4) was approximately 10 times that for pyruvate (Table 4.5) in enterocytes from 0- to 21-day-old pigs. Increasing the extracellular concentrations of alanine, palmitate, propionate or butyrate from 0.5 to 5 mM did not affect ($P > 0.05$) the rates of production of lactate or pyruvate from glucose. However, glutamate, glutamine, and aspartate decreased ($P > 0.05$) the rates of production of lactate or pyruvate from glucose by enterocytes of pigs at each age. Increasing the extracellular concentrations of glutamate, glutamine, and aspartate from 0.5 to 5 mM decreased ($P > 0.05$) the rates of production of lactate or pyruvate from glucose by enterocytes of pigs at each age. The rates of production of lactate or pyruvate decreased by enterocytes from 0- and 7-day-old pigs, did not differ ($P > 0.05$) between 7- and 14-day-old pigs, and increased ($P < 0.05$) thereafter in 21-day-old pigs by 146% and 181%, respectively compared with 14-day-old pigs.

Table 4.4. Production of lactate by pig enterocytes in the presence of 5 mM glucose[1]

Substrate in medium	Concen-tration (mM)	Age of pigs (days)			
		0	7	14	21
Glutamate	0.5	48.6 ± 1.0[c]	83.2 ± 4.2[b]	79.4 ± 2.3[b]	198 ± 5.5[a]
	2.0	45.1 ± 2.1[c]	81.7 ± 4.5[b]	76.1 ± 2.7[b]	200 ± 5.5[a]
	5.0	43.6 ± 1.0[c]	78.8 ± 5.1[b]	72.1 ± 1.7[b]	197 ± 4.0[a]
Glutamine	0.5	48.6 ± 2.7[c]	82.9 ± 4.7[b]	78.1 ± 3.0[b]	198 ± 6.8[a]
	2.0	47.0 ± 2.4[c]	81.6 ± 7.1[b]	75.4 ± 3.2[b]	200 ± 9.5[a]
	5.0	43.9 ± 2.1[c]	78.5 ± 1.5[b]	72.5 ± 1.9[b]	196 ± 5.1[a]
Aspartate	0.5	50.0 ± 1.6[c]	85.3 ± 1.9[b]	80.4 ± 2.3[b]	202 ± 6.9[a]
	2.0	47.5 ± 2.9[c]	82.3 ± 1.5[b]	75.1 ± 1.6[b]	206 ± 4.3[a]
	5.0	44.5 ± 0.53[c]	80.6 ± 3.5[b]	72.9 ± 0.92[b]	203 ± 2.6[a]
Alanine	0.5	52.5 ± 1.8[c]	90.8 ± 9.3[b]	85.3 ± 1.8[b]	210 ± 7.2[a]
	2.0	49.2 ± 2.7[c]	85.1 ± 3.1[b]	85.8 ± 1.1[b]	208 ± 4.9[a]
	5.0	45.9 ± 1.1[c]	83.9 ± 4.4[b]	84.7 ± 2.7[b]	204 ± 6.4[a]
Palmitate	0.5	177 ± 3.2[b]	95.5 ± 3.3[c]	89.9 ± 3.3[c]	216 ± 11.3[a]
	2.0	174 ± 10.4[b]	93.6 ± 1.6[c]	85.9 ± 3.1[c]	223 ± 7.1[a]
Glucose	5.0	173 ± 16.2[b]	96.3 ± 3.7[c]	89.3 ± 3.5[c]	220 ± 8.6[a]
Propionate	0.5	172 ± 4.1[b]	95.3 ± 5.6[c]	88.8 ± 6.5[c]	217 ± 3.7[a]
	2.0	176 ± 9.1[b]	96.6 ± 5.2[c]	87.6 ± 2.2[c]	220 ± 9.8[a]
	5.0	171 ± 9.2[b]	94.2 ± 2.3[c]	86.6 ± 2.2[c]	221 ± 7.0[a]
Butyrate	0.5	170 ± 6.2[b]	97.1 ± 6.6[c]	87.8 ± 3.6[c]	214 ± 13.6[a]
	2.0	172 ± 6.1[b]	97.1 ± 4.1[c]	87.1 ± 6.3[c]	218 ± 14.8[a]
	5.0	171 ± 7.9[b]	97.8 ± 3.1[c]	85.9 ± 2.6[c]	215 ± 6.6[a]

[1]Data, expressed as nmol lactate/10^6 cell per 30 min, are means ± SEM, n = 6 pigs/age group. Enterocytes were incubated at 37°C for 30 min in the KHB buffer, as described in text. The medium contained 5 mM D-glucose and one of the following: 0.5-5.0 mM [U-^{14}C]glutamate, 0.5-5.0 mM [U-^{14}C]glutamine, 0.5-5.0 mM [U-^{14}C]aspartate, 0.5-5.0 mM [U-^{14}C]alanine, 0.5-2.0 mM [U-^{14}C]palmitate, 0.5-5.0 mM [U-^{14}C]propionate, and 0.5-5.0 mM [1-^{14}C]butyrate.
a-d: Within a row, means not sharing the same superscript letters differ ($P < 0.05$).

Table 4.5. Production of pyruvate by pig enterocytes in the presence of 5 mM glucose[1]

Substrate in medium	Concentration (mM)	Age of pigs (days)			
		0	7	14	21
Glutamate	0.5	4.31 ± 0.39[c]	7.90 ± 0.61[b]	7.30 ± 0.62[b]	19.2 ± 1.0[a]
	2.0	4.07 ± 0.25[c]	7.51 ± 0.85[b]	7.00 ± 0.74[b]	19.2 ± 1.4[a]
	5.0	3.63 ± 0.40[c]	7.46 ± 0.52[b]	6.71 ± 0.39[b]	19.5 ± 1.7[a]
Glutamine	0.5	4.47 ± 0.35[c]	8.32 ± 0.45[b]	7.04 ± 0.43[b]	19.6 ± 1.9[a]
	2.0	4.07 ± 0.34[c]	7.08 ± 0.18[b]	6.53 ± 0.59[b]	18.8 ± 1.3[a]
	5.0	3.81 ± 0.29[c]	7.37 ± 0.46[b]	7.26 ± 0.43[b]	18.7 ± 1.9[a]
Aspartate	0.5	4.43 ± 0.33[c]	8.49 ± 0.84[b]	7.35 ± 0.44[b]	19.0 ± 0.60[a]
	2.0	4.19 ± 0.38[c]	6.35 ± 0.58[b]	6.77 ± 0.62[b]	18.7 ± 1.4[a]
	5.0	4.07 ± 0.45[c]	7.43 ± 0.41[b]	6.99 ± 0.52[b]	18.0 ± 1.0[a]
Alanine	0.5	4.56 ± 0.39[c]	7.74 ± 0.42[b]	7.15 ± 0.72[b]	19.3 ± 0.80[a]
	2.0	5.17 ± 1.12[c]	6.97 ± 0.33[b]	6.68 ± 0.65[b]	20.3 ± 1.6[a]
	5.0	4.80 ± 1.03[c]	7.82 ± 0.34[b]	6.96 ± 0.40[b]	18.3 ± 1.2[a]
Palmitate	0.5	16.4 ± 0.87[b]	9.05 ± 0.64[c]	8.70 ± 0.83[c]	23.6 ± 2.0[a]
	2.0	22.5 ± 2.2[b]	9.61 ± 0.52[c]	8.79 ± 0.60[c]	23.0 ± 1.7[a]
Glucose	5.0	17.6 ± 1.7[b]	9.13 ± 0.57[c]	8.19 ± 0.87[c]	23.0 ± 1.6[a]
Propionate	0.5	17.6 ± 1.4[b]	9.48 ± 0.76[c]	8.60 ± 0.65[c]	21.9 ± 2.1[a]
	2.0	18.8 ± 1.4[b]	9.12 ± 0.61[c]	8.83 ± 0.11[c]	20.7 ± 2.0[a]
	5.0	17.4 ± 1.0[b]	9.06 ± 0.69[c]	7.87 ± 0.71[c]	22.5 ± 2.2[a]
Butyrate	0.5	17.6 ± 0.92[b]	9.00 ± 0.91[c]	8.59 ± 0.27[c]	20.4 ± 2.1[a]
	2.0	18.1 ± 1.7[b]	8.93 ± 0.53[c]	8.69 ± 0.48[c]	21.1 ± 2.0[a]
	5.0	16.5 ± 1.0[b]	8.78 ± 0.33[c]	7.98 ± 0.66[c]	22.8 ± 2.0[a]

[1]Data, expressed as nmol pyruvate/10^6 cell per 30 min, are means ± SEM, n = 6 pigs/age group. Enterocytes were incubated at 37°C for 30 min in the KHB buffer, as described in text. The medium contained 5 mM D-glucose and one of the following: 0.5-5.0 mM [U-^{14}C]glutamate, 0.5-5.0 mM [U-^{14}C]glutamine, 0.5-5.0 mM [U-^{14}C]aspartate, 0.5-5.0 mM [U-^{14}C]alanine, 0.5-2.0 mM [U-^{14}C]palmitate, 0.5-5.0 mM [U-^{14}C]propionate, and 0.5-5.0 mM [1-^{14}C]butyrate.

a-d: Within a row, means not sharing the same superscript letters differ ($P < 0.05$).

Utilization of amino acids. Rates of utilization of glutamate, glutamine, aspartate and alanine by enterocytes from all age groups of pigs increased ($P < 0.05$) progressively with increasing their extracellular concentrations from 0.5 mM to 5 mM (Table 4.6). About 39% of utilized glutamate, 22% of utilized glutamine, 38% of utilized aspartate, and 22% of utilized glucose were oxidized to CO_2 in cells from 0-day-old chickens. The corresponding values were reduced to 14% for glutamate, 7% for glutamine, 8% for aspartate, and 3% for glucose for enterocytes from 21-day-old pigs.

In cells from 0- to 21-day-old pigs, the rate of utilization of glutamine was the highest among all the tested amino acid substrates, followed by glutamate, aspartate, and alanine. The rates of utilization of 5 mM glutamate, 5 mM glutamine, and 5 mM aspartate by enterocytes decreased ($P < 0.05$) progressively with increasing the age of pigs from 0 to 21 days. The rate of utilization of 0.5 and 2 mM alanine decreased ($P < 0.05$) as pigs grew from 0- to 14-day-old, and did not differ ($P > 0.05$) between 14- and 21-day-old pigs.

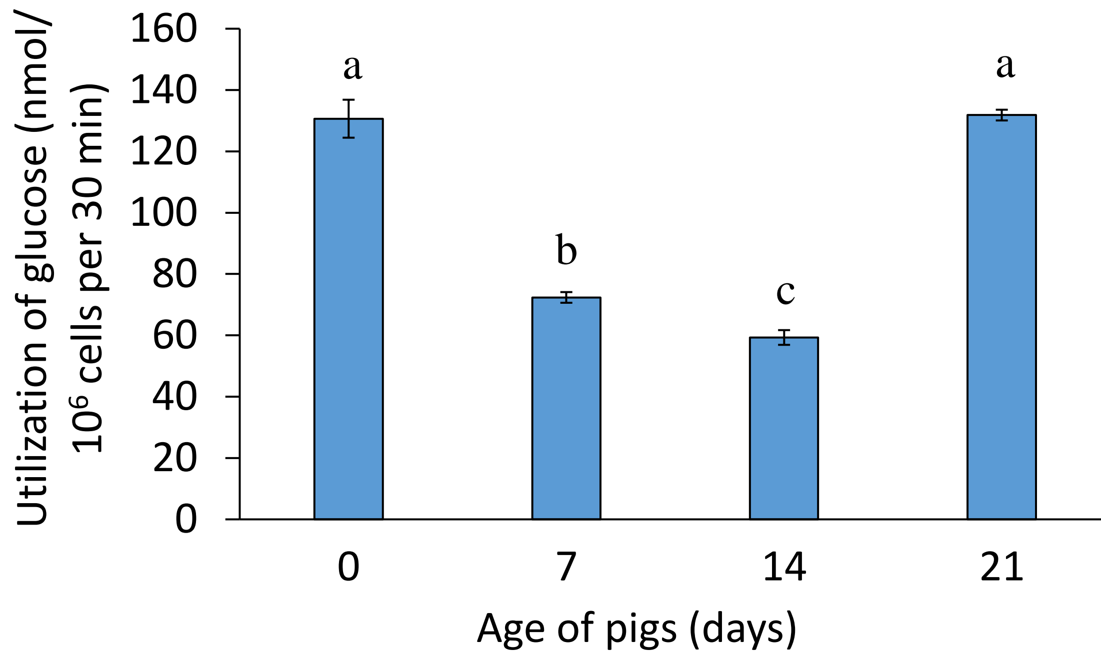
Table 4.6. Utilization of amino acid substrates by pig enterocytes[1]

Substrate in medium	Concentration (mM)	Age of pigs (days)			
		0	7	14	21
Glutamate	0.5	75.7 ± 5.9[a]	60.2 ± 3.5[b]	33.5 ± 1.3[c]	29.8 ± 2.5[c]
	2.0	114 ± 6.0[a]	85.4 ± 3.9[b]	61.5 ± 1.9[c]	41.8 ± 3.9[d]
	5.0	152 ± 8.4[a]	118 ± 7.7[b]	72.0 ± 3.5[c]	56.3 ± 5.2[d]
Glutamine	0.5	122 ± 1.4[a]	58.2 ± 4.2[b]	43.9 ± 2.5[c]	31.8 ± 2.7[d]
	2.0	181 ± 6.5[a]	85.1 ± 6.0[b]	63.3 ± 5.0[c]	53.5 ± 2.5[d]
	5.0	221 ± 6.9[a]	138 ± 10.1[b]	97.0 ± 4.4[c]	83.3 ± 3.4[d]
Aspartate	0.5	56.3 ± 3.0[a]	37.1 ± 2.0[b]	24.5 ± 2.3[c]	20.0 ± 1.4[c]
	2.0	86.2 ± 6.5[a]	65.2 ± 4.1[b]	43.9 ± 4.0[c]	32.9 ± 2.6[d]
	5.0	110 ± 5.0[a]	88.4 ± 5.5[b]	55.1 ± 2.8[c]	47.9 ± 3.3[c]
Alanine	0.5	31.8 ± 1.1[a]	24.5 ± 1.1[b]	17.0 ± 1.2[c]	14.6 ± 0.79[c]
	2.0	48.9 ± 1.8[a]	34.9 ± 1.2[b]	22.0 ± 0.82[c]	18.6 ± 1.2[c]
	5.0	71.0 ± 2.4[a]	54.2 ± 2.3[b]	30.4 ± 1.2[c]	24.7 ± 1.5[c]

[1]Data, expressed as nmol/10^6 cell per 30 min, are means ± SEM, n = 6 pigs/age group. Enterocytes were incubated at 37°C for 30 min in the KHB buffer, as described in text. The medium contained 5 mM D-glucose and one of the following: 0.5-5.0 mM [U-^{14}C]glutamate, 0.5-5.0 mM [U-^{14}C]glutamine, 0.5-5.0 mM [U-^{14}C]aspartate, and 0.5-5.0 mM [U-^{14}C]alanine. a-d: Within a row, means not sharing the same superscript letters differ ($P < 0.05$).

Utilization of glucose. The rates of glucose utilization by enterocytes from 0-, 7-, 14-, and 21-day-old pigs are shown in Figure 2.1. The rate of utilization of glucose by enterocytes decreased ($P < 0.05$) by 45% in 7-day-old versus 0-day-old pigs, decreased ($P > 0.05$) by 18% in 14-day-old versus 7-day-old pigs, and increased ($P > 0.05$) thereafter by 122% between 14- and 21-day-old pigs.

Figure 4.1. Utilization of glucose by pig enterocytes in the presence of 5 mM glucose[1]



[1]Data, expressed as nmol glucose/10^6 cell per 30 min, are means ± SEM, n = 6 pigs/age group. Enterocytes were incubated at 37°C for 30 min in the KHB buffer, as described in text. The medium contained 5 mM D-glucose.
a-c: Means not sharing the same superscript letters differ ($P < 0.05$).

Enzyme activities. Activities of key enzymes involved in the metabolism of glutamate, glutamine, aspartate, alanine, glucose and fatty acids in pig enterocytes are summarized in Table 4.7. Glutamate dehydrogenase activity was very low in the cells from 0- to 21-day-old pigs. There were high glutaminase, GOT, and GPT activities in pig enterocytes. The activity of glutamine synthetase was low in enterocytes of pigs at each postnatal age. The activities of GOT and GPT in the cells did not differ ($P > 0.05$) between 0- and 7-day-old pigs, decreased ($P < 0.05$) thereafter in 21-day-old pigs. In contrast, glutaminase activity decreased ($P < 0.05$) between 0 and 7 days of age and remained at the elevated level in 21-day-old pigs.

The activities of PFK and pyruvate kinase were high in enterocytes from 0- to 21-day-old pigs, whereas the hexokinase activity was lower. The activities of hexokinase, pyruvate kinase, and PFK increased ($P < 0.05$) by 184%, 96%, and 74% in 7-day-old versus 0-day-old pigs, decreased ($P < 0.05$) by 60%, 63%, and 58% between 7- and 14-day-old pigs, increased ($P < 0.05$) thereafter in 21-day-old pigs compared with 14-day-old pigs. The activity of CPT-I in enterocytes increased ($P < 0.05$) progressively with increasing the age of pigs from 0 to 21 days.

Table 4.7. Maximal activities of some key enzymes in carbohydrate and amino acid metabolism as well as the transport of long-chain fatty acids in pig enterocytes[1]

Enzyme	Age of pigs (days)			
	0	7	14	21
	nmol/min/mg protein			
GDH	0.55 ± 0.04[b]	1.15 ± 0.13[a]	1.28 ± 0.22[a]	1.08 ± 0.17[a]
GOT	23.3 ± 1.9[a]	21.7 ± 0.40[a]	14.7 ± 1.1[b]	6.71 ± 0.28[c]
GPT	8.19 ± 0.58[a]	7.85 ± 0.33[ab]	7.37 ± 0.68[b]	2.55 ± 0.10[c]
PAG	45.2 ± 2.9[a]	26.3 ± 1.6[b]	23.9 ± 1.1[b]	24.4 ± 1.8[b]
GS	0.41 ± 0.11[b]	0.40 ± 0.05[b]	0.53 ± 0.07[a]	0.66 ± 0.06[a]
Hexokinase	1.64 ± 0.14[d]	4.66 ± 0.25[a]	1.90 ± 0.09[c]	3.23 ± 0.47[b]
PFK	17.1 ± 1.3[b]	29.7 ± 1.0[a]	12.5 ± 1.7[c]	16.4 ± 1.3[b]
Pyruvate kinase	3.67 ± 0.42[b]	7.18 ± 0.64[a]	2.63 ± 0.23[c]	3.57 ± 0.38[b]
CPT-I	0.71 ± 0.02[d]	1.06 ± 0.02[c]	1.69 ± 0.02[b]	1.84 ± 0.05[a]

[1]Data, expressed as nmol/min/mg protein, are means ± SEM, n = 6 pigs/age group.
a-c: Within a row, means not sharing the same superscript letters differ ($P < 0.05$).
CPT-I = carnitine palmitoyltransfrease I; GDH = glutamate dehydrogenase; GOT = glutamate-oxaloacetate transaminase; GPT = glutamate-pyruvate transaminase; GS = glutamine synthetase; PAG = phosphate-activated glutaminase; PFK = phosphofructose kinase.

Discussion

Metabolic fates of amino acids, glucose, and fatty acids in pig enterocytes. This research quantified the metabolic fates of amino acids, glucose, and fatty acids in enterocytes of developing pigs, and evaluated the contributions of the substrates to ATP production by these cells. Our results indicated that in pig enterocytes, 1) CO_2, aspartate, and alanine were the major metabolites of glutamate, 2) CO_2, glutamate, and alanine were the major metabolites of aspartate, and 3) ammonia, CO_2, glutamate, aspartate and alanine were the major metabolites of glutamine. Our results indicate that the oxidation of glutamate and glutamine accounted for 11~49% and 6~28% of the total utilized glutamate and glutamine, respectively, in enterocytes of 0- to 21-day-old pig. The finding that there was no formation of ammonia from glutamate, alanine and aspartate in pig enterocytes is consistent with the very low activity of GDH from the cells and indicates that transamination via high activities of GPT and GOT initiated the catabolism of these three amino acids. Of note, the rate of glutamine utilization was similar to those of ammonia production, which indicated that glutamine oxidation was initiated by glutaminase to form glutamate and ammonia, followed by the degradation of glutamate to α-ketoglutarate via transamination.

Previous work has shown that compared with glucose, glutamine was a preferential energy substrate for enterocytes of 0- to 21-day-old pigs (Wu 1995). This conclusion was re-evaluated in the present study. Surprisingly, glutamate was oxidized to CO2 at a greater rate than the same concentration of glutamine in piglet enterocytes during their postnatal growth (Table 4.1). The rate of ATP production from glutamate is 13 to 20% higher than that from glutamine by enterocytes of pig on each day after birth (Table 4.2). Overall, glutamate and glutamine were the two leading metabolic fuels among all tested substrates for enterocytes of pigs, which is

consistent with the data on GPT, GOT, and glutaminase activities in these cells (Table 4.7). Due to the high rates of productions of glutamate, aspartate, and alanine from glutamine in pig enterocytes, the concentration of glutamine in the plasma of pigs and humans is about half of that in chickens (Wu 2018). In addition, the pattern of catabolism of glutamine differed from that of glutamate. Specifically, 17 ~ 32% of the utilized glutamine was oxidized to CO_2 in enterocytes of 0-day-old and 7-day-old pigs, while only 6 ~ 9% of the utilized glutamine was oxidized to CO_2 in enterocytes of 14- to 21-day-old pigs (Table 4.1, 4.6). These results suggest the compartmentation of intracellular glutamine degradation in that mitochondrially-derived glutamate via glutaminase may not be coupled efficiently with glutamate transaminase in the mitochondria. The carbon skeleton of glutamine can be spared for the synthesis of glutamate, aspartate, and alanine, as the production of glutamate, glutamine, and aspartate accounted for 70 to 90% of the glutamine utilized by pig enterocytes (Tables 4.3 and 4.6). The rate of production of alanine from glutamine by enterocytes of 0- to 21-day-old pig was ~2 times that of aspartate, suggesting that the oxidation of glutamine-derived glutamate was initiated mainly by glutamate-pyruvate transaminase rather than glutamate-oxaloacetate transaminase. These metabolic data are again consistent with the results on the activities of these two enzymes in pig enterocytes (Table 4.7).

Glucose is known to be actively utilized by many tissues of all animals. This is achieved because of the wide distribution of diverse glucose transporters in different animal tissues [e.g., GLUT4 in skeletal muscle (except for chickens), heart, and adipose tissue; and GLUT2 in the small intestine, liver and pancreas]. We observed a high rate of lactate production as well as high activities of hexokinase, phosphofructokinase and pyruvate kinase in enterocytes of pigs at each age, further sustaining a high rate of glycolysis in these cells. Glutamate, glutamine, aspartate,

and alanine reduced markedly the production of lactate and pyruvate from glucose perhaps via different mechanisms, and ultimately spared glucose. It is possible that the extensive oxidation of glutamate, glutamine, aspartate generates citrate [which inhibits PFK, a key enzyme of glycolysis (Wu 2018)] and that the formation of alanine from glutamate, glutamine, aspartate yields alanine [which inhibits pyruvate kinase, another key enzyme of glycolysis (Wu 2018)]. Enhancing the extracellular concentration of alanine can increase its intracellular concentration, leading to an inhibition of pyruvate kinase and therefore, the production of lactate and pyruvate from glucose.

It appears that glucose-derived pyruvate (occurring in the cytosol) is preferentially converted into lactate locally by lactate dehydrogenase rather than being transported into the mitochondria possibly due to low expression of mitochondrial pyruvate transporter. In contrast, the metabolism of glutamate in the mitochondria yields pyruvate that may be directly decarboxylated into acetyl-CoA by the mitochondrial pyruvate dehydrogenase. Likewise, glutamine-derived pyruvate (occurring in the mitochondria) may be directly channeled to the mitochondrial pyruvate dehydrogenase for the production of acetyl-CoA. Collectively, these findings indicate that the oxidation of glucose is limited in pig enterocytes and also suggest compartmentation of pyruvate metabolism in these cells.

A novel finding of this study is that among all the potential energy substrates, the oxidation of short- and long-chain fatty acids in enterocytes of 0- to 21-day-old pigs was very limited (Table 4.1). Long-chain fatty acids are activated by the cytosolic acyl-CoA synthase to form acyl-CoAs, which enter the mitochondria via the CPT transport system. The low rate of oxidation of fatty acids in enterocytes allows efficient absorption of dietary oils into the lymphatic vessel. Interestingly, oxidation of short-chain fatty acids (propionate and butyrate)

was also limited in pig enterocytes (Table 4.1). It is possible that acyl-CoA synthase activity for short-chain fatty acids is low in pig enterocytes, thereby limiting the activation of short-chain fatty acids into acyl-CoAs for oxidation in the mitochondria. Taken together, fatty acids are not major metabolic fuels for those cells.

Developmental changes of amino acid, glucose, and fatty acid metabolism in pig enterocytes. Results of the present study revealed developmental changes in the metabolism of amino acids, glucose, and fatty acids in pig enterocytes. Glutamate, glutamine and aspartate were extensively oxidized by enterocytes of pigs in each age group. As pigs grew from 0 to 21 days of age, the rates of oxidation of glutamate and glutamine by enterocytes decreased by 87% (Table 4.1). This is consistent with the decreases in the rates of utilization of glutamine and glutamate by 65-75% and 60%, respectively (Table 4.6), in the rates of production of glutamate, aspartate, and alanine from glutamine by 55~75%, and in the rates of production of aspartate and alanine from glutamate by 65~75% (Table 4.3). There was little change in the rates of alanine oxidation by enterocytes between 0- and 21-day-old chickens (Table 4.1). However, when enterocytes were incubated with 0.5 to 5 mM of aspartate, its oxidation decreased by 90% in 21-day-old vs 0-day-old pigs. Rates of alanine utilization by these cells decreased by 45~50% as pigs grew. These results are consistent with the large changes in activities of glutaminase, GPT and GOT in the cells between 0 and 21 days of age.

Compared with 0-day-old pigs, the rate of oxidation of glucose by enterocytes of 21-day-old pigs decreased by 87% (Table 4.1), while both rates of production of lactate and pyruvate by these cells increased by 22% (Tables 4.4 and 4.5). Thus, as pigs grew during the suckling period, less glucose carbons were irreversibly oxidized to CO_2 and more glucose carbons were

conserved as lactate, corresponding to decreasing metabolic rate. Overall, the rate of glucose utilization by enterocytes did not differ between 0 and 21 days of age.

In contrast to amino acids and glucose, pig enterocytes had a limited ability to oxidize short- and long-chain fatty acids to CO_2. As pig grew from 0 to 21 days of age, the rate of oxidation of the fatty acids by the cells changed little (Table 4.1). These nutrients contribute only a small amount of energy to enterocytes in any developmental stage.

Energetic roles of amino acids, glucose, and fatty acids in pig enterocytes. A salient observation of the present study is that glutamate was quantitatively the most important metabolic fuel for enterocytes in all age groups of pig. For 0-day-old pigs, the contribution of glutamate and glutamine to ATP production by enterocytes accounted for 60~70% of total ATP production by all the tested substrates (Table 4.2). Consistent with an age-dependent decrease in metabolic rate, the contribution of these three amino acids to ATP production by enterocytes also decreased with age (e.g., by more than 88% in 21-day-old pigs, compared with 0-day-old pigs) (Table 4.2). Aspartate contributed much less ATP to enterocytes at each age than glutamate or glutamine. Particularly, the rate of ATP production from glutamate and glutamine by enterocytes of 0-day-old pigs was 2 and 1.8 times, respectively, that from aspartate. Together, glutamate plus glutamine plus aspartate provide 70-80% of energy to the cells from 0- to 21-day-old pigs.

Glucose is a significant energy substrate for pig enterocytes at each age. The rate of ATP production from the oxidation of glucose to CO_2 by the cells was the third highest among all the tested substrates. In contrast, the roles of propionate and butyrate as energy substrates were minor for enterocytes, and these two short-chain fatty acids are negligible in sow's milk. (Table 4.2). Interestingly, although CPT-I activity in enterocytes increased between 0 and 14 days of age (Table 4.7), the rate of oxidation of palmitate by the cells did not change during this

postnatal period likely because a low activity of cytosolic acyl-CoA synthase. Compared with glutamate, glutamine, aspartate and glucose, palmitate was also a minor source of energy for enterocytes of 0- to 21-day-old pigs.

Physiological significance of amino acids in pig enterocytes. Glutamate, glutamine and aspartate not only serve as important energy substrates for pig enterocytes, but also contribute carbon and nitrogen precursors to biosynthetic processes (Wu 2013). The rates of oxidation of glutamate and glutamine by the enterocytes of 0- to 21-day-old pigs were high (Table 4.1). This finding confirms the previous reports that these two amino acids were important energy sources for the cells (Blachier et al. 1999; Wu 1995). Furthermore, glutamate and glutamine are important substrates for the synthesis of glutathione and alanine in the pig small intestine (Hou and Wu 2018). These physiological processes account for the extensive utilizations of glutamate and glutamine by pig enterocytes. Thus, although glutamate is the most abundant amino acid in the diets of pigs, glutamate is among the least abundant amino acids in their plasma (50-100 μM) (Wu 2018). Likewise, due to the extensively oxidation of glutamine by pig enterocytes, dietary supplementation with 1% glutamine increases its concentration in plasma by only 46% at 1 h after feeding (Wang et al. 2008). Moreover, the high rate of catabolism of aspartate by enterocytes results in a low concentration of aspartate (~15 μM) in the plasma of pigs, compared with a much higher concentration of aspartate (~100 μM) in the plasma of chickens whose enterocytes had a low rate of aspartate utilization (Chapter I). Thus, most of the circulating glutamate, glutamine and aspartate in pigs must be derived from endogenous synthesis (Wu 2013). Because arterial glutamate and aspartate are not taken up by enterocytes, dietary glutamate, glutamine and aspartate as well as arterial glutamine provides the bulk of ATP to the cells and play an important role in maintaining the intestinal integrity and health of pigs.

In conclusion, results of this study demonstrate that 1) glutamate plus glutamine plus aspartate provide most ATP to enterocytes of 0- to 21-day-old pigs, 2) oxidation of alanine by pig enterocytes was limited, 3) the contribution of glucose to ATP production by enterocytes was the third highest after glutamate and glutamine, 4) glutamate, glutamine, aspartate and alanine greatly reduced the utilization of glucose via glycolysis, and 5) short- and long-chain fatty acids were only minor metabolic fuels for pig enterocytes. Our results indicated that glutamate, glutamine, and aspartate were extensively oxidized in enterocytes of developing pigs to serve as major energy substrates for the cells. This knowledge aids in understanding the role of dietary amino acids in intestinal metabolism, growth and health. Our findings also provide a biochemical basis for dietary supplementation with glutamate, glutamine, and aspartate to improve piglet nutrition. The results may also have important implications for feeding human infants.

CHAPTER V

SUMMARY AND CONCLUSIONS

Novel and important data were generated from the present research. This chapter provides an overall summary of the findings and an integrated discussion to highlight a significant contribution of the work to the field of animal biochemistry and nutrition.

Summary

The enterocyte, the columnar absorptive cell, is responsible for nutrient absorption. Enterocytes account for more than 85% of the total mucosal epithelial cell population in the small intestine (Klein and McKenzie 1983). During the first day after birth, the weight of the small intestine of newborn piglets increases by up to 70%, which is associated with increases in the length and diameter of the small intestine by 20% and 15%, respectively (Widdowson et al. 1976; Xu et al. 1992). The fast growth rate of the small intestine in newborns is also characterized by increased villus volume and the ratio of enterocyte number to villus volume by 5-fold and 2-fold, respectively. Because the enterocytes absorb dietary nutrients into the portal circulation for utilization by other tissues, the high rate of their growth is crucial for enhancing the digestive capacity in neonates. A high rate of protein turnover has been reported for these cells, and thus enterocytes must have high requirements for energy. All these findings suggest that a better understanding of energy sources for neonatal enterocytes is of tremendous significance for optimizing the supply of dietary nutrients to animals.

In the small intestine of adult rats, the rates of utilization of glutamate, glutamine, and aspartate are particularly high (Windmueller et al. 1982). Previous studies with rats have also revealed that in the presence of glutamine, glutamate, and aspartate, oxidation of glucose is

limited in the small intestine. These three amino acids contribute to 80% of the total carbon dioxide produced by the small intestine (Jungas et al. 1992). Glutamine contributes 100% more ATP than glucose does through glycolysis plus Krebs cycle in enterocyte of 0- to 7-day-old piglets in the presence of both of substrates (Wu et al., 1995). Because of the high activity of phosphate-activated glutaminase in the mitochondria of rat enterocytes (Pinkus and Windmueller 1977), L-glutamine can be extensively degraded to ammonia and L-glutamate. Thus, L-glutamate and L-glutamine may contribute equally to ATP production in rat enterocytes. Existing data demonstrate that butyrate serves as a major energy substrate for rat colonocytes and accounts for almost 80% of the total oxygen consumption (Hoogenraad et al. 1985). The rate of oxidation of butyrate in rat colonocytes is 10 and 70 times that of glutamine and glucose, respectively (Weber and Veach 1979). This foundational knowledge gives a better understanding of intestinal metabolism in animals.

Glucose and fatty acids are now considered to be minor sources of energy for the mammalian enterocytes (Wu 2018). It had long been assumed that glucose is actively utilized by many tissues in animals as a major metabolic fuel. So far, only a few studies have been conducted to quantify the contribution of amino acids and glucose to ATP production by newborn enterocytes, and little is known about the metabolic fate of short- and long-chain fatty acids in these cells. There are many differences in metabolism between mammalian and avian species, including different pathways for the utilization of nutrients by their enterocytes and other cell types. For example, uric acid has a physiological role in chickens as a potent antioxidant to protect them from high concentrations of glucose (12-15 mM) in the blood. Because glutamine serves as a precursor for uric acid synthesis in avian species, oxidation of this amino acid may be limited in enterocytes, so as to maximize the entry of dietary glutamine into

the portal circulation. This characteristic of glutamine metabolism may allow chickens to conserve dietary glutamine and to maintain a high circulating level of glutamine for hepatic synthesis of uric acid.

By quantifying the contributions of diverse nutrients to ATP production by enterocytes, more optimal proportions and amounts of amino acids in diets can be established to improve the efficiency of nutrient utilization and reduce the excretion of wastes to the environment. To evaluate the energetic role of amino acids, glucose, and fatty acids, the study outlined in Chapter III determined the oxidation of these nutrients by enterocytes from 0- to 42-day-old chickens. The rate of oxidation of glutamate by enterocytes from 0- to 42-day-old chickens was the highest among all the tested substrates (Table 3.1). The rate of ATP production from glutamate by chicken enterocytes was much higher than any other tested substrates (Table 3.2). Thus, glutamate is the major and preferential metabolic fuel for chicken enterocytes. This novel result is consistent with the high activities of GPT and GOT and the high rates of production of alanine and aspartate from glutamate in the cells (Table 3.7). However, compared with glutamate and glucose, glutamine, aspartate, and alanine were only minor energy sources for chicken enterocytes, as previously reported for glutamine (Wu et al. 1995). The limited oxidation of glutamine was consistent with low glutaminase activity in the cells (Tables 3.1 and 3.7). While glutamine synthetase activity in enterocytes of 7- to 42-day-old chickens was high (Table 3.7), we could not detect the formation of glutamine from glutamate by chicken enterocytes because the incubation medium lacked ammonia (Table 3.7). Thus, enterocytes from 0- to 42-day-old chickens are metabolically different than enterocytes from 0- to 21-day-old pigs that actively oxidize glutamine (Wu et al. 1995).

Second to glutamate, glucose was quantitatively a significant contributor to ATP production by chicken enterocytes. Although the contribution of glucose to ATP production by the cells decreased as chicken grew, the rates of production of lactate and pyruvate were constant in the cells from 0- to 21-day-old chickens (Tables 3.2, 3.4, 3.5). These results suggest that the enterocytes constantly produced significant amounts of ATP from glucose through the glycolysis pathway as chicken grew. This suggestion is substantiated by the result that the high activities of hexokinase, PFK, and pyruvate kinase were present in the cells (Table 3.7). As a potent inhibitor of pyruvate kinase (Wu 2018), alanine reduced the production of both lactate and pyruvate from glucose in chicken enterocytes (Tables 3.4 and 3.5). In addition, the present study indicated, for the first time, that none of the short-chain fatty acids and palmitate was a significant metabolic fuel for enterocytes of post-hatching chickens. This result is further established by the low CPT-I activity in the cells (Table 3.7). It is possible that chicken enterocytes have a low activity of acyl-CoA synthase for both short- and long-chain fatty acids. Further research is warranted to test this hypothesis.

Considering metabolic differences between mammals and avian species, the research presented in Chapter IV was conducted to identify the metabolic fates of amino acids, glucose, and fatty acids in pig enterocytes and to compare the energetic roles of these nutrients in the cells. At each age, pig enterocytes actively utilized glutamate, glutamine, glucose, and aspartate to produce ATP (Table 4.2). Among the nutrients, glutamate and glutamine were the major metabolic fuels, with glutamate providing 14%, 20%, 25% and 28% more ATP than glutamine on days 0, 7, 14 and 21, respectively. This is consistent with previous reports that in the small intestine of adult rats, the rates of utilization of glutamate, glutamine, and aspartate are high and with the high abundant phosphate-activated glutaminase activity in the mitochondria of rat

enterocytes (Windmueller et al. 1982; Pinkus and Windmueller 1977). In the present study, we also found high activities of phosphate-activated glutaminase, GPT, and GOT in enterocytes from 0- to 21-day-old pigs (Table 4.7). These results show that glutamine can be hydrolyzed into glutamate by pig enterocytes easily, and transamination of glutamate into either aspartate or alanine was rapid. Our data are further substantiated by the high rates of production of aspartate and alanine from both glutamate and glutamine in the cells via the transamination of glutamate with oxaloacetate and pyruvate, respectively (Table 4.3). Of note, the rates of glutamine utilization by pig enterocytes were similar to those of ammonia production (Tables 4.3 and 4.6), suggesting that glutamine is metabolized via the hydrolysis of glutamine to glutamate plus ammonia by glutaminase and the subsequent degradation of glutamate via transamination pathways. As in chicken enterocytes, there was low GDH activity and no detectable production of ammonia from glutamate, alanine and aspartate in pig enterocytes.

Third to glutamate and glutamine, pig enterocytes utilized glucose to provide energy through both the glycolysis pathway and the Krebs cycle (Tables 4.2, 4.4 and 4.5). The activity of glycolysis is dramatically inhibited by amino acids (glutamate, glutamine, aspartate, and alanine) in the cells, especially 0-day-old pigs, suggesting that pig enterocytes preferentially oxidize glutamate, glutamine, and aspartate to produce ATP when incubated in the presence of 5 mM glucose. These results are consistent with the previous report that a high rate of glycolysis occurs in pig enterocytes and a role of glutamine in inhibiting glycolysis in the cells (Wu et al. 1995).

Much evidence shows that butyrate is a major energy substrate for rat colonocytes and accounts for almost 80% of their total oxygen consumption (Hoogenraad et al. 1985). In contrast, fatty acids are considered as a minor source of energy for mammalian enterocytes (Wu 2018).

This notion is confirmed by results of the present study. Propionate, butyrate and palmitate were poor metabolic fuels for pig enterocytes and did not affect glycolysis in the cells. This is likely due to limited oxidation of the short- and long-chain fatty acids by enterocytes from 0- to 21-day-old pigs.

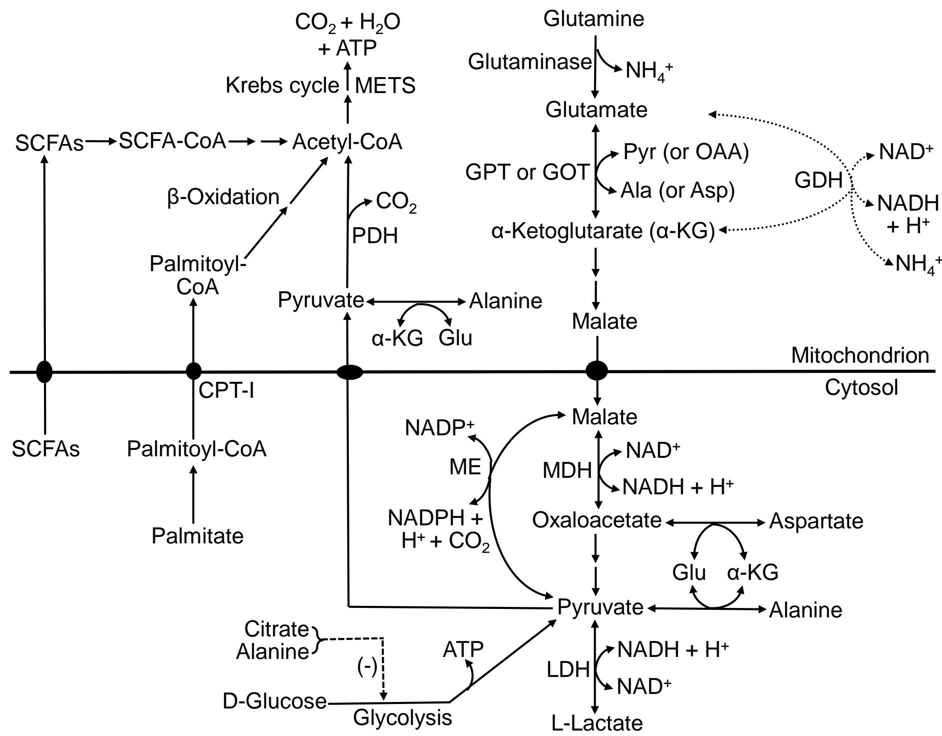
Complete oxidation of substrates to CO_2 occurs after they are converted into acetyl-CoA (Figure 5.1). Results of this present thesis support the notion that both chicken and pig enterocytes actively metabolize glutamate to produce ATP. Compared with glutamate, the rates of ATP production from other tested nutrients (glucose, glutamine, aspartate, alanine, propionate, butyrate and palmitate) were lower in the cells. The important role of glutamate as a major metabolic fuel for chicken enterocytes was not identified in the previous studies (Porteaus 1980; Watford et al. 1979). Because of the lack of arterial glutamate uptake by enterocytes, dietary provision of sufficient glutamate is vital for the metabolism, function and health of the chicken and pig intestine. In addition, glutamine contributes almost an equal amount of ATP to enterocytes of newborn pigs as glutamate and only slightly a lesser amount of ATP in cells from 7-21-day-old pigs than glutamate. The differences in the energetic roles of diverse nutrients in pig vs chicken enterocytes provide a biochemical basis for differential supplementation with glutamate and glutamine to poultry and swine diets.

Conclusions

The experiments described in this thesis determined energetic roles of diverse substrates as metabolic fuels for the enterocytes of developing chickens and pigs. Glutamate was the major source of ATP in the cells from both species. Except for glutamate, glucose was also a significant energy substrate for chicken and pig enterocytes. Compared with glutamate and

glucose, other tested nutrients (glutamine, aspartate, alanine, short-chain fatty acids, and palmitate) made only a minor contribution to ATP production by chicken enterocytes. In contrast, glutamine and aspartate were quantitatively important metabolic fuels for pig enterocytes. As for chicken enterocytes, palmitate, propionate, and butyrate contributed only a small amount of ATP to pig enterocytes. Taken together, the differences in energy sources for pig and chicken enterocytes underscore species differences in requirements for dietary glutamate and glutamine by the small intestine. Because glutamate in arterial blood is not taken up by avian and pig enterocytes, dietary glutamate must be provided to both chickens and pigs to maintain the metabolism, function and health of their small intestines. Thus, our findings not only advance the field of amino acid nutrition but also help to guide the design of new diets for improving the efficiency of producing high-quality animal proteins for human consumption.

Figure 5.1. Oxidation of amino acids, glucose and fatty acids in chicken and pig enterocytes[1]



[1]Glutamate, glutamine, aspartate, and alanine are metabolized to pyruvate through a series of pathways that involve both the cytosol and mitochondria. In contrast, glucose is converted into pyruvate via the cytosolic glycolysis pathway, and the glucose-derived pyruvate is either reduced to lactate in the cytosol or oxidized to acetyl-CoA in the mitochondria. Alanine and citrate (an intermediate of the Krebs cycle) inhibit glycolysis by inhibiting pyruvate kinase and phosphofructokinase-1, respectively. Palmitate, a long-chain fatty acid, is activated to palmitoyl-CoA in the cytosol by long-chain acyl-CoA synthase, and palmitoyl-CoA is transported into the mitochondrial matrix via carnitine palmitoyltransferase-I for oxidation to acetyl-CoA. Short-chain fatty acids (propionate and butyrate) are transported from the cytosol into the mitochondria for activation by short-chain acyl-CoA synthase into acyl-CoAs. The latter are metabolized to acetyl-CoA. Thus, glutamate, glutamine, aspartate, alanine, glucose, short-chain fatty acids, and palmitate are ultimately converted into acetyl-CoA, which is oxidized to CO_2 and H_2O via the Krebs cycle and the electron transport system in the mitochondria.
Abbreviations: Ala, alanine; GDH, glutamate dehydrogenase (absent from chicken and pig enterocytes); Glu, glutamate; GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase; LDH, lactate dehydrogenase; MDH, NAD^+-linked malate dehydrogenase; ME, malic enzyme; METS, electron transport system; OAA, oxaloacetate; PDH, pyruvate dehydrogenase; PFK-1, phosphofructokinase-1; Pyr, pyruvate; SCFAs, short-chain fatty acids.

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APPENDIX A

Corn- and soybean meal-based diets for chickens

		Starter	Grower	Finish1	Finish2*
Feeding period days		0 – 8	9 – 18	19 – 28	> 29
Feed structure		Crumble	Crumble	pellet	pellet
Crude protein (%)		21 – 22	19 – 20	18 – 19	17 - 18
Metabolizable energy	(MJ/kg)	12.45	12.66	12.97	13.18
(AMEn†)	(Kcal/kg)	2,975	3,025	3,100	3,150
	(Kcal/lb)	1,349	1,372	1,406	1,429
Digestible lysine	(%)	1.22	1.12	1.02	0.97
Digestible methionine	(%)	0.46	0.45	0.42	0.40
Digestible Met + Cys	(%)	0.91	0.85	0.80	0.76
Digestible tryptophan	(%)	0.20	0.18	0.18	0.17
Digestible threonine	(%)	0.83	0.73	0.66	0.63
Digestible arginine	(%)	1.28	1.18	1.07	1.02
Digestible valine	(%)	0.89	0.85	0.76	0.73
Digestible isoleucine	(%)	0.77	0.72	0.67	0.64
Calcium	(%)	0.90	0.84	0.76	0.76
Available phosphorus	(%)	0.45	0.42	0.38	0.38
Sodium	(%)	0.16 – 0.23	0.16 – 0.23	0.16 – 0.23	0.16 – 0.23
Chloride	(%)	0.16 – 0.30	0.16 – 0.30	0.16 – 0.30	0.16 – 0.30
Potassium	(%)	0.60 – 0.95	0.60 – 0.95	0.60 – 0.95	0.60 – 0.95
Linoleic acid	(%)	1.00	1.00	1.00	1.00

† Energy system is based on the apparent metabolizable energy corrected by nitrogen (AMEn).
* The diet recommendation is provided by Cobb-Vantress, Inc.

APPENDIX B

Supplementary levels of vitamins and trace elements (per tonne basal diet)

		Starter	Grower	Finisher 1 & 2
Vitamin A	(MIU)	10-13	10	10
Vitamin D3	(MIU)	5	5	5
Vitamin E	(KIU)	80	50	50
Vitamin K	(g)	3	3	3
Vitamin B1	(g)	3	2	2
Vitamin B2	(g)	9	8	6
Vitamin B6	(g)	4	3	3
Vitamin B12	(mg)	20	15	15
Biotin	(mg)	150	120	120
Choline	(g)	500	400	350
Folic Acid	(g)	2	2	1.5
Nicotinic Acid	(g)	60	50	50
Pantothenic Acid	(g)	15	12	10
Manganese	(g)	100	100	100
Zinc	(g)	100	100	100
Iron	(g)	40	40	40
Copper	(g)	15	15	15
Iodine	(g)	1	1	1
Selenium	(g)	0.35	0.35	0.35

† Preferably Choline is added directly into the mixer rather than via a premix because of its hygroscopic nature. Vitamin and trace mineral levels may vary depending on the source and supplier. The numbers above refers to e.g. usage of inorganic minerals and a vitamin D3 source. MIU = million international units, KIU = thousand international units, g = grams, mg = milligrams.
* The supplementary levels of vitamins and trace elements is provided by Cobb-Vantress, Inc.

APPENDIX C

CARNITINE PALMITOYLTRANSFERASE I ACTIVITY ASSAY

Principle: CPT-I

Palmitoyl-CoA + carnitine ----------------> palmitoyl-carnitine + CoASH

Materials:

1. 20 mM Palmitoyl-CoA [MW: 1005.94]: Dissolve 1 g in 50 mL DD water.
2. 100 mM L-carnitine [MW: 161.199]: Dissolve 162 mg in 10 mL DD water.
3. L-[N-methyl-^{14}C] carnitine-HCl is obtained from American Radiolabeled Chemicals (ARC 308) at 0.1 mCi/ml in 50 % EtOH and stored at 4℃.
4. 1.2 M HCl: Add 5 mL 6 N HCl in to 20 mL DD water.
5. 150 mM KCl [MW: 74.5513]/5 mM Tris-HCl [MW: 157.60], pH 7.2: Dissolve 560 mg KCl, 40 mg Tris-HCl in 45 mL DD water. Adjust pH with HCl and make it to 50 mL by DD water, then store at 4°C.
6. 4 M $MgCl_2$ [MW: 95.211]: Dissolve 3.808 g MgCl2 in 10 mL DD water. Stable at room temperature.
7. Rotenone [MW: 394.41] (Sigma, R-8875): Dissolve 40 mg in 1 mL acetone. Store in a tightly capped tube at 4°C.
8. 210 mM Tris-HCl [MW: 157.60], pH 7.2: Dissolve 1.66 g Tris-HCl in 45 mL DD water. Adjust pH with HCl and make it to 50 ml by DD water. Store at 4°C.
9. 1-Butanol.
10. Solid reagents: bovine serum albumin, essentially fatty acid free.
11. Adenosine triphosphate (ATP). (Sigma. A-2283).

12.KCN [MW: 65.12] (Aldrich, 20,781-0).

13.Substrate Mix (5x): To 4.75 ml water, add the following:

62.5 μL of 20 mM palmitoyl-CoA, 125 μL of unlabeled 100 mM L-carnitine, and 62.5 μL of [^{14}C] carnitine.

14.Assay Cocktail (2x): To 50 ml of 210 mM Tris-HCl, add 1 g bovine serum albumin (BSA) (mix gently).

Then, add the following:

203 mg ATP, 7.7 mg glutathione, 13 mg KCN (as solids), 100 ul of 4 M $MgCl_2$, and 100 μL of 40 mg/mL rotenone.

Steps:

1. Add the following to 2 mL plastic tubes:

100 μL of 5x substrate mix, 50 μL of 150 mM KCl/5 mM Tris, pH 7.2, and 250 μL of 2x Assay Cocktail.

2. Add 100 μL of enzyme sample. Vortex briefly. 3. Place the tubes in a 26°C shaking water bath. 4. After 15 min, add 500 μL of 1.2 M HCl and vortex. 5. Add 500 μL of 1-butanol. Vortex each tube for 30 s. 6. Centrifuge at 10,000 g for 1 min. Obtain the upper butanol phase. 7. Transfer 300 μL of the upper, butanol phase to a 1.5-mL microfuge tube containing 500 μL of water. Cap the tubes and vortex the tube. Repeat Step 6.

3. Transfer 250 μL of the upper phase to a scintillation vial. Add 5 mL Aqueous cocktail for counting.

For blanks, add:

100 μL of 5x substrate mix, 50 μL of 150 mM KCl/5 mM Tris, pH 7.2, 250 μL of 2x Assay Cocktail, and 500 μL 1.2 N HCl and vortex.

Add 100 μL of enzyme sample. Wait 5 min.

Repeat steps 3-8 above.

Preparation of Mitochondria

The liver was first minced with scissors and homogenized with 10 volumes of 0.25 M sucrose in a Dounce homogenizer (Kontes). After initial centrifugation for 15 min at 600 x R in a refrigerated Sorvall centrifuge the supernatant fraction was subjected to further centrifugation for 15 min at 7,700 x g. The supernatant was discarded and the pellet was resuspended to the original volume in 0.25 M sucrose. After further centrifugation for 15 min at 7,700 x g the pellet was resuspended in 150 mM KCl. The mitochondria were used for experiments at this stage when functionally intact organelles were required.