" Nature does nothing uselessly."

Aristotle

University of Alberta

Regulation of Lysine Catabolism

by

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Your file Votre référence
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To my parents James & Mary Pink,

& To my grandmothers Alice Simms & Margaret Pink

Whose love, sacrifice and enduring belief permitted me the strength to complete the tasks I set for myself.

And for whose only request of my future was that I not go to sea.

Abstract

Cereal grains represent the main protein source for livestock, and humans in many parts of the world. However, the low lysine content of these grains is a main limiting factor for protein synthesis; hence lysine must be supplemented to the diet. Lysine utilization in the body also includes degradation via lysine α-ketoglutarate reductase (LKR), the first enzyme, and primary regulatory step in the catabolic pathway. The control of lysine degradation is poorly understood and therefore the regulatory parameters that influence LKR activity were the focus of this thesis.

The first hypothesis was that LKR activity was not confined to the liver. Extra-hepatic tissues in both the pig and chicken were shown to catabolize lysine at significant rates. Most notably, epithelial cells (IEC) of the intestine and muscle possessed the catalytic machinery necessary to degrade lysine to saccharopine. The production of $^{14}CO_2$ from radioactively labeled lysine in liver, IEC and muscle mitochondria demonstrated that the entire degradative pathway was present in these tissues.

LKR activity and sensitivity to inhibition was determined at times of significant nutritional transition in the growing pig. Significant changes in LKR activity and lysine oxidation in muscle mitochondria were observed in the first week of life, in contrast to the stable activity of LKR in other tissues like the liver and IEC. LKR inhibition studies suggested three periods of regulatory transition: birth to 1 week of life, pre-weaning to weaning (7-14 days) and post weaning. Age-related regulation of LKR activity was linked with major nutrition transitions, was tissue specific and most likely represented major metabolic shifts within the animal.

Broiler poults were fed lysine at 0.8% -1.6% (of the diet) for optimal protein synthesis; there was no significant change in liver, kidney or IEC LKR activities. Birds fed lysine below requirement demonstrated poor performance and higher plasma lysine catabolite levels than control (100% requirement) birds, but LKR activity was not decreased. Birds fed lysine above requirement had increased plasma lysine concentrations, but lower lysine catabolite concentrations; and no increase in LKR activity in liver, kidney or IEC compared to controls. These results demonstrate that control of lysine degradation does not follow a simple dose-response mechanism in growing broilers; LKR activity is not necessarily proportional to dietary lysine intake.

Activity of the second catalytic enzyme saccharopine dehydrogenase followed Michaelis-Menten kinetics, whereas LKR was susceptible to substrate inhibition. Substrate inhibition of LKR was age and tissue dependent, but was demonstrated in both pig and poultry tissues. The function of a substrate-inhibited lysine-catabolic pathway remains unclear, but may be important for maintaining the cellular levels of lysine and tryptophan catabolites, particularly aminoadipate, ketoadipate and kynurenine and kynurenate. Furthermore, the inhibitory effects of compounds like aminoadipate on LKR activity were similarly affected by tissue, age and dietary level of lysine.

Lysine degradation was concluded to be:

1. at physiologically significant levels in extra-hepatic tissues (i.e. muscle, brain, intestinal epithelium);
2. regulated in coordination with age and major nutritional transitions (i.e. birth and weaning);

3. regulated in coordination with dietary lysine intake, except at very deficient lysine intake;
4. an apparently important and indispensable function in extra-hepatic tissues, which is yet to be described.

Considering lysine to only be a substrate for protein synthesis is an archaic idea, one that needs to be reformulated to include the potential contributions of lysine catabolites to metabolism.

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   Proceedings of 9th International Symposium on Digestive Physiology in Pigs, Vol 2 p131-133. 2003

MANUSCRIPTS IN PREPARATION

1. **Branched Chain Amino Acid Catabolism in the Porcine Small Intestine**
   Raja Elango, **Desmond Pink**, Walter T. Dixon, Paul P. Pencharz and Ronald O. Ball
   Manuscript submitted and accepted with revisions to *American Journal of Physiology*

2. **Lysine Catabolism in the Adult Pig is not Limited to the Liver**
   Desmond Pink, Rajavel Elango, Joan Turchinsky, Walter T. Dixon and Ronald O. Ball. Manuscript submission to *American Journal of Physiology* : draft available if required.

3. **Effect of Age and Nutritional Transition on Lysine Catabolism in Growing Pigs**
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4. **Effect of Dietary Lysine Intake on Lysine α-Ketoglutarate Reductase in Broiler Chickens D Pink**, R Kirschenman, E Tong, S Tan, WT Dixon, RO Ball and D Korver
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ABSTRACTS

1. **Regulation of Lysine α-Ketoglutarate Reductase in Growing Pigs**
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Canadian Society of Animal Science Annual meeting; Edmonton, Alberta Canada; July 18-21, 2004 *Poster presentation in Graduate Student competition (presented by Raven Kirschenman)*

3. **Regulation of Lysine α-Ketoglutarate Reductase Varies During Postnatal Stages of Growth and Development in the Pig**
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FASEB: Experimental Biology 2004 Abstract 364; Washington, DC, USA; April 17-21, 2004 FASEB J 18:A539.
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4. **Regulation of branched-chain amino acid (BCAA) catabolism in the pig small intestine**
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FASEB: Experimental Biology 2004; Washington DC, USA; April 17-21, 2004
Abstract presented as poster presentation

5. **Regulation of Lysine Degradation During the Postnatal Stages of Growth and Development in the Pig**
D Pink, R Elango, WT Dixon and RO Ball
ABS #16 in Advances in Pork Production, volume 15 edited by RO Ball
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6. **Small Intestinal Utilization of Branched Chain Amino Acids (BCAA) in Market Weight Pigs**
Rajavel Elango, **Desmond Pink**, Walter T. Dixon, Paul B. Pencharz & Ronald O. Ball
ABS#7 in Advances in Pork Production, volume 15 edited by RO Ball.
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7. **Branched Chain Amino Acid Catabolism in the Neonatal Piglet Gut**
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Digestive Physiology in Pigs: 9th International Symposium on Digestive Physiology in Pigs Volume 2, Pg 207.
Banff, Alberta, Canada; May 14-17, 2003
Poster presentation

8. **Lysine Catabolism in the Neonatal Piglet During Postnatal Stages of Development**
D Pink, R Elango, WT Dixon and RO Ball

FASEB: Experimental Biology 2003 Abstract 5304; San Diego, California, USA April 11-15, 2003 FASEB J 17: A702.
Oral presentation

9. **Branched Chain Amino acid Catabolism in the Neonatal Piglet During Postnatal Stages of Development**
R Elango, **D Pink**, PB Pencharz and RO Ball
FASEB: Experimental Biology 2003 Abstract 431.1 San Diego California, USA; April 11-15, 2003
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10. **Lysine degradation in swine**
D Pink, R Elango, WT Dixon and RO Ball
Banff Pork Seminar 2003; Banff, Alberta, Canada; January 20-23, 2003
ABS#13 in Advances in Pork Production volume 14 edited by RO Ball.
Poster presentation and *oral presentation by WT Dixon*

11. **Small intestinal utilization of branched chain amino acids (BCAAs) in piglets**
R Elango, **D Pink**, WT Dixon and RO Ball
Banff Pork Seminar 2003; Banff, Alberta, Canada; January 20-23, 2003
ABS#14 in Advances in Pork Production volume 14 edited by RO Ball.
Poster presentation

12. **Lysine Catabolism in Swine: An Enzymatic Approach**
DBS Pink, J Turchinsky, WT Dixon and RO Ball
FASEB: Experimental Biology 2002 Abstract 214.7; New Orleans, LA, USA; April 20 - 24, 2002 FASEB J 16: A258.
Poster presentation

13. **Lysine Ketoglutarate Reductase Activity in Extra-Hepatic Tissues of the Pig**
D Pink, R Elango, WT Dixon and RO Ball
Canadian Federation of Biological Societies 45th Annual Meeting; Montréal, Québec, Canada June 14, 2002
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14. **Branched Chain Aminotransferase (BCAT) Activity Exists in Isolated Pig Enterocytes**
R Elango, **D Pink**, PB Pencharz and RO Ball
Canadian Federation of Biological Societies 45th Annual Meeting Abstract 14, pg 80; Montréal, Québec, Canada June 14, 2002
Poster presentation

Abbreviations

AAA	α-aminoadipate
AADAT	α-aminoadipate aminotransferase
AAAS	α-aminoadipate semialdehyde
AASS	α-aminoadipate semialdehyde synthase
BH_4	tetrahydropterin
CDNA	complementary DNA
CoA	Coenzyme A
$DMPH_4$	6,7-dimethyltetrahydrobiopterin
DTT	dithiothreitol; Cleland's reagent
EDTA	ethylenediaminetetraacetic acid
EDGA	ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
KAA	α-ketoadipate
KADH	α-ketoadipate dehydrogenase
α-KG	α-ketoglutarate (same as 2-oxoglutarate)
KHB	Krebs-Henseleit buffer
LB	Lineweaver-Burke
LKR	lysine α-ketoglutarate reductase (same as lysine 2-oxoglutarate reductase)
MM	Michaelis-Menten
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NAD	nicotinamide adenine dinucleotide
NO	nitric oxide
MT	metric ton (1000 kilograms)
ODC	ornithine decarboxylase
PBS	phosphate buffered saline
PQQ	pyrroloquinoline quinone
RT-PCR	reverse transcription-polymerase chain reaction
SAM	S-adenosylmethionine
SDH	saccharopine dehydrogenase
SI	substrate inhibition
TCA	citric acid cycle
TEA	triethanolamine
w/w	weight / weight
w/v	weight / volume
v/v	volume / volume
[x]	concentration of compound in brackets

1 Introduction

1.1 The Fundamental Problem: Lysine - The First limiting Amino Acid

The primary nutritional goal of the livestock industries, and in particular the swine industry, is to increase the rate and efficiency of protein accretion. The ultimate goal of any animal producer is to generate the optimal market weight animal in the fastest, most cost efficient manner possible.

Feeding pigs for an optimal cost and rate of growth is a multivariate system, encompassing factors such as genotype (110, 111, 181, 379), gender (164, 487, 494), age (314, 492), environmental temperature (86, 87, 246, 480), dietary protein levels (165, 246, 270, 360), and nutrient bioavailability and digestibility (24, 25, 38, 41, 97, 201, 278, 283, 296, 396, 427, 449, 506). When considering the typical grain diet fed to growing pigs, the chief factor regulating protein accretion is intake of the first limiting amino acid – which is most often lysine (277, 457).

1.1.1 Lysine in Livestock Diets

For many years, pig diets were formulated to meet crude protein requirements, rather than the requirements of specific amino acids (277). Subsequently there was much research investigating the specific amino acid requirements of swine (83, 250, 251, 282, 427, 457, 493), the amino acid composition (and bioavailability) of different feedstuffs (38, 39, 245, 255, 277, 278, 339, 464) and the relatively wide availability of various feedstuffs. The formulation of pig diets based upon amino acid requirements has become

much more practical and accurate than formulation based on a crude protein basis. Cereal grains (corn/maize, wheat, barley, oats) form the basis of most swine diets, typically supplying approximately half of the dietary protein for (growing-finishing) pigs. Hence, the amino acid composition of cereals is very important (277). Table 1.1 lists the most limiting amino acids for growing pigs, in several of these grains. The low content of lysine in cereal grains makes it the most important amino acid in swine nutrition. Producers use complementing grain mixtures and protein feed, supplementation protocols or a combination thereof, to meet the lysine requirements of their animals and thus ensure that the diet is not a limiting factor for optimal protein synthesis.

Table 1.1 Limiting Amino Acids in Cereal Grains for Swine Diets

	Limiting Amino Acids		
Cereal Grain	**First**	**Second**	**Third**
Barley	Lysine	Threonine	Histidine
Corn	Lysine and Tryptophan*		Threonine
Oats	Lysine	Threonine	ND**
Sorghum	Lysine	Threonine	Tryptophan
Triticale	Lysine	Threonine	ND
Wheat	Lysine	Threonine	ND

Taken from Lewis (277); *co limiting (279), **not described.

Because lysine is the first limiting amino acid in these grains, (grower-finisher) pig diets are formulated based on lysine content. After meeting the requirement of the first limiting amino acid, the diet must be formulated to ensure requirement is met for the 2nd and 3rd limiting amino acids, typically threonine and tryptophan. Supplementation

with synthetic threonine and tryptophan, to reduce protein supplements, is becoming more affordable as the cost of producing these amino acids becomes more economical.

Amino acids that are absorbed in excess of the demands of protein synthesis cannot be stored and are deaminated. The amino-nitrogen is released as urea and the remaining carbon skeleton is metabolized via the citric acid cycle to yield energy. Excess protein in the diet, or improper balances of amino acids lead to inefficient utilization of dietary protein and amino acids for pig growth. Hence the concept of an ideal protein has arisen to help define amino acid requirements that incorporate dynamic patterns of maintenance, new tissue accretion, milk synthesis during lactation and tissue catabolism (16, 277, 278, 331). Using these patterns, diet formulations can incorporate amino acid profiles that more closely approximate an animal's actual requirement and thereby decrease catabolism due to amino acid excess.

Feed costs account for approximately 60% of production costs (see Appendix Figure 8.2 for a breakdown of production costs) (244), however supplementation with synthetic amino acids accounts for only a small portion of this amount (~5%). However, to meet the amino acid requirements for optimal performance in the absence of supplementation, significant additions of protein supplements would have to occur, increasing the cost of feed and thus increasing total production costs overall. Thus formulation of pig diets by incorporating synthetic amino acids is both a cost efficient and metabolically efficient means of meeting the amino acid and protein requirements for optimal protein accretion.

1.1.1.1 Economics of Lysine Supplementation in the Livestock Industry

Synthetic amino acids have been used as industrial feed sources for more than 40 years. Synthetically produced DL-Methionine was introduced in the late 1950's for application in the poultry industry. Production of L-Lysine by fermentation processes started during the 1960's in Japan (458). As technologies have improved, the cost of producing synthetic amino acids has declined, permitting greater expansion of use of synthetic amino acids in animal feed.

Today about 600,000MT of feed-grade L-Lysine-HCl is produced per year (458). Utilization of synthetic lysine-HCL saves the equivalent of 18 million tons of soybean meal – roughly equivalent to 15% of soybean meal production worldwide (4). The cost of L-Lysine-HCl is approximately 1.85-2.50 CDN/Kg (1.5-2 USD)[*] (458). In the Canadian market, this represented more than $60 million in trade imports of L-Lysine-HCl for supplementation to livestock feed in 2003 and 2004 (57) (Figure 1.1). The province of Alberta's share of this amounted to almost $9 million in 2004 (Figure 1.2).

Lysine imports have increased linearly since 1999; extrapolation of this trend indicates that by 2006, Alberta will import more than $12 million worth of feed-grade lysine. Nationally, this total will exceed $83 million. Clearly, research directed towards reducing lysine requirements of animals, or improving efficiency of utilization has great economic potential.

Several aspects of lysine utilization have previously been considered in such research, including: 1) lysine content of feed sources, 2) digestibility and availability of lysine from these sources, 3) lysine requirement for optimal protein accretion, and 4)

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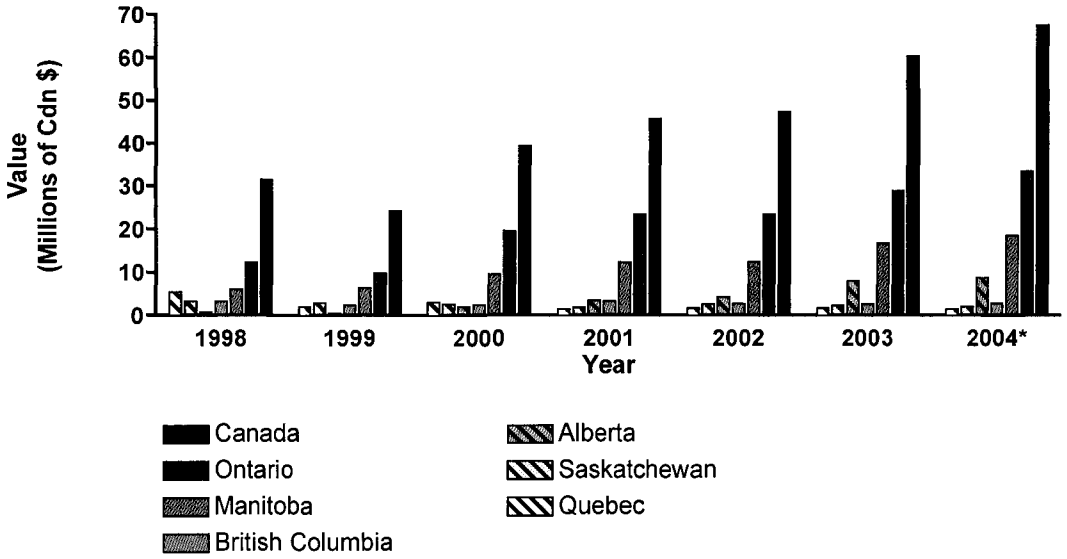
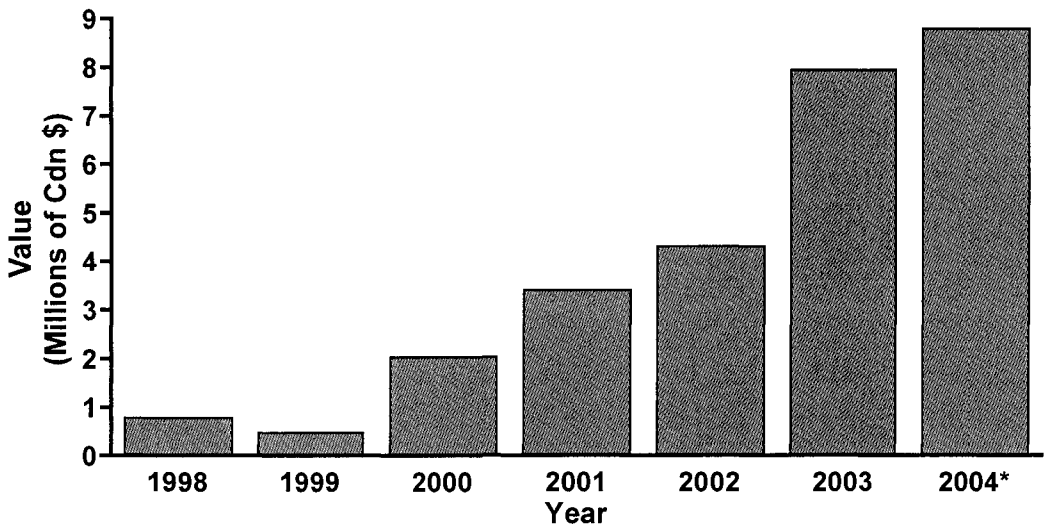
[*] Currency exchange as of January 28, 2005.

Figure 1.1 Canadian Imports of Lysine for Manufacture of Animal and Poultry Feeds



Data presented as absolute dollars, no rounding; *values for 2004 were extrapolated from previous years (57).

Figure 1.2 Alberta Imports of Lysine for Manufacture of Animal and Poultry Feeds



Data presented as absolute dollars, no rounding; *values for 2004 were extrapolated from previous years (57).

factors that influence the preceding aspects of lysine utilization. However, the cellular metabolism and utilization of lysine has not been extensively investigated in pigs. Major advances have been made toward understanding the total requirement of lysine at different stages of growth, but little information exists regarding the biochemical aspects of porcine lysine metabolism.

A more complete elucidation of the biochemistry of lysine will provide the understanding and knowledge required to improve utilization, reduce catabolism, or at the very least, better manage supplementation of grain-based with synthetic lysine.

1.2 Biochemistry of Lysine Metabolism

Lysine, in addition to being the most limiting amino acid in pig diets, is also an indispensable amino acid; thus it cannot be synthesized by the mammalian (or avian) body and must be derived from dietary sources. Therefore the degradation of dietary lysine really becomes the focal point of understanding its metabolism. This point is more strongly emphasized when one considers that basal lysine degradation rates appear not to change when the dietary intake falls below requirements for protein synthesis (313). In this study by Moehn et al (313), pigs were initially offered a purified diet providing all nutrients in excess of requirements to determine maximum protein deposition rates. Following this period, pigs were allocated to treatment groups corresponding to lysine intakes of 60, 70, 80, and 90% of estimated requirements for maximal protein deposition. Lysine catabolism was determined either directly (oxidation) using a primed, constant infusion of l-^{14}C-lysine or indirectly (disappearance) using the nitrogen balance method. Lysine catabolism decreased with increasing growth potential, but quite surprisingly lysine disappearance and lysine oxidation were independent of lysine intake, except for

the lowest lysine intake level, where the rates were lower. Therefore, the pig does not conserve lysine, by reducing lysine catabolism, even when the intake of lysine is limiting protein synthesis. This is a paradox. The body is degrading an essential amino acid when it is seemingly very limiting.

In mammals, the principal lysine catabolic pathway, known as the saccharopine or α-aminoadipic acid semialdehyde pathway proceeds via a sequence of seven reactions, the last of which feeds into the pathway for fatty acid catabolism (via crotonyl CoA) and toward the citric acid cycle. The products of lysine catabolism are thus exclusively ketogenic; i.e., under starvation conditions, lysine can be used for the synthesis of ketone bodies, β-hydroxybutyrate and acetoacetate, but not for the net synthesis of glucose (99, 182, 292, 490). This is important when one considers the adaptive shift by the brain to use ketone bodies as the primary metabolic fuel during starvation. The relative toxicity of excess lysine or excess lysine catabolites in humans has been associated with severe genetic diseases causing mental retardation and neuromotor conditions (99, 109, 392) (further discussion in section 1.4.4). In other species however, excess lysine appears to be tolerated to various degrees. Decreased performance by an unexplained arginine antagonism in dogs fed excess (4%) lysine was noted by Latshaw and coworkers (271) similar to the decreased growth at 3-4 times requirement observed in pigs (134, 136), arginase was unaffected in these studies. Laying hens were able to tolerate 1% additional lysine (above requirement) without any adverse effects (257), but chicks appeared to have decreased performance when fed excess lysine well (276). Cognitive impairments were not scored in any of these studies.

These points, taken together, suggest that there may be metabolic reasons for lysine catabolism during deficiency. Clearly there is a necessity for a more complete understanding of lysine catabolism and the factors influencing the rate of catabolism.

1.2.1 Lysine Catabolic Enzymes

Enzymes of the saccharopine or α-aminoadipic acid pathway catalyze the reactions in the principal route of lysine degradation (108). Unlike most amino acids, the first step of lysine degradation is not deamination and transfer of the α-amino group to an acceptor molecule like α-ketoglutarate. Lysine α-ketoglutarate reductase (LKR, EC 1.5.1.8), the first enzyme of the catabolic pathway (203) acts upon lysine in the presence of α-ketoglutarate to form saccharopine (ε-N-(L-glutaryl-2)-L-lysine). Saccharopine is then cleaved to α-aminoadipic 6-semialdehyde and glutamic acid by saccharopine dehydrogenase (SDH, EC 1.5.1.9). These two enzymes are collectively known as α-aminoadipic acid semialdehyde synthase (AASS, ECs 1.5.1.7, 1.5.1.8, 1.5.1.9). Alpha-aminoadipic 6-semialdehyde is converted to α-aminoadipic acid (AAA) by L-α-aminoadipate-6-semialdehyde dehydrogenase (AASADh, EC 1.2.1.31) and then via the catalytic action of α-aminoadipate aminotransferase (AADAT, EC 2.6.1.39), α-ketoadipic acid (KAA) is produced. The alpha carboxyl group of lysine is removed via the oxidative steps orchestrated by the α-ketoglutarate dehydrogenase complex. This multiple enzyme complex includes α-ketoglutarate dehydrogenase (EC 1.2.4.2), of which multiple copies are bound to dihydrolipoyllysine-residue succinyl transferase (EC 2.3.1.61), which also tightly binds dihydrolipoyl dehydrogenase (EC 1.8.1.4)(294, 362, 410). The catalytic actions of this complex result in the formation of glutaryl-CoA and

carbon dioxide. Several additional reactions then lead into the citric acid cycle via glutaconyl-Coa, trans-crotonyl-CoA, and finally acetyl-CoA. The unique and key steps in the pathway are illustrated in Figure 1.3 (a more complete illustration of lysine metabolic pathways (243) is shown in Figure 8.5).

1.2.2 The Saccharopine and Pipecolic Acid Pathways of Lysine Degradation

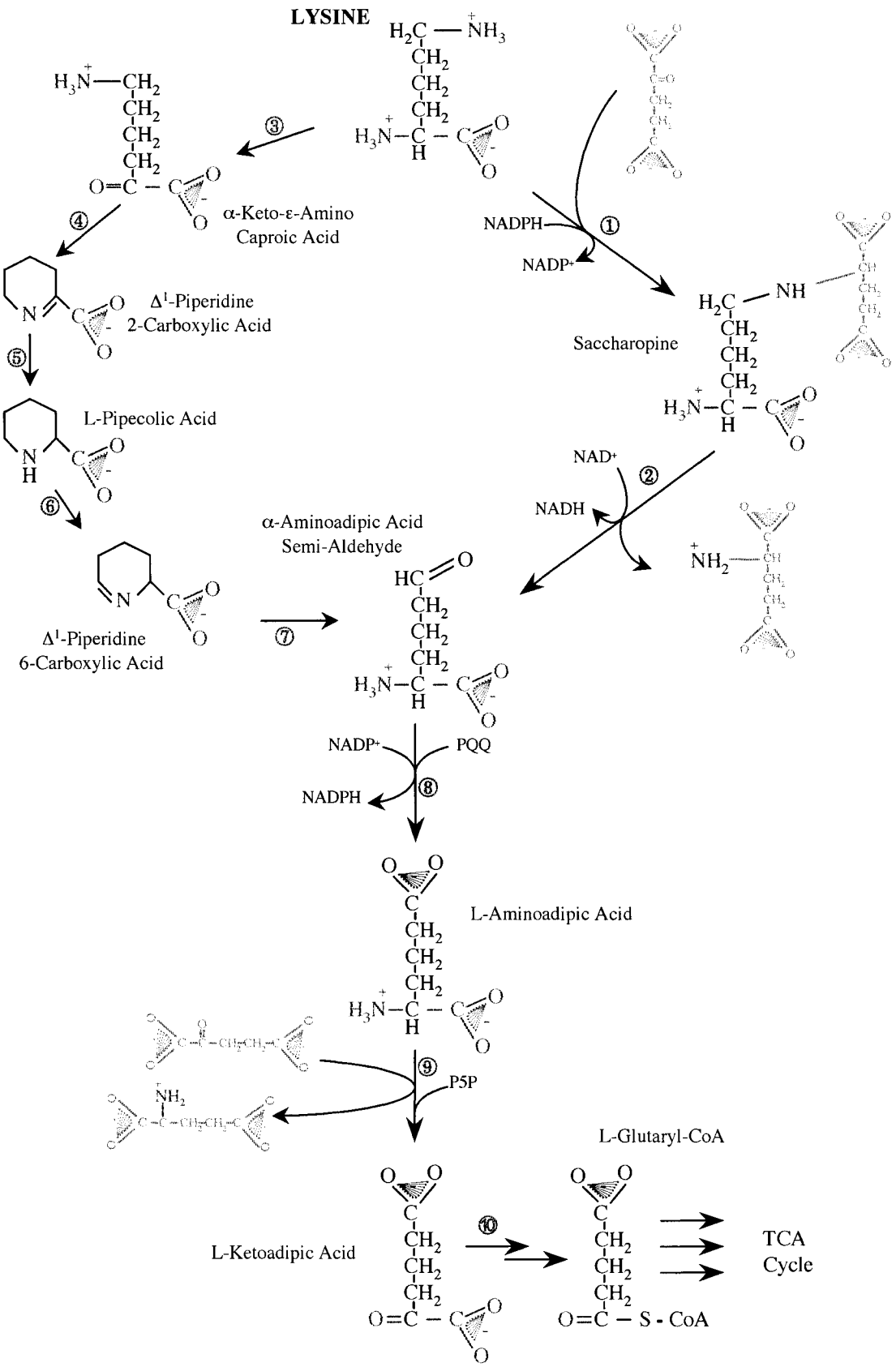
Early investigations examining the metabolism of lysine showed that lysine was degraded to glutaric acid (42, 332, 381) and α-aminoadipic acid (43, 332), although the metabolic route to arrive at these catabolites remained obscure (387). Work in the early 1950s showed that lysine could also be converted to glutamate (6, 311). These studies

Following page:

Figure 1.3 Lysine Catabolic Pathways

Lysine is degraded via two metabolic pathways, the main route of catabolism – the saccharopine or α-aminoadipate semialdehyde pathway and the overflow pathway – the pipecolic acid pathway. The enzymes, as shown in the figure are:

① Lysine α-ketoglutarate reductase (EC 1.5.1.8);
② Saccharopine dehydrogenase (EC 1.5.1.9);
①+ ② α-Aminoadipic acid semialdehyde (EC 1.5.1.10);
③L-Lysine oxidase (EC 1.4.3.14);
④ Spontaneous rearrangement;
⑤ Δ^1-Piperidine-2-carboxylate reductase (EC 1.5.1.21);
⑥ L-Pipecolate dehydrogenase (EC 1.5.99.3);
⑦ Spontaneous rearrangement;
⑧ α-Aminoadipic acid semialdehyde dehydrogenase (EC 1.2.1.31);
⑨ α-Aminoadipic acid aminotransferase (EC 2.6.1.39);
⑩ α-Ketoglutarate dehydrogenase complex also known as α-ketoadipate dehydrogenase
1. α-Ketoglutarate dehydrogenase (EC 1.2.4.2),
2. Dihydrolipoyllysine-residue succinyltransferase (EC 2.3.1.61),
3. Dihydrolipoyl dehydrogenase (EC 1.8.1.4)



pointed toward the, as yet unknown saccharopine pathway. Rothstein and Miller (385-387) built upon the work of Weissman and Schoenheimer (488) and concluded that pipecolic acid was a major metabolite of lysine degradation in the rat. In the late 1960s, several studies revealed saccharopine as a central and key intermediate in the degradation of lysine (187-189, 204, 220). The secondary route of lysine degradation, via pipecolic acid, is currently accepted as an overflow pathway, activated when the lysine load overwhelms the capacity of LKR (99). This pathway is also recognized as having a prominent role in the brain (374), although recent evidence suggests a major role for LKR as well (354).

1.3 Structural and Sequence Components of the Bifunctional Protein

Many parameters of lysine degradation were revealed by research examining the clinical manifestations of genetic defects of lysine-catabolic enzymes resulting in hyperlysinemia (496, 499), however much remained unknown about the proteins responsible for the catalytic activities of the initial steps. Two separate enzymes were identified in rat liver, one with a reductase activity and the other a dehydrogenase activity (337). Other investigations similarly identified the two activities, but after using a variety of chromatographic methods, could not separate the activities of the two enzymes – suggesting perhaps that the activities resided within a single polypeptide moiety (107, 155). Later, the activities of LKR and SDH were shown to reside on a single polypeptide in baboon and bovine livers (100, 291, 292) and more recently in human cells (392). The mouse has been shown to utilize both bifunctional and monofunctional lysine-degrading enzymes; more specifically a bifunctional hepatic LKR/SDH enzyme and monofunctional SDH have been localized (353). The bifunctional protein has been

referred to as α-aminoadipic semialdehyde synthase (AASS) (292); the amino-terminal portion has the LKR activity and the carboxy-terminal portion provides the SDH activity. Structurally, the protein appears to be a homotetramer of bifunctional proteins, each having a mass of 115 kDa (combined mass of 468 kDa) (292). Multiple alignments of genomic gene sequences demonstrate similarities across several species (Figure 1.5, 8.3).

Figure 1.4 Amino Acid Sequence of the Human AASS Protein

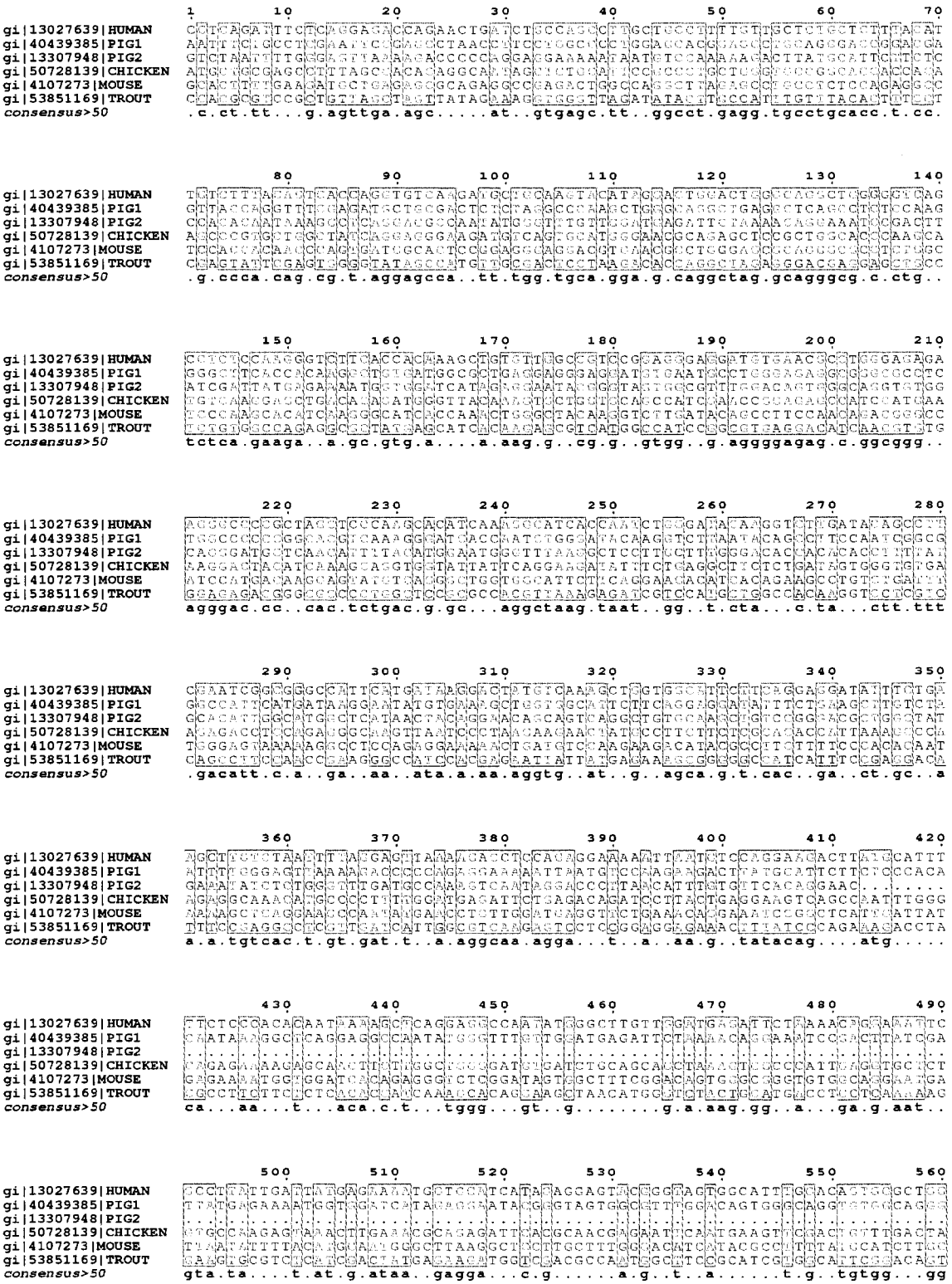
	1	11	21	31	41	51	
1	MLQVHRTGLG	RLGVSLSKGL	HHKAVLAVRR	EDVNAWERRA	PLAPKHIKGI	TNLGYKVLIQ	60
61	PSNRRAIHDK	DYVKAGGILQ	EDISEACLIL	GVKRPPEEKL	MSRKTYAFFS	HTIKAQEANM	120
121	GLLDEILKQE	IRLIDYEKMV	DHRGVRVVAF	GQWAGVAGMI	NILHGMGLRL	LALGHHTPFM	180
181	HIGMAHNYRN	SSQAVQAVRD	AGYEISLGLM	PKSIGPLTFV	FTGTGNVSKG	AQAIFNELPC	240
241	EYVEPHELKE	VSQTGDLRKV	YGTVLSRHHH	LVRKTDAVYD	PAEYDKHPER	YISRFNTDIA	300
301	PYTTCLINGI	YWEQNTPRLL	TRQDAQSLLA	PGKFSPAGVE	GCPALPHKLV	AICDISADTG	360
361	GSIEFMTECT	TIEHPFCMYD	ADQHIIHDSV	EGSGILMCSI	DNLPAQLPIE	ATECFGDMLY	420
421	PYVEEMILSD	ATQPLESQNF	SPVVRDAVIT	SNGTLPDKYK	YIQTLRESRE	RAQSLSMGTR	480
481	RKVLVLGSGY	ISEPVLEYLS	RDGNIEITVG	SDMKNQIEQL	GKKYNINPVS	MDICKQEEKL	540
541	GFLVAKQDLV	ISLLPYVLHP	LVAKACITNK	VNMVTASYIT	PALKELEKSV	EDAGITIIGE	600
601	LGLDPGLDHM	LAMETIDKAK	EVGATIESYI	SYCGGLPAPE	HSNNPLRYKF	SWSPVGVLMN	660
661	VMQSATYLLD	GKVVNVAGGI	SFLDAVTSMD	FFPGLNLEGY	PNRDSTKYAE	IYGISSAHTL	720
721	LRGTLRYKGY	MKALNGFVKL	GLINREALPA	FRPEANPLTW	KQLLCDLVGI	SPSSEHDVLK	780
781	EAVLKKLGGD	NTQLEAAEWL	GLLGDEQVPQ	AESILDALSK	HLVMKLSYGP	EEKDMIVMRD	840
841	SFGIRHPSGH	LEHKTIDLVA	YGDINGFSAM	AKTVGLPTAM	AAKMLLDGEI	GAKGLMGPFS	900
901	KEIYGPILER	IKAEGIIYTT	QSTIKP				

Selected features of Alpha-aminoadipic semialdehyde synthase Accession Number Q9UDR5 (AASS_HUMAN). Mitochondrial precursor (LKR/SDH) [Includes: red highlight-mitochondrial target sequence; green highlight-Lysine ketoglutarate reductase (EC 1.5.1.8); grey highlight-linker region with possible phosphorylation sites (bold text); yellow highlight-Saccharopine dehydrogenase (EC 1.5.1.9) (SDH)].

1.3.1 Mechanism of Catalytic Activity

A large body of research (168, 169, 430, 448, 518) in the plant sciences, largely under the guidance of Gad Galili (Weizmann Institute of Science) has revealed much about the role of lysine catabolism in plants. Described as "one of the most highly regulated metabolic pathways in plants" (169), the saccharopine pathway has provided illuminating ideas on mechanisms regulating catalytic activity of AASS and the various

Figure 1.5 Sequence Alignment of AASS Proteins from Different Species

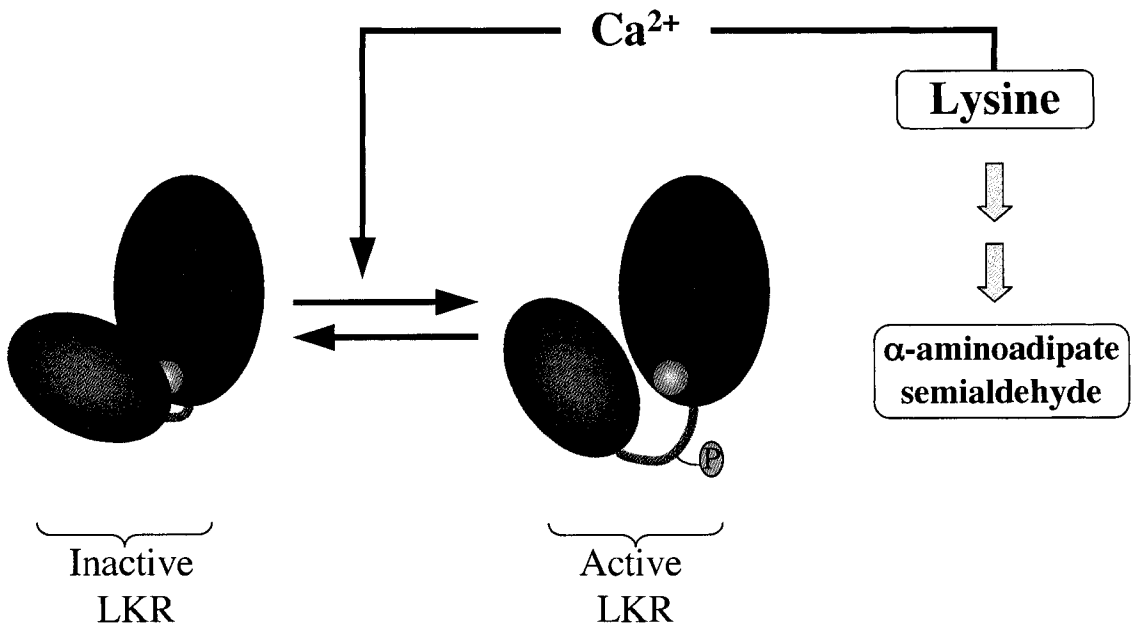


Multiple sequence alignment of human, separate pig sequences, chicken, mouse and Rainbow trout gene sequences. Complete sequences are described in appendix 8.1.3, Figure 8.3 with complete legend descriptions.

roles of lysine in plants. A recently proposed plant model (169, 518) suggests that high lysine levels initiate a Ca^{2+}-dependent intracellular signaling cascade that phosphorylates the LKR/SDH enzyme (perhaps in the linker region) (Figure 1.4) and shifts LKR/SDH enzyme activity from low to high. Sequence analysis suggests that the two enzyme domains are joined by a linker region (448). This linker is likewise apparent in the mouse, maize and *C.elegans* proteins (353). Thus in the non-phosphorylated stage, the SDH domain presumably interacts with the LKR domain masking the LKR active site, but upon phosphorylation, the two domains dissociate to reveal the LKR active site, allowing for a higher level of activity. Analysis of the linker region has identified several amino acids (serine, threonine, tyrosine) that would potentially be available for phosphorylation (Figure 1.4). While plants and mammals share many similarities within the scheme of lysine catabolism, this catalytic mechanism has not been confirmed in any mammalian system. However, the presence of the phosphorylation sites in the linker region does strongly suggest that such a mechanism may play a role in regulating mammalian lysine degradation.

The bifunctional nature of the AASS (LKR/SDH) protein may be an evolutionarily conserved attribute (99) for the purposes of substrate "channeling" – in essence, a more efficient utilization of substrate (324, 362). Saccharopine then, is bound by the AASS protein as an intermediate, and can be "held" until acted upon by the SDH moiety in a sequential fashion. In this way, the "saccharopine pool" within the mitochondrial milieu can be maintained, especially if this compound can provide feedback regulation of LKR. Papes et al. (353) have similarly discussed the possibility that protein phosphorylation may be a means of regulating the activity of the enzyme.

Figure 1.6 Proposed Mechanism for the Lysine-Dependent Stimulation of LKR Activity in Plants



Proposed reaction mechanism of lysine α-ketoglutarate reductase activity. An increase in available cellular lysine causes an increase in cellular Ca^{2+} concentrations. This rise in intracellular calcium presumably signals the phosphorylation of key amino acids in the linker region between the LKR and SDH domains of the bifunctional protein. This phosphorylation event causes a conformational shift that opens or reveals the catalytic site of LKR. Colour code: LKR, blue oblong; SDH, green oblong; active site of LKR, yellow sphere; linker region, red curved line; phosphorylated amino acid, bright green-blue oblong with capital P.

1.3.2 Tissue Localization of the Lysine Degrading Enzymes

One of the well-established dogmas of amino acid biochemistry is that the degradation of indispensable amino acids is primarily a hepatic event (27). While the liver does appear to be the primary tissue of lysine degradation (374), this dogma has led most researchers to focus on the hepatic compartment and neglect other tissues as possibly important sites of degradation. However, the primary enzymes of the catabolic pathway (LKR and SDH; AASS) have been described in various extra-hepatic tissues of several mammalian species including rat (147), mouse (203), dog (175), rabbit (191), pig

(147), sheep (145) and human (220) Table 1.2). Additionally, lysine catabolic enzymes have been described in several avian tissues (190, 289, 485) and in rainbow trout (483). Tissue distribution of LKR activity in rats was demonstrated primarily in liver, but significant activity in kidney, pancreas, brain and heart was also noted (81). In human tissues, the activity of LKR was localized to the liver, kidney, heart and skin (221), but in different relative proportions to the rat (81). When comparing the capacity for lysine oxidation (by the liver, as it was considered to be the major site of oxidation) to the intake of lysine, it is clear that the capacity of the liver to oxidize lysine exceeds daily intake (Table 1.3). However, this rate of oxidation ignores extra-hepatic contributions to the total lysine oxidized. Recent work in 21 day old chicks has described LKR activity and lysine oxidation in several tissues; of particular note, LKR activity was measured in homogenates of breast muscle and intestine (289) (Table 1.4). These tissues have not traditionally been considered sites of lysine degradation, but this work shows clearly that extra-hepatic tissues account for significant lysine oxidation. These authors also showed, using RT-PCR, that all tissues (except pancreas) showed positive signals for LKR/SDH mRNA. In agreement, Northern blot analysis revealed that AASS RNA products were present in a variety of human tissues, with the major portion residing in liver (392) agreeing with earlier enzymatic data of Hutzler and Dancis (220, 221). Contrary to these results, the mouse LKR/SDH gene was found only in liver and kidney (353), yet Manangi (289) notes that work by several teams has shown that the rat has enzyme activity in the liver (35, 204, 403), kidney (320, 321) and brain (374).

The presence of enzyme protein or mRNA, or the detection of enzyme activity *in vitro* does not represent the *in vivo* situation. To gain access to the enzymic, the

Table 1.2 Species Comparison of LKR and SDH Activities

	Specific Activity of Enzymes		
	LKR	SDH	
Species	*(nmol/min/mg)*	*(nmol/min/mg)*	*reference*
Human	4.43	4.70	(147)
Rat	4.77	5.03	(147)
Rat	2.71	2.77	(403)
Mouse	11.08	6.48	(353)
Pig	5.40	5.82	(147)
Cat	4.23	6.90	(147)
Dog	3.50	5.50	(147)
Ox	4.50	4.51	(147)
Sheep	5.33	5.43	(147)
Chicken	0.65	---	(289)

Values derived from liver mitochondria protein in all references except chicken where data are presented as μmol/min/g tissue obtained from a 25% tissue homogenate.

Table 1.3 Capacity of Lysine Degradation by the Liver in Different Species

Species	Estimated Lysine Intake (g/day)	Lysine Degraded[1] (g / whole liver per day)	Capacity for Lysine Degradation as Percent of Intake[6]
Human[2]	2.78	41.39	1489
Rat[3]	0.022	0.10	455
Pig[4]	16.00	18.94	118
Dog[5]	3.72	4.07	109

[1]Data adapted from Table 5, Fellows and Lewis (147). Subjects do not match with those used to estimate intake.
[2]Calculated from estimated mean requirement for 75 kg adult male (37 mg/kg/day x 75kg) (514).
[3]Calculated for a 300g rat at maintenance consuming a synthetic diet containing 1.1% lysine and feed consumption of 20g/day (329).
[4]Calculated for a 120 kg pig fed a 0.52% lysine diet *ad libitum* and 3075 g feed consumption/day (331).
[5]Calculated for a 20 kg adult dog at maintenance consuming 400 g/day of a 0.93% lysine diet (413).
[6]Calculated as the potential amount of lysine that can be degraded by the liver in excess of lysine intake, therefore in the human, the liver has capacity to degrade ~1500% of lysine intake.
Degradation refers to either flux or LKR activity, please see each reference for particular details.

experimenter very often manipulates environmental conditions that affect enzyme regulation (e.g. pH, osmotic pressure, substrate concentration, etc). In other words, results are frequently taken out of the context of the cellular environment. However, that being said, the detection of gene transcription, protein and measured activity is strong evidence that an enzyme is functioning *in vivo*. In swine, the liver has been considered to be the

Table 1.4 Capacity of Chicken Tissues to Oxidize Lysine

	Lysine α-Ketoglutarate Reductase			*Lysine Oxidation*		
Tissue[1]	μmol/min• g tissue	μmol/min• tissue	% of Total	nmol/min•g tissue	nmol/min• tissue	% of Total
Liver	0.65^{a}	1.94	20.5	0.88^{a}	2.63	16.7
Kidney	0.28^{abc}	0.31	3.3	0.43^{bc}	0.47	2.9
Pancreas	0.22^{bc}	0.13	1.4	0.45^{bc}	0.27	1.7
Heart	0.19^{c}	0.19	2.0	0.51^{b}	0.51	3.2
Brain	0.23^{bc}	0.21	2.2	0.53^{b}	0.48	3.0
Lung	0.42^{abc}	0.34	3.6	0.25^{cd}	0.20	1.2
Spleen	0.10^{c}	0.01	0.1	0.20^{d}	0.03	0.2
Muscle[2]	0.15^{c}	3.15	33.3	0.47^{b}	9.87	62.7
Intestine	0.58^{ab}	3.77	39.8	0.20^{d}	1.28	8.1
SEM	0.14			0.07		

[1]Data are taken from Manangi et al. 2005 (289). Values are means, n=10 of tissues isolated from 3-wk-old chicks. Means in a column without a common superscript letter differ, $P< 0.05$. Lysine oxidation was based upon [U-^{14}C]L-lysine.
[2]Muscle refers to breast muscle.

prominent tissue of lysine degradation; extra-hepatic tissues have shown little or negligible LKR/SDH activity (147). However, several groups have recently provided *in vivo* data describing intestinal *utilization* of lysine. *In vivo* data (434-438, 468) from several portal balance studies have suggested that the porcine small intestine contributes significantly to the utilization of dietary essential amino acids, most notably lysine, leucine, threonine and methionine. These reports estimated that the intestine utilized as much as 30% of the available dietary intake of lysine. The complete fate of this utilized lysine remains unclear, however, Stoll and colleagues (438) suggested that no more than 40% of the enterally utilized lysine was directed to protein synthesis by the intestine. Thus the possibility of the remaining lysine being catabolized, presumably as an energy source, is plausible. However, direct evidence to support the presence of lysine catabolic enzymes in the mammalian intestine had not been demonstrated (501) at that time.

1.3.3 Subcellular Localization of Lysine Degradation

The activities of both LKR and SDH have been localized to the mitochondria (145, 337), specifically the mitochondrial matrix (35). However, LKR activity has been reported in both mitochondrial and cytosolic fractions of mouse liver extracts (203). Papes et al. (353) used two computational methods to predict the mitochondrial localization of the mouse liver enzyme after examination of the protein sequence deduced from a cDNA-LKR/SDH clone. They discussed the possibility that the "cytosolic isoform" may be due to activity of the monofunctional SDH, but also state that the method of protein purification did not permit resolution of this argument. Comparable data on the localization of LKR/SDH in swine tissues could not be found in the literature.

Other enzymes involved in lysine degradation have similarly been found in both the cytosol and mitochondria. Aminoadipate semialdehyde dehydrogenase (aminoadipate semialdehyde ⇒ aminoadipate) was found in the cytoplasm using subcellular fractionation techniques for both human (70) and rat liver (461). Indeed, subcellular localization prediction, performed at the PSORT II (325, 326) server (University of Tokyo), predicted a cytoplasmic localization for the mouse (Mus musculus; SWISS-PROT accession #Q80WC9) (238) amino acid sequence. However, it is unlikely that the α-aminoadipate semialdehyde generated in the mitochondria would be transported to the cytoplasm for degradation by the cytosolic isoform. It is more likely that a mitochondrial dehydrogenase, closely resembling the cytoplasmic enzyme described above, mediates the conversion of the α-aminoadipate semialdehyde to α-aminoadipate in a fashion similar to the cytoplasmic ornithine decarboxylase degradation of lysine (358, 363). Such a protein has not yet been specifically identified. BLAST

analysis of the mouse sequence (238) did not reveal any likely candidate proteins. Oxidation of L-[^{14}C]-lysine to $^{14}CO_2$ by isolated washed hepatic mitochondria (204) reinforces the hypothesis that lysine degradation via the saccharopine pathway is entirely a mitochondrial event. Subcellular fractionation of organelles and additional washing of mitochondria will remove any residual cytosolic enzymes capable of utilizing aminoadipate semialdehyde. Therefore one must conclude that a dehydrogenase capable of converting the semialdehyde to aminoadipate is resident in the mitochondria, but as of yet remains unidentified.

Aminoadipate aminotransferase isoforms have been found in both the cytosolic and mitochondrial fractions of human liver isolates (345). AADAT-I, the mitochondrial isoform, was shown to convert α-aminoadipate to α-ketoadipate, whereas AADAT-II , the cytosolic isoform, was shown to convert α-ketoadipate produced by tryptophan catabolism, to α-aminoadipate. This tryptophan-derived α-aminoadipate can then pass through the mitochondrial membrane, via the oxodicarboxylate carrier (149) and be reconverted to α-ketoadipate by AADAT-1. α-Ketoadipate is then oxidized by the α-ketoglutarate dehydrogenase complex to produce glutaryl-CoA and CO_2. This complex has been localized to mitochondria in pig heart and kidney (376, 393, 508), mouse skeletal muscle (51), rat liver and brain (228) and human brain (410) tissues.

The subcellular compartmentilaztion of enzymes often provides one means of regulating flow of particular metabolites through related pathways (233, 345). Hence, defining the organelle(s) in which the enzymes of lysine degradation reside and the relative position of interacting pathways (e.g. tryptophan catabolism) should provide

clues toward a clearer understanding of the regulation of the enzymes themselves and the pathway as a whole.

1.4 Effects of Analogues, Catabolites & Metals on Lysine Catabolism

The pathway responsible for lysine degradation has many similarities across species. Regulation of the pathway however, appears to be species dependent. In human tissues, activity of LKR appears to be sensitive to a variety of metal ions, L-glutamate, L-ornithine, L-homocitrulline, DL-pipecolic acid, cadaverine, L-lysylglycine, carbamyl phosphate and hydroxylamine(221). In the same study, human LKR accepted DL-hydroxylysine, S-2-aminoethyl-L-cysteine or DL-α,ε-diaminopimelic acid as alternative substrates.

Experiments in rats have demonstrated that although the N-acetylated derivatives of methionine and threonine were bioavailable and supported growth of the animal, neither the α- nor the ε-, monoacetylated derivative of lysine nor the α,ε-diacetyl derivative of lysine were effective in significantly promoting the growth of rats (40). Many other derivatives of lysine have been assessed as potential substitutes for lysine including ε-N-methyl-L-lysine, ε-N-dimethyl-L-lysine and ε-N-trimethyl-L-lysine; these compounds were not found to promote growth in mice (162). Finot et al (150) categorized potential sources of lysine (27 α-N- and ε-N-substituted derivatives of lysine belonging to eight different classes: 1) natural dipeptides, 2) α-N-acyl-, 3) ε-N-acyl-, 4) ε-N-α-amino acyl-, 5) ε-N-omega-amino acyl-, 6) α-N-ε-N-di-amino acyl-, 7) ε-N-acylglycyl- and 8) Schiff's bases) on the ability of tissue homogenates to hydrolyze the test compounds to biologically active lysine. While several compounds were completely (natural dipeptides: glycyl-lysine, alanyl-lysine, glutamyl-lysine, lysyl-alanine) or

partially hydrolyzed (ε-N-α-amino acyls: where α-amino group included glycyl, phenylalanyl, glutamyl, and argininyl residues), none were as effective as L-lysine itself for promoting growth.

In the context of swine, many compounds have been added to the diet to enhance performance at some level. For example, metals have been added to the diets of pigs for decades (20). In particular, a dietary excess of zinc has been added to prevent diarrhea, improve immune function and enhance overall performance (469). The most important question still remains: – Does the addition of these types of compounds affect the metabolism or improve or reduce the utilization of lysine? Metals are widely accepted as key components of many enzymatic reactions. Indeed, many aminopeptidases, enzymes which cleave amino acids from the amino-terminus of proteins or peptides, are actually zinc metalloenzymes (453), thus the levels of these metals can affect amino acid metabolism. Supplementation with zinc is often greater than recommended (331), although improvements in growth performance have not been consistently reported (73, 207, 397, 469). Similarly, tissue (Zn) levels were increased following zinc supplementation (73, 397), however sometimes no effects were seen (469). The different supplementation levels of zinc may account for these differences. Copper, added as Cu sulphate (125 – 250 ppm), has also been shown to improve growth in pigs (13, 103, 135, 207), but no data on tissue levels was found. Interestingly, (hepatic) intracellular levels of zinc did not change following supplementation of zinc (sulphate, 80 or 160 ppm) (469). These authors also noted no change in tissue levels of zinc; other studies, which reported increased tissue levels of zinc following supplementation, did not report intracellular

levels. The effects of supplementing these metals on lysine metabolism or utilization have not been reported in the literature and deserve further attention.

Van Kempen and colleagues (471) fed adipic acid, a structural analogue of α-aminoadipic acid, in an effort to reduce lysine degradation and hence improve lysine utilization, as proposed by Cerdan et al.(65) and van Kempen(470). Although animals fed a nursery diet supplemented with adipic acid showed an 18% increase in plasma lysine, no significant change in plasma aminoadipic acid was noted ($P<0.10$) although there were numerical decreases. These promising results led to secondary experiments in which the authors fed a low-lysine diet (70% of the NRC requirement (331)) supplemented with 1% adipic acid. When compared to controls (adequate lysine), or to low lysine fed animals (no adipic acid supplement), the adipic acid-supplemented group did not show any improvement in performance. In fact, these animals actually demonstrated inferior performance to the non-supplemented group. Unfortunately, these authors did not conduct any tissue metabolism research to help explain their results and thus point the way for future research.

Taken together, these experiments highlight that while structural analogues may closely resemble lysine, the biological activity may not be as comparable. Additionally, those analogues that do not inhibit catabolic enzyme activity directly may exert their influence at some other level. Therefore, additional research should focus on pathway regulation across several tissues.

Certain steps of the lysine catabolic pathway are reversible and thus some catabolites can be "synthesized". Therefore, many studies have used pathway intermediates and metabolically related compounds of lysine as potential catabolic

inhibitors. Saccharopine was shown to competitively inhibit LKR activity in human liver homogenate (221). Bovine liver LKR (ammonium sulphate purified and crude homogenate) activity was similarly inhibited by saccharopine (8). Scislowski et al. (403) noted a significant reduction in the conversion of labeled lysine to $^{14}CO_2$ in isolated rat liver mitochondria when saccharopine was added to the incubation medium. In studies using purified LKR from human placenta, Fjellestedt and Robinson (155) showed that saccharopine was a competitive inhibitor with respect to α-ketoglutarate, non-competitive with respect to lysine, and finally uncompetitive with respect to NADPH. These authors further demonstrated that the enzyme was susceptible to substrate inhibition by high concentrations (> ~5mM) of α-ketoglutarate. Therefore, from these last studies, it is apparent that all components of the reaction mechanism become important in the potential product (and substrate) inhibition of LKR.

Lysine and saccharopine, both structurally similar to arginine and arginosuccinate respectively, have been shown to inhibit enzymes of the urea cycle (7). Hence the effects of the urea cycle intermediates on LKR activity are similarly possible. Citrulline was found to moderately inhibit semi-purified LKR (8). A 20% reduction in activity at 10mM citrulline was noted. The normal [citrulline] (concentration) in cells is estimated to be less than 50 μM and plasma [citrulline] to be about 20μM. These are far below the [citrulline] (concentration) required to elicit inhibition of LKR in vitro (8).

Homocitrulline, structurally very similar to lysine and a natural product of lysine metabolism (171, 172, 262, 391), was not metabolized in the intact, perfused rat model. In these studies the liver failed to concentrate the infused homocitrulline, suggesting that homocitrulline was transported with difficulty either into or from the liver to the

perfusate. Homoarginine, the subsequent metabolite of homocitrulline, can however, be metabolized to urea and lysine via the catalytic action of ornithine transcarbamylase (OTC) (61, 212, 390). Given that OTC is found in the pig intestine (114, 119), it is possible that homocitrulline absorbed by the intestine (141) can be converted to homoarginine and transported to the liver or kidney. Once in these tissues, homoarginine can be acted upon by arginase and metabolized to lysine and urea (390). Furthermore, both homocitrulline and homoarginine can be derived from (injected) lysine (391). While homoarginine has been described as a non-metabolizable marker for measuring endogenous amino acid flow in the gut (209, 319, 339), there have been reports of dietary homoarginine having some benefits. Addition of 1.0% DL-homoarginine to a lysine-free rat diet resulted in a limited growth response after a 6-12 day delay (431). In poultry, ingested homoarginine was distributed throughout the body, but depressed feed intake (10). In another study, dietary homoarginine did not improve feed intake or weight gain above the unsupplemented diet provided to control chicks (12). Human liver LKR (partially purified) activity was decreased by 49% in the presence of 3 mM homocitrulline (221). Data for the inhibitory potential of homoarginine was not found.

These experiments demonstrate that lysine α-ketoglutarate reductase activity can be affected by a wide variety of compounds. Unfortunately, similar experiments have not been performed in a wide variety of species (rat, cow, human) or in all tissues of potential interest. No data describing porcine LKR and SDH inhibition by any of these compounds was found in the literature.

1.4.1 Dietary Effects

One of the primary goals of lysine research in the livestock industry is to improve the lysine utilization by the animal. One of the goals of the work described in this thesis was to investigate how to better manage the lysine requirement of the animal. Implementing dietary regimes encompassing a range of lysine concentrations provides direct examination of the relationship between LKR/SDH activity and dietary levels of lysine. Diets providing an excess of dietary lysine to rats have been shown to cause an increase in LKR activity in rats (160, 219, 321). Muramatsu and colleagues (321) showed that when dietary lysine levels exceeded the (apparent) lysine requirement for protein synthesis in rats, hepatic LKR activity increased relatively quickly (within 1-2 days), and plateaued at ~10 days. Following this plateau, LKR activity appeared to decrease in those animals being fed the highest level of lysine. The increase in activity was similarly noted in kidney homogenates, but the activity of LKR did not decrease after 10 days. In comparison, when rats were fed diets deficient in lysine (~ 90% requirement), the activity of LKR was reduced to presumably basal levels after 2 days (160). Unfortunately, the authors did not perform a crossover study to investigate how long the LKR activity would remain at the low (basal) level if the diet was switched to lysine-adequate or lysine-excess. Similarly, hepatic (10% homogenate) LKR activity decreased significantly when rats were fed a lysine-free diet, and increased by more than three-fold when fed a dietary excess of lysine (81). Rats fed protein-free diets did not demonstrate a decrease in LKR activity compared to controls, however consumption of graded wheat gluten diets showed that as the gluten concentration increased (and presumably the availability of lysine) the activity of LKR increased. However at low

levels of wheat gluten (10%), the activity of LKR was decreased below that seen in a protein-free diet (81). This may be due in part to the faster absorption kinetics of synthetic amino acids versus protein bound amino acids. The concentration of amino acids, and thus lysine, entering the portal blood and the liver will more quickly reach higher concentrations, high enough perhaps to elevate the LKR activity. That LKR activity was more dependent on dietary lysine level rather than protein level or source was likewise acknowledged by Muramatsu (321). Using a mouse model, Papes et al. (353) demonstrated that both the enzyme activities and mRNA levels for LKR and SDH (encoded by Mus-Lor/Sdh) increased when L-lysine was injected intraperitoneally. Conversely, 48 hours after intraperitoneal injection of saccharopine, a 25% decrease in LKR activity, but no decrease in mRNA levels was seen. Additionally, no change in SDH activity was seen in these mice. If the saccharopine were acting as an inhibitor of LKR, interacting at the binding site for lysine, it is plausible that saccharopine bound in this fashion may be able to interfere with LKR activity, but not be available for SDH catalytic activity. The authors reasoned that this decrease in LKR activity was the result of a post-translational modification of the enzyme *in vivo* and not an *in vitro* effect. They cited unpublished data from their laboratory referring to protein phosphorylation modulation of LKR activity in maize and also similar effects seen in Arabidopsis (237). Thus saccharopine may act simply as a competitive inhibitor of LKR for lysine, rather than causing enzyme induction, which is in agreement with the early enzyme data of Hutzler and Dancis (221).

During starvation experiments, the activities of both LKR and SDH were increased approximately 50% in mice (353). This increase in activity was paralleled with

an 80% increase in the Mus-Lor/Sdh mRNA levels. The catalytic activities of LKR and SDH in mice thus appear to be influenced at the level of transcriptional regulation. Similar increases in mRNA levels have been reported for other amino-acid degrading enzymes during starvation (459). Lysine is a ketogenic amino acid and so its degradation can provide carbon skeletons to fuel the citric acid cycle in situations of limited carbon supply such as starvation. In this way, lysine degradation can be coupled to the energetic balance of the cell and the organism.

1.4.2 Transport of Lysine

A broad range of transporters have evolved to facilitate the movement of amino acids across the many membranes within different cell types depending upon the physiological state. These transporters have been classified into distinct systems based upon various criteria including substrate specificity and transport mechanism involved as well as regulatory properties (for more complete discussions please see the following reviews (46, 54, 80, 120, 156, 224, 249, 254, 263, 287, 290, 295, 301, 318, 425, 481)).

The transport of cationic amino acids was thought, for a long time, to occur via a single transporter, system y+ (124). Today, through the use of new molecular and kinetic approaches, several systems have been described detailing unique and distinct cationic amino acid transporters with differences in structure, substrate specificity, mechanism, distribution and regulation. These systems have been summarized in Table 1.3. In short, cationic (lysine) transport is mediated by both sodium dependent and independent active transport systems (for an in-depth discussion on cationic amino acid transporters, please see especially Devés and Boyd (124).

1.4.2.1 System y^+: the Na+-Independent Cationic Amino Acid Transporter

System y^+ was initially described, or at least the concept of the system was first described, as the transporter for cationic amino acids (with a lesser affinity for neutral amino acids in the presence of Na^+) during the early work of Christensen et al (76, 77) in Ehrlich tumor cells and reticulocytes. These descriptions became the paradigm for cationic amino acid transport and were extended to many other cells and tissues. More recent evidence (472) has shown that some systems recognize both cationic and neutral amino acids with high affinity whereas other systems recognize distinctly cationic amino acids. These observations have led to the question of whether the observations of system y^+, described earlier, reflect one transport system or the sum of two separate systems (124). Originally, Christensen (75) noted that lysine transport was partially (60-70%) inhibited by phenylalanine, compared to only 20% inhibition of phenylalanine uptake by lysine. The authors referred to this "lysine accepting system" that could also recognize neutral amino acids as a "positive-charge tolerant variant of the L system... system L^+." Later, a second system referred to as the "lysine-preferring agency", named Ly+, had been distinguished from the original "lysine-accepting agency (L^+) (79)." When lysine transport was investigated in rabbit reticulocytes (76), Christensen described a saturable process for lysine, arginine and ornithine that was different from the Ehrlich cells. Unfortunately, instead of recognizing two distinct systems in Ehrlich cells and reticulocytes, the observations were unified into one paradigm – system Ly^+ (78). It was more than a decade later before evidence was presented describing a transport system in

cultured human fibroblasts with marked selectivity toward cationic amino acids (491). This was where *system* y^+ was first used to indicate that the transport system was not used exclusively for lysine transport, but also included arginine, ornithine and other cationic analogs.

The proteins encoding system y^+ transporters have been named Cationic Amino acid Transporters, or CAT for short. The first member of the CAT family, mCAT-1, was identified as the receptor for murine ecotropic leukemia viruses (5). Similarities between the receptor and certain permeases from *Saccaromyces cerevisiae* led to the discovery that the virus receptor was actually a Na^+-independent transporter of cationic amino acids (198). The other CAT proteins (CAT-2A, CAT-2B & CAT-3) have been identified in different mammalian species including humans (89, 90, 216, 478).

Characteristically, the CATs demonstrate concentration-dependent stimulation of transport by substrate at the opposite side of the membrane – *trans* stimulation (198). While CAT-1 has the most pronounced trans stimulation of the family of proteins, CAT-2A is relatively insensitive to this type of simulation.

1.4.2.2 Systems $B^{0,+}$ and $b^{0,+}$: the Na^+-Dependent & Na^+-Independent Broad-Scope Transporters

System $B^{0,+}$ was originally described in mouse blastocysts and represents the only sodium (and chloride) dependent transport system for cationic amino acids (473). The transporter protein, $ATB^{0,+}$, has a substrate specificity similar to system $b^{0,+}$, but can also accept small neutral amino acids such as L-alanine and L-serine. $ATB^{0,+}$ belongs to a family of sodium and chloride-dependent neurotransmitter transporters (416). The mouse

homologue has been detected in the cecum and colon and transports a variety of nitric oxide synthase (NOS) inhibitors (197). This may be important for regulating bioavailability of these compounds to particular areas in the hindgut, including regions of the enteric nervous system in this area. Work by Van Winkle and colleagues (472, 473) further identified the transport capacity of a sodium-independent and broadscope transporter that showed high affinity for leucine and lysine and accounted for the majority of transport of these amino acids at low concentrations (1μM). This system was designated system $b^{0,+}$. This transporter, $b^{0,+}$AT preferentially utilizes cationic and bulky neutral amino acids, that do not branch at the α-carbon or the β-C-atom (contrasting the preferences of system $B^{0,+}$). The $b^{0,+}$ system has high affinity (K_m in the μM range) in the apical membrane of small intestinal and renal proximal tubule (S3 segment) epithelial cells and a low affinity occurring predominantly in the S1/S2 segments of proximal tubules (124). The $b^{0,+}$AT functional unit is a heteromer and functions as an obligatory exchanger like y^+L (88).

1.4.2.3 System y+L: The Cation-Modulated Broad-Scope Transporter

Devés and co-workers (125) identified a transport system in human erythrocytes in which neutral amino acids (leucine) inhibited the influx of lysine (held at μM in these particular studies) to 50% of the original rate. They went on to demonstrate that lysine enters erythrocytes via two transport systems: 1) a high-affinity low capacity transporter, which recognizes leucine and lysine similarly (y^+L) and 2) a lower affinity, high capacity transporter specific for the cationic amino acids (system y^+).

Table 1.5 Mammalian Systems of Lysine Transport

System	Protein	Gene	Tissue Expression	K_m (lysine)	Notes
Neutral amino acid transporter: sodium-dependent					
A	ATA3	SCL38A4	Liver cell line(196)	300μM for L-Arg, no Lys done (196)	First report of a subtype of System A to transport cationic amino acids
Cationic-amino-acid transporters: sodium dependent					
$B^{0,+}$	$ATB^{0,+}$	SLC6A14	Blastocysts, Xenopus laevis oocytes, fibroblasts (303)	140 μM (473)	Blastocysts and possibly brush border membrane. Broad specificity for neutral and cationic amino acids. Accepts BCH.
y^+L^*	y + LAT1 y + LAT2	SLC7A7 SLC7A6	Placenta, erythrocytes	~10-13.9 μM (125)	Na^+-independent cationic / Na^+-dependent neutral-amino-acid exchanger. Electroneutral.
Cationic-amino-acid transporters: sodium independent					
$b^{0,+**}$	$b^{0,+}AT$	SLC7A9	Blastocysts, Xenopus laevis oocytes, fibroblasts (303)	100μM (88)	Broad specificity cationic- and neutral amino acid exchanger.
y^+	CAT-1	SCL7A1	All but liver (198)	70-250μM (198)	Cationic-amino-acid (and Na+-dependent neutral amino acid) transport.
	CAT-2	SCL7A2			Variable degree of *trans*-stimulation.
	2A		Liver, muscle, skin (352)	2A: 2-5 mM (198)	CAT-2 also contains splice variants (42 amino acid difference) CAT-2A and CAT-2B (89)
	2B		T cell, lung, testis, macrophage	2B:38-380μM(198)	
	CAT-3	SCL7A3	Brain (352) Pancreas, heart, skeletal (352)	3:40-450μM (198)	
	CAT-4	SCL7A4	muscle, placenta >>>brain, lung, liver, kidney (352)	4: ?	

Transmembrane transport of lysine is catalyzed by three main systems and five subsystems.
*Holotransporter formed upon association with the CD98 glycoprotein encoded by the gene SLC3A1;
**Holotransporter formed upon association with the rBAT glycoprotein encoded by the gene SLC3A2. BCH, α-aminoendobicyclo -[2,2,1]heptane-2-carboxylic acid.
Table adapted from Table 1 (224).

1.4.2.4 Lysine Transport as a Regulator of Lysine Oxidation

Lysine α-ketoglutarate reductase and saccharopine dehydrogenase have, as mentioned earlier, been localized to the mitochondrial matrix of the rat liver. The authors suggested that transport of lysine into the matrix was a prerequisite for lysine catabolism (35). Their arguments were based on findings in ornithine catabolism which demonstrated a transport limitation (91, 302) into the mitochondrial matrix and also that the ornithine degrading enzymes, ornithine transcarbamylase and ornithine aminotransferase are localized to the mitochondrial matrix. Blemings et al. (35) listed four factors in support of their transport regulation premise: i) The enzymes LKR and SDH are localized to the mitochondrial matrix; ii) Fibroblasts from hyperlysinemic patients had normal enzyme activities of LKR and SDH but demonstrated a 50% decrease in the oxidation of lysine (350); iii) Uptake of ornithine, a cationic amino acid like lysine, was a controlling feature of ornithine metabolism; iv) Transport rates of lysine into mitochondrial matrices were not increased when the rats were fed a high protein diet (60% casein), unlike the LKR and SDH activities, which were significantly increased (36). Using a variety of calculations, Blemings et al. (35) (Table 3 therein) showed that as dietary protein content increased, the amount of oxidized lysine increased but did not differ from the amount (of lysine) transported into the mitochondria. However, the activities of LKR and SDH under these parameters, predicted a significantly greater flux of lysine through the pathway, and hence a greater amount of lysine oxidized. Therefore, the authors (35) concluded, that the oxidation of lysine was limited by the amount of lysine being transported into the mitochondrion. These data

suggest that lysine uptake into mitochondria may provide a regulatory limit on the potential catabolism and subsequent oxidation of lysine.

While the importance of lysine transport as a significant regulator of lysine catabolism is acknowledged, the studies were limited to the liver of the rat. The same transport limitation may not be as prevalent in other tissues such as the kidney, muscle or brain. Unlike rats, pigs and chickens as well as other organisms (trout) have been selected for optimal performance parameters, including a high rate of protein synthesis. These selection parameters may influence the impact of transport regulation on lysine catabolism. Furthermore, growing animals have very different lysine requirements than animals at maintenance and therefore, the transport limitation may be a function of age. These questions indicate that while transport of lysine to the environment of the lysine catabolic enzymes is important, there are many more questions about the interactions of age, tissue and organism on this regulatory mechanism.

1.4.3 Hormonal Influences on Lysine Metabolism

Hormones, by affecting the turnover rates of key proteins through signal cascades or direct interaction, can modulate cell differentiation and growth (118). Thus hormones can direct the flux of particular substrates through metabolic pathways. De Feo (118) reviewed the hormonal influences on human protein metabolism. Table 1.4 provides a summary of the reviewed data on leucine, with an additional column for lysine.

These data indicate that different hormones play different roles in protein synthesis and hence in the oxidation of amino acids. Relatively few studies have been performed examining hormonal influences on lysine oxidation, a surprising deficiency,

Table 1.6 Hormonal Effects on Whole-body Protein Synthesis and Breakdown and the Rates of Leucine and Lysine Oxidation in Humans.

Hormone	Protein		Amino Acid Oxidation	
	Synthesis	Degradation	Leucine	Lysine
Adrenaline	=[1]	↓	↓	?
Androgens	= / ↑	= / ↑	↓	?
Glucagon	= / ↓	= / ↑	↑	↓
Glucocorticosteroids	=	↑	↑	?
IGF-1	↓	↓	= / ↓	?
Insulin	↓	↓	= / ↓	?
Thyroid hormones	↑	↑	↑	?
Somatotropin	↑	↓	?	↓

[1]Symbols : = , no change; ↑, increase or up-regulation; ↓,decrease or down regulation.

given the nutritional importance of lysine. Glucagon, has been shown to increase oxidation of leucine (118) and has been shown to similarly influence lysine oxidation. Scislowski and colleagues (403) demonstrated that the activity of the first three enzymes in the lysine oxidative pathway in rat liver mitochondria were increased 107%, 80% and 23% respectively by glucagon treatment (rats were killed 30 minutes following a single injection of glucagon). In contrast, Hussein and Muller (219) injected glucagon twice daily for three days and found a 40% decrease in LKR activity after 24 hours but normal activity after 72 hours. After 168 hours (one week) the daily glucagon administration caused a significant (150%) increase in LKR activity compared with control rats (219). Others showed that glucagon stimulated LKR activity by 2-3 fold in both the adult rat liver and brain (374). Somatotropin (growth hormone), used to increase protein synthesis in the absence of dietary manipulation, reduced whole body lysine oxidation in female rats by 40% (166). No research was found in the literature reporting the effects of the

other hormones listed in Table 1.4 on lysine oxidation. Very clearly there is a need to provide this information.

1.4.4 Aberrant Lysine Metabolism

Genetic-based aberrations and clinical problems in lysine catabolism have been described and a concise review is available (99). Investigations examining the metabolic loci, to explain clinical manifestations of hyperlysinemia, eventually led to the conclusion that the saccharopine pathway was the main route of lysine degradation (108) and that the pipecolic pathway was a secondary, overflow pathway (99). To date, defects of each of the enzymes in the pathway have been linked to various disease states (Table 1.5). Of note, there is a description of defective mitochondrial transport of lysine leading to hyperlysinemia (350). A similar defect in an ornithine mitochondrial transporter has likewise been described(56, 183, 213, 227, 259, 349, 351).

Table 1.7 Abnormalities in Lysine Catabolism

Enzyme name	Adverse Effects	Associated with Inborn Error	Reference
Lysine-ketoglutarate reductase	Challenged mental development (nil to severe), convulsions, poor growth, anemia, muscular & ligament asthenia	Hyperlysinemia	(108, 467, 496, 498)
Saccharopine dehydrogenase	Challenged mental development	Saccharopinuria, Hyperlysinemia	(146)
Aminoadipate-semialdehyde dehydrogenase	Degenerative neurological disease and hepatomegaly	Hyperpipecolatemia	(170)
Aminoadipic aminotransferase	Challenged mental development	α-Aminoadipic aciduria	(153)
Ketoadipic dehydrogenase	Challenged mental development, psychomotor and speech retardation, seizures, hypotonia, dysmorphic features	Ketoadipic aciduria	(361, 371, 489, 495)
Glutaryl-CoA decarboxylase	Neurodegeneration, psychomotor retardation	Glutaric aciduria	(210, 258, 272, 443, 507)

Adapted from Dancis et al, 1976 (109).

The aberrations in lysine catabolism illustrate that lysine is primarily catabolized via the saccharopine pathway. That other animals are susceptible to similar inborn errors of metabolism is presumed, although finding clinical examples in these animals would be highly unlikely. Most of the defects in lysine catabolism lead to neural degeneration, dysfunctional motor control and delayed development and growth; these phenotypes would ultimately mark any domestic animal as substandard. Because domestic animals with these defects demonstrate a low growth rate and abnormal behaviour they would be culled or die before reaching market weight or breeding age. Research on lysine catabolism would benefit from an animal model of genetic defects in lysine catabolism. Domestic animals raised for meat would have a very low prevalence for any of the defects due to intense selection pressure. Therefore "wild-type" or unselected populations would present an increased chance of success. Simple urine testing for abnormal lysine or lysine catabolite concentrations would be a diagnostic.

1.4.5 Functions of Lysine Other than Protein Synthesis

The majority of the research on nutrition of domestic animals relates primarily to the goal of improved utilization of lysine for protein synthesis. However, several studies elucidated alternate roles for lysine. One of the most important roles for lysine is the synthesis of the mitochondrial fatty acid carrier carnitine. Lysine-HCl supplementation to swine diets and to human populations consuming mainly grain-based diets has been shown to reduce stress (419, 429). The possible link between lysine and hypercholesterolemia is also very interesting.

1.4.5.1 The Carnitine Connection

Carnitine (L-3-hydroxy-4-N,N,N-trimethylaminobutyrate), an essential metabolite in intermediary metabolism, plays a number of significantly important roles. Most prominent perhaps is the transport of long chain fatty acids from the cytosol into the mitochondrial matrix, facilitating the process of β-oxidation. Products of peroxisomal β-oxidation (acetyl-CoA) are also transferred to the mitochondria by carnitine-facilitated transport for further catabolism in the Krebs's cycle (for further details on mammalian carnitine metabolism, the review by Vaz and Wanders (477) is recommended).

Lysine forms the backbone of carnitine and methionine provides the 4-*N*-methyl groups (98, 214, 450). Carnitine synthesis begins when the ε-amino group of protein-bound lysine is trimethylated; this occurs as a post-translational event in particular proteins such as calmodulin, myosin, actin, cytochrome c and histones (477). Methyltransferases (specific to the protein in which lysine is bound) catalyze the transfer of a methyl group from S-adenosylmethionine (SAM) to the ε-amino group of the protein-bound lysine moiety. Following the formation of the trimethylated lysine, a series of reactions proceed to generate carnitine (Appendix 8.2); the reactions in the pathway have been extensively reviewed elsewhere (60, 375, 477). Dietary sources account for approximate 75% of carnitine in omnivorous man, the remaining 25% being derived from *de novo* synthesis from protein-bound lysine (477). In comparison, strict vegetarians synthesize ~90% of carnitine *de novo*; thus most growing animals who are normally fed a primarily vegetarian diet, would have to synthesize the vast majority of required carnitine. The synthesis of carnitine from free lysine has been explored in different models (11, 215, 451) and under "normal" conditions has been considered to be very

small proportion of total lysine requirement, < 1% of the total lysine intake (451). However, a lysine-deficient diet (126, 261, 451) or genetic or medical aberration (leading to a requirement for total parenteral nutrition for example) eventually leads to decreased levels of carnitine in several tissues of the body (31, 130, 447) (with the exception of the liver). This decreased tissue carnitine concentration suggests that the synthesis of carnitine is sensitive to dietary lysine intake. When lysine intake was deficient, release of lysine from protein breakdown was conserved for required protein synthesis rather than carnitine synthesis in extra-hepatic tissues. Furthermore, excess lysine has been shown to actually decrease plasma carnitine but to increase the carnitine precursor trimethyllysine (113). The authors suggested that in this scenario, the excess dietary lysine would spare more endogenous lysine for carnitine synthesis, while maintaining protein synthesis.

Several studies have shown the beneficial effects of supplementing pig diets with L-carnitine (30, 199, 200, 322, 348). Other studies have shown contrasting results. For example, Cho et al (74) observed no appreciable improvement in the performance of newly weaned pigs when 1000 mg/kg carnitine was supplemented. Benefits from added carnitine are most likely due to improved lipid oxidation and hence energy metabolism by the animal as opposed to sparing of dietary lysine for protein synthesis.

1.4.5.2 Lysine and the Stress Response

The exact nature of the relationship between gastrointestinal and metabolic function and stress remains unclear, yet it is known that stress can exacerbate complications of either (423). Many housing and farming practices inherent to the modern swine industry increase the stress levels of pigs. Restricted floor space, regrouping, weaning, air quality, and transportation are among some of the several

stressors that can lead to compromised endocrine and immune function in pigs; ultimately detrimentally affecting growth, meat quality and the welfare of these animals (429). Several lines of evidence are now pointing towards possible roles for lysine to counter these various stressors.

Acting as an inhibitor of arginine uptake, lysine supplementation in excess of requirement has been shown to limit nitric oxide (NO) overproduction in endotoxic shock in rats (281). Similar scenarios have been documented in neonatal piglets (62). During periods of stress, the requirement for L-arginine by inducible nitric oxide synthase (iNOS) may exceed availability from intracellular depots and may depend upon the extracellular availability of L-arginine. Regulation of iNOS then is dependent upon L-arginine transport into the cell via CAT (y+) proteins (281). Because L-lysine is transported by similar transport systems as arginine (Table 1.2) it may actively inhibit extracellular arginine uptake.

A series of investigations have demonstrated plausible links among lysine and several stress hormones. Chronically administered lysine in mice acted as a partial agonist on central benzodiazepine receptors (71) resulting in an anticonvulsant effect that diminished after ten days. Lysine imbalances have also been shown to affect central neurotransmitter systems (norepinephrine and serotonin) (177, 420, 421) and brain areas (the amygdala and the hypothalamic region) (420) that are implicated in regulation of stress and anxiety.

Using restraint stress on rats (appendages wrapped in adhesive tape), Smriga and colleagues showed that lysine administration could depress pathologies of stress, including incidence of diarrhea (422) and fecal output (422). Following restraint stress in

rats, plasma corticosterone was reduced using a mixture of lysine and arginine (424). Following these experiments, Smriga and Torii (422) began to decipher the mechanism by which lysine affected the stress response. Noting that aberrant serotonin (5-hydroxytryptamine, 5HT) responses were involved in stress-induced anxiety and gastrointestinal disorders, and that the 5-HT_4 receptors were localized throughout the intestine, these researchers thought an "interesting candidate" to blunt these effects would be lysine. Their results suggest that lysine may indeed be a partial 5-HT_4 antagonist, blocking the binding of 5-HT by almost 10%. Based on the results of the earlier work implicating lysine and arginine in reducing stress responses, it was hypothesized that fortifying a finishing-pig diet with lysine and arginine in the week preceding slaughter would reduce the stress associated with transportation to market (429). The results demonstrated that the fortified diet reduced plasma cortisol levels (8h) prior to and (8h) following transportation. In addition, the pigs demonstrated a reduction in stress-induced anxiety behaviour that translated into improved/safer handling of the animals. The animals receiving the lysine-arginine fortified diet were larger (~1 kg) but this increase was not significant. If additional supplementation of lysine and arginine in the week preceding slaughter serves to improve animal welfare (via stress reduction), then this practice would appear to be important for producers of domestic livestock to consider

1.4.5.3 Lysine and Hypercholesterolemia

Animal proteins such as casein are more hypercholesterolemic than soy protein or other plant proteins (58, 442). This fact is readily apparent in rabbits fed low-fat, cholesterol free, semi-purified diets (268). A series of studies by Carroll and co-workers (59, 179, 180, 217, 266-268) have suggested a role for essential amino acids, notably

lysine, arginine and methionine, in the regulation of hypercholesterolemia. Summarizing the results of several experiments (265, 266), it was shown that when rabbits were fed diets enriched (3-fold over control) with selected essential amino acids, all essential amino acids, except arginine, had hypercholesterolemic potential; lysine and methionine seemed to be the most potent. Later work by these authors showed that although dietary methionine enhanced the effect of lysine (268), causing a further increase in serum cholesterol and liver phospholipids, addition of arginine to the lysine plus methionine-enriched diet partially prevented the increase in serum cholesterol and liver phospholipids (180). Mechanistically, these effects may be explained by a down-regulation by amino acids of hepatic LDL receptors (72, 265-267) and increased synthesis of LDL apolipoprotein B (247).

The effects of dietary lysine on cholesterol represent another facet of lysine metabolism outside of its role as a substrate for protein synthesis although the mechanism is unclear. Also highlighted is the fact that many of the essential amino acids participate in various metabolic functions as groups rather than individual facilitators.

1.5 Summary

Highlighted and emphasized throughout the *Introduction* is the complexity that is associated with the control of lysine utilization, metabolism and catabolism. The bifunctional nature of the LKR enzyme protein controlling the rate-limiting step* in the degradative pathway indicates that the regulation of lysine catabolism is under tight

* LKR is understood to be a metabolic control point in the catabolism of lysine, based upon the K_M of the enzyme for lysine compared to the K_M of the other enzymes in the pathway. Other factors, including transport of lysine are also *rate-limiting*, we shall use this term in accordance with convention recognizing the above restrictions.

control. Mixed with the demands of the industrial sector for improved lysine economics, is the academic quest for a greater understanding of how amino acids, and their metabolism, allow the rates of protein synthesis and degradation to achieve a homeostatic balance. To say that we do not understand fully the roles of amino acids, and lysine in particular is an understatement. More research is needed to delineate both general and specific aspects of lysine metabolism including: intracellular transport mechanisms, inter-organ metabolism, the influence of transcriptional and translational controls, the effects of various hormones, the effects of catabolites (both from lysine and other amino acids (e.g. tryptophan), regulatory changes during age and development (e.g. growth versus maintenance), gender, species, genetic selection (especially in domestic livestock).

2 General Hypothesis of the Thesis

Lysine, an essential dietary amino acid, is found in low concentrations in the cereal grains that constitute the major portion of diets fed to growing pigs. More specifically, the low concentration of lysine in these grains is low enough to limit the rate of protein synthesis. This causes several problems. Initially, the low lysine concentration results in all other amino acids being in excess, hence they must be degraded by the animal. This results in wasted nutrients and energy by the growing animal. These wastes spill into the environment, a fact that must be considered by the livestock industry. Any practice that does not, in some way, improve the performance of the animal costs the producer money. To combat this problem, synthetic lysine is added to the diet to meet the demands of protein synthesis. Unfortunately, the lysine requirements of individual pigs varies quite substantially. The old adage that more is better does not apply; excess lysine does not improve performance in a linear fashion. After a critical point, excess lysine will have a negative effect on performance. Furthermore, synthetic lysine is expensive and as such it is more likely that lysine, in a typical pig diet is very seldom in excess. Experiments using radioactive tracers to measure lysine oxidation have shown that the basal rate of lysine degradation does not decrease until lysine falls below 60%-70% of requirement. Not only is this counter-intuitive, but contrasts with the degradation of other indispensable amino acids which decrease rapidly to a lower rate when the intake of the amino acid is below requirement. This difference indicates that the breakdown of lysine is tightly controlled and quite possibly the catabolites may be important for alternate, as yet unknown, metabolic roles. Mechanisms regulating the breakdown of lysine in mammals remain poorly understood.

At the cellular level, lysine degradation is controlled by both lysine transport into the mitochondria, and by those enzymes catalyzing its breakdown. The so-called rate-limiting enzyme is the bifunctional protein, α-aminoadipic semialdehyde synthase, constituting the activities of α-lysine ketoglutarate reductase and saccharopine dehydrogenase. Once acted upon by these enzymes, lysine cannot be utilized for protein synthesis because this step is irreversible in animals. As with mechanisms indirectly affecting the rate of lysine degradation, the mechanism of enzymatic catalysis is unclear. Research investigating the plant isoform of this enzyme-pair has revealed a complex network of regulation. Mechanistically, catalysis is facilitated by a Ca^{2+}-driven phosphorylation activation of the enzyme. The activation mechanism for the mammalian isoforms has not been described.

Delineation of the mechanisms that regulate activation and catalysis would provide the necessary knowledge and insight to begin manipulation of key components of the pathway. Understanding the metabolic responses of lysine catabolism under key environmental stressors (e.g. lysine limitation, weaning, transportation) will enable researchers to define strategies to reduce lysine supplementation or at least to better manage lysine supplementation to livestock. These strategies may take advantage of, for example, potential inhibitory compounds that slow the rate of activity of the key enzymes in the degradative pathway. In this way, feeding regimes that incorporate addition of candidate lysine catabolic regulators may be devised.

Typically, the liver has been considered to be the sole organ of importance when discussing lysine breakdown. This premise was built upon techniques that were not sufficiently sensitive to adequately measure lysine degradation at the cellular and

organelle level in all tissues. However, based upon more current *in vivo* and *in vitro* data, we hypothesize that extra-hepatic tissues including kidney, muscle and intestine can significantly contribute to the overall degradation of dietary lysine. These contributions need to be considered when discussing whole-body lysine utilization and degradation. Our goal was to examine the regulation of lysine catabolism at the cellular level in different tissues to provide a more complete understanding of mechanisms that play key roles in defining lysine utilization in growing animals.

Hypotheses

- Lysine catabolism is regulated by LKR activity.
- Extra-hepatic tissues in pigs and chickens can catabolize lysine.
- Catabolized lysine in extra-hepatic tissues is a relatively important component of whole body capacity for lysine degradation.
- Age affects lysine catabolic capacity in the pig.
- Increasing dietary lysine intake increases lysine catabolism.
- Metabolically and structurally related compounds can affect the capacity for lysine catabolism.

3 Enzyme Kinetics and Cellular Regulation of Lysine Catabolism in Tissues of the Pig

3.1 Abstract

One of the key regulators of lysine catabolism in pigs is the activity of lysine α-ketoglutarate reductase (LKR), the rate-limiting enzyme in the lysine catabolic pathway. Past research indicated that activity of this enzyme was predominantly hepatic; more current *in vivo* data suggest that other tissues can also significantly catabolize lysine. We sought to describe the extra-hepatic activity of LKR in various tissues of the pig. Using mitochondria from various tissues of market age pigs, we measured the activity of LKR and saccharopine dehydrogenase (SDH). Liver mitochondria had the highest LKR activity, but appeared to be subject to substrate inhibition. Mitochondria from muscle, kidney, heart and intestinal epithelial cells all had measurable LKR activity. LKR activity was significantly inhibited by a variety of compounds including saccharopine, α-aminoadipate, α-ketoadipate, 5-hydroxy-L-lysine, and different metals. Oxidation of lysine to $^{14}CO_2$ was demonstrated in mitochondria isolated from liver, muscle and intestinal epithelial cells. Antibodies against the enzyme provided evidence of the protein localization in different tissues. Our results demonstrate that lysine degradation in extra-hepatic tissues, most notably in cells of the intestine and muscle, should be considered significant when discussing whole-body lysine utilization.

3.2 Introduction

Efficient utilization of the indispensable amino acids is of great importance in swine and poultry production. This is especially true for lysine, often the first limiting

amino acid in cereal grains, which are used as the major constituent of most animal feedstuffs. Consequently, crystalline lysine (Lysine-HCl) is added to the diet to meet the dietary requirements for optimal growth and protein deposition, demanded by animal production. Until recently, nearly all of the lysine research focused on dietary manipulations (e.g. optimizing grain mixtures, inclusion of synthetic amino acids) to enhance and optimize parameters like growth, feed efficiency or carcass weight. In fact, very few investigations have examined the regulation of dietary lysine utilization. Determination of cellular factors and molecular pathways controlling the metabolic fate of this limiting essential amino acid may be the key to truly optimize future lysine supplementation and utilization.

Lysine ketoglutarate reductase (LKR; EC 1.5.1.8) and saccharopine dehydrogenase (SDH; EC 1.5.1.9) catalyze the initial reactions in the main metabolic route of lysine catabolism in mammalian tissues (Figure 1.3). Known as the saccharopine or α-aminoadipate semialdehyde pathway, the degradation of lysine begins with the condensation of the amino acid with α-ketoglutarate, yielding saccharopine [ε-N-(L-glutaryl-2)-L-lysine]. The dehydrogenase activity then catalyzes the oxidation of saccharopine to α-aminoadipate δ-semi-aldehyde and glutamate. These initial reactions have been ascribed to a single polypeptide entity, α-aminoadipate δ-semialdehyde synthase (292), and have been demonstrated in a variety of tissues (221) and species including humans (221), cattle (8, 292), rats (34-36), mice (203, 353), poultry (289) and trout (483).

The main site of lysine degradation is generally accepted to be the liver; LKR activity in most other tissues is considered to be lower or negligible (220, 221). However

in vivo data (434-438, 468) from portal balance studies suggested that the small intestine contributes significantly to the utilization of dietary essential amino acids, most notably lysine, leucine, threonine and methionine. These reports estimated that the intestine utilizes as much as 30% of the available dietary intake of lysine. The complete fate of this utilized lysine remains unclear, however Stoll and colleagues (438) suggest that no more than 40% of the enteral lysine utilization was directed to protein synthesis by the intestine. Thus the possibility of the remaining lysine being catabolized, presumably as an energy source, is plausible. However, no direct evidence to support the presence of lysine catabolic enzymes in the intestine has been demonstrated (501).

Past research indicating no or negligible LKR activity in the intestine has typically utilized mucosal scrapings or homogenates of intestine as the primary source of intestinal tissue for enzyme analysis (221). The gel-like properties of the mucosa within these types of samples, especially when goblet cells are induced to secrete mucin, make sub-cellular fractionation and hence the determination of mitochondrial enzyme activity, extremely difficult. Hence, it is possible that the activity of LKR measured in these experiments was greatly underestimated.

Our main objective was to compare the extra-hepatic and hepatic activities of lysine α-ketoglutarate reductase, the primary enzyme of lysine catabolism, in the pig. As the primary site of lysine degradation, we described the kinetic characteristics of hepatic LKR and SDH. For comparison, we investigated hepatic LKR activity as well as mammary gland LKR activity in the lactating pig. In addition, we improved the procedure for isolating intestinal-cell mitochondrial-protein and determined that mucosal

epithelial cells of the intestine could indeed catabolize lysine via the saccharopine pathway.

3.3 Materials and Methods

3.3.1 Animals and tissue sampling

Adult market size pigs (110-120kg) from a commercial abattoir (Edmonton Custom Packers, Edmonton, AB, Canada) were used for tissue sampling. Animals were killed by normal commercial practice; application of high voltage directly to the brain area, followed by immediate exsanguination. Jejunal sections from the small intestine were excised from the animals and stored in cold PBS/5mM DTT (pH 7.4). Samples of liver, kidney, muscle and heart were also obtained from the same animals and stored in ice cold 0.3M mannitol /1mM EDTA. The samples were immediately (~45 min) transferred to the laboratory on ice for further processing.

Tissues from lactating gilts at the Swine Research and Technology Centre (SRTC), were obtained at 19-21 days of lactation, after animals were killed by captive bolt administration to the cranial area, followed by exsanguination. Tissues were placed in ice-cold buffer and transported to the laboratory on ice. These animals were killed as part of ongoing research at the SRTC; all protocols were approved by the Faculty Animal Care committee, and were in accordance with procedures outlined by the Canadian Council on Animal Care.

3.3.2 Isolation of intestinal mucosal epithelial cells

Intestinal epithelial cells were prepared using modifications of previously described methods (195, 293, 486) to investigate LKR activity in the intestine. A section of small intestine was obtained and transported to the laboratory in ice cold phosphate buffered saline with protease inhibitor cocktail added (PBS: 137mM NaCl / 2.7mM KCl / 4.3 mM Na_2HPO_4 / 1.4 mM KH_2PO_4 / 5 mM dithiothreitol (DTT). The section was rinsed (1X) with fresh cold PBS to remove any remnants of digesta and then rinsed (3X) with fresh oxygenated (19:1 O_2:CO_2; Carbogen) Krebs-Henseleit Ca^{2+}-Free buffer (121mM NaCl, 4.7mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 25.2mM $NaHCO_3$) containing 20 mM Hepes (pH 7.4), 5 mM EDTA, 20 mM DL-glucose, 2.1 mM lactate and 0.3 mM pyruvate (KHB buffer) / 5 mM DTT. The intestinal section was then filled with pre-warmed (37°C) oxygenated KHB (Ca^{2+}-free)/DTT, sealed with clamps and immersed in a container filled with the same buffer. This system, with continuous Carbogen gassing, was then shaken for 45 minutes at 37°C with gentle massaging of the sealed intestinal sections at regular intervals. The resulting cell suspensions were filtered through 2 layers of cheesecloth and centrifuged for 3 minutes at 400x *g*, 4°C. Cells were washed 3 times in fresh oxygenated KHB buffer. At this time the cell viability was assessed by Trypan blue exclusion; preparations routinely demonstrated ≥90% viability. See also appendix 8.4.1 for a complete description of the methods.

3.3.3 Isolation of mitochondria

The re-suspended mucosal epithelial cell pellet [in 250mM sucrose/5mM HEPES/5mM DTT (pH 7.4)] was then transferred to a cold Dounce homogenizer

(Wheaton Science Products, Millville, NJ) and the cell suspension disrupted by 8 passes of pestle A followed by 8 passes of pestle B. Mitochondria from the cells were then obtained by differential centrifugation according to previously published methods (293). The final mitochondrial pellet was re-suspended in 0.3M mannitol/2mM DTT.

All other tissues were weighed and then homogenized (VirTis tissue homogenizer, VirTis, Gardiner, NY) (1.0 g tissue / 5 mLs of buffer). Mitochondria were then isolated by differential centrifugation (221, 323, 403). Mitochondria from lactating mammary gland were similarly isolated according to published methods (304). Mitochondrial pellets were re-suspended in 0.3M mannitol. See also appendix 8.4.2 for a complete description of the method.

3.3.4 Lysine Ketoglutarate Reductase Assay

The initial step in lysine degradation was measured according to published methods (403) and modified to a 96 well microplate format (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA). Oxidation of NADPH to $NADP^+$ was measured spectrophotometrically at 30°C using an extinction coefficient of 6.22 $M^{-1}cm^{-1}$ (221). The reaction mixture contained 0.125 mM NADPH, 10 mM L-lysine, 7.5 mM α-ketoglutarate, 100 mM HEPES, pH 7.8 and 300 μg of solubilized mitochondrial protein (0.2% (v/v) Non-Idet P-40). The reaction commenced with the addition of NADPH. The assay was linear with time and protein concentration and optimized for substrate concentrations of lysine and α-ketoglutarate. See also appendix 8.4.3 for a complete description of the assay.

The conversion of lysine to saccharopine was confirmed by HPLC analysis (Appendix 8.5.4.2, Figure 8.7, Table 8.1). Mitochondria (disrupted) were incubated (as described above) for 15 minutes. Samples were deproteinized with 20% (v/v) perchloric acid, centrifuged (15000xg, 5 minutes) and aliquots taken for HPLC analysis. Amino acids were separated and quantified using a pre-column o-phthaldialdehyde procedure (298); ethanolamine and β-amino-n-butyric acid were used as internal standards. L-saccharopine and α-aminoadipate were added to the amino acid standard profile (Sigma #AAS18) for direct comparison.

3.3.5 Saccharopine Dehydrogenase Assay

The activity of saccharopine dehydrogenase was measured in a similar fashion to LKR (403) and adapted to the microplate. The reduction of NAD^+ to NADH was measured in a reaction mixture containing 2.0 mM L-saccharopine, 2.0 mM NAD^+, 50 mM Tris/HCl, pH 9.4 and using an extinction coefficient of 6.22 $M^{-1}cm^{-1}$. Addition of saccharopine was used to start the reaction. Activity of SDH was linear with time and protein concentration at 30°C. See appendix 8.4.4 for a complete description of the assay.

3.3.6 Inhibition Assays

For inhibition studies of either enzyme, potential inhibitors were added prior to the addition of protein. Controls were treated in exactly the same manner with the exception of substituting an equal volume of deionized water for inhibitor.

3.3.7 Protein Determination

Protein concentrations of mitochondria and intact mucosal epithelial cells were determined by the Bicinchoninic acid (BCA) technique (Sigma-Aldrich, BCA1). BSA (Fraction V, Sigma-Aldrich) was used as a standard.

3.3.8 Lysine oxidation

Intact intestinal epithelial cells or IEC mitochondria, as well as liver, heart and muscle mitochondria were used to examine the oxidation of radiolabeled lysine to $^{14}CO_2$. Assay conditions were based upon previously described methods (403); each data point was performed in duplicate for a minimum of three separate experiments. The oxidation media contained 60 mM KCl, 25 mM $KHCO_3$, 25 mM TEA-HCl (pH 7.6), 10 mM NaCl, 5 mM $MgCl_2$, 5 mM K_2HPO_4, 2 mM ADP, 1 mM EGTA, 5 mM sodium succinate, 5 mM sodium citrate, 3 mM α-ketoglutarate, 0.1 mM malate (sodium salt), 130 mM mannitol, 40 mM sucrose, 0.5 mM lysine and 0.5 μCi of either 1-^{14}C-lysine or U-^{14}C-lysine per reaction. Oxidation media was titrated to pH 7.6 and made fresh each day immediately prior to use. The oxidation was performed in 25ml Erlenmeyer flasks using 2ml of oxidation media and 1ml of protein added to start the reaction. Flasks were sealed with rubber stoppers, which had a center well containing 300μL of CO_2 absorber (ethylene glycol monomethyl ether: ethanolamine, 2:1, (v/v)).

Oxidation assays were performed for 3 hrs at 30°C in a shaking water bath, at the end of which the reaction was stopped by injecting 1ml of ice cold 20% (v/v) $HClO_4$. The flasks were then allowed to continue shaking in the water bath for an additional 60 min to ensure complete collection and trapping of $^{14}CO_2$. Complete center wells were then

transferred to scintillation vials, mixed with 5 ml of Atomlight (Packard BioScience B.V., Groningen, The Netherlands) and the radioactivity counted in a liquid scintillation counter (Beckman LS 5801, Beckman Coulter Canada Inc., Mississauga, ON).

Perchloric acid (20% (v/v), ice cold) was added to flasks prior to protein addition for zero time controls and non-radioactive (cold) lysine controls were performed with each experiment. The oxidation assay was validated for linearity with respect to time and protein concentration.

3.3.9 Lysine oxidation in different mucosal epithelial cell preparations

The epithelial cell isolation procedure yielded adequate numbers of viable, intact cells. However, the quantity of mitochondrial protein recovered from the epithelial cells was not sufficient to perform all necessary oxidation experiments*; thus intact mucosal epithelial cells were used for the lysine oxidation assays. In order to compare the differences in oxidation rates between tissues on the same basis (mitochondria) and between the epithelial cell preparations and IEC mitochondria, we conducted lysine oxidation studies in intact epithelial cells, cell homogenates and epithelial cell mitochondria.

3.3.10 Chemicals

L-[1-^{14}C]-lysine (Specific activity 54mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc., St. Louis, MO. L-[U-^{14}C]-leucine (Specific activity

*We were conducting experiments measuring $^{14}CO_2$ production from labeled lysine, valine, leucine, isoleucine and ketoisocaproic acid (Elango et al., submitted). To meet the demands, it was necessary to utilize whole intact IEC. To obtain sufficient additional mitochondria, the numbers of animals required would have more than doubled, with no increase in the experimental n.

58mCi/mmol) was purchased from Amersham Biosciences Corp., Piscataway, NJ. All other laboratory chemicals were purchased from Sigma (Oakville, Ontario) unless specified otherwise.

3.3.11 Statistical analysis

Data are presented as means ± SEM. One-way and repeated measures ANOVA (SAS/STAT version 8.01, SAS institute, Cary, NC) was performed employing PROC GLM procedure with the specific activity of enzyme rates assessed by least square means procedure. For inhibition experiments, all treatments were compared to controls using Dunnett's multiple comparison test. Data was considered significant at $P < 0.05$. Enzyme kinetic regression was assessed using classical Michaelis-Menten and substrate inhibition kinetic equations (equation details in section 3.4.1) using Prism 4 for Macintosh, version 4.0b.

3.4 Results

3.4.1 Kinetic Analysis of Lysine α-Ketoglutarate Reductase and Saccharopine Dehydrogenase

Assay conditions (pH, time, protein) were optimized for measuring LKR and SDH activities in porcine tissues, and activities were measured in the linear range of time and protein concentration (Figure 3.1A-D). Assays were routinely terminated after 15 minutes.

The data for hepatic LKR activity did not demonstrate classical Michaelis-Menten kinetics; rather substrate inhibition was noted at lysine concentrations greater than 15

mM (Figure 3.2A). Lineweaver-Burke analysis of the data gave a graphical solution representative of substrate inhibition (Figure 3.2B). Using kinetic data obtained at lysine concentrations less than 15 mM, analysis by the Lineweaver-Burke regression estimated V_{max} at 4.2 nmol/min/mg; K_m was estimated at 12.7 mM (the linear regression solution r^2= 0.9130).

Saccharopine dehydrogenase activity was examined in the liver and demonstrated classical Michaelis-Menten solutions (Figure 3.2 C,D). V_{max} was estimated at 11.22 nmol/min/mg ; the K_m was estimated at 1.41 mM using the Lineweaver-Burke analysis (the linear regression solution r^2= 0.9737).

LKR kinetic analysis was conducted in IEC mitochondria as well. The effect of high lysine was very subtle and whether IEC LKR was substrate inhibited was not clear (Figure 3.3). The Michaelis-Menten kinetic analysis, based on the classical equation, *velocity* = $(V_{max} \bullet X)/(K_m + X)$, provided a significantly better fit of the data (r^2 = 0.4923) than the substrate inhibition curve, *velocity* = $(V'_{max} \bullet X/(K'_m+X+X^2/K_{si}))$ where K_{si} represents the point of substrate inhibition (set to 12mM), r^2 = 0.5149. The Michaelis constant was estimated at 1.03 mM for IEC LKR using Michaelis-Menten kinetics and 1.73 mM using substrate inhibition.

Following Page: Figure 3.1

Representative graphs to illustrate enzyme activity linearity with protein content and time elapse. Panel A: LKR; Panel B: SDH; enzyme activity was linear with protein content in the incubation mixture (0.1 - 0.8 mg). Panel C: LKR; Panel D: SDH; rate of enzyme activity illustrated as change in absorbance versus time. Absorbance readings were measured at nine-second intervals in 96 well microplates (~200 measures/well/15 minutes). Data are presented as Mean ± SEM for 3 separate experiments.

Figure 3.1 Hepatic LKR and SDH Activity is Linear with Protein Concentration and Time

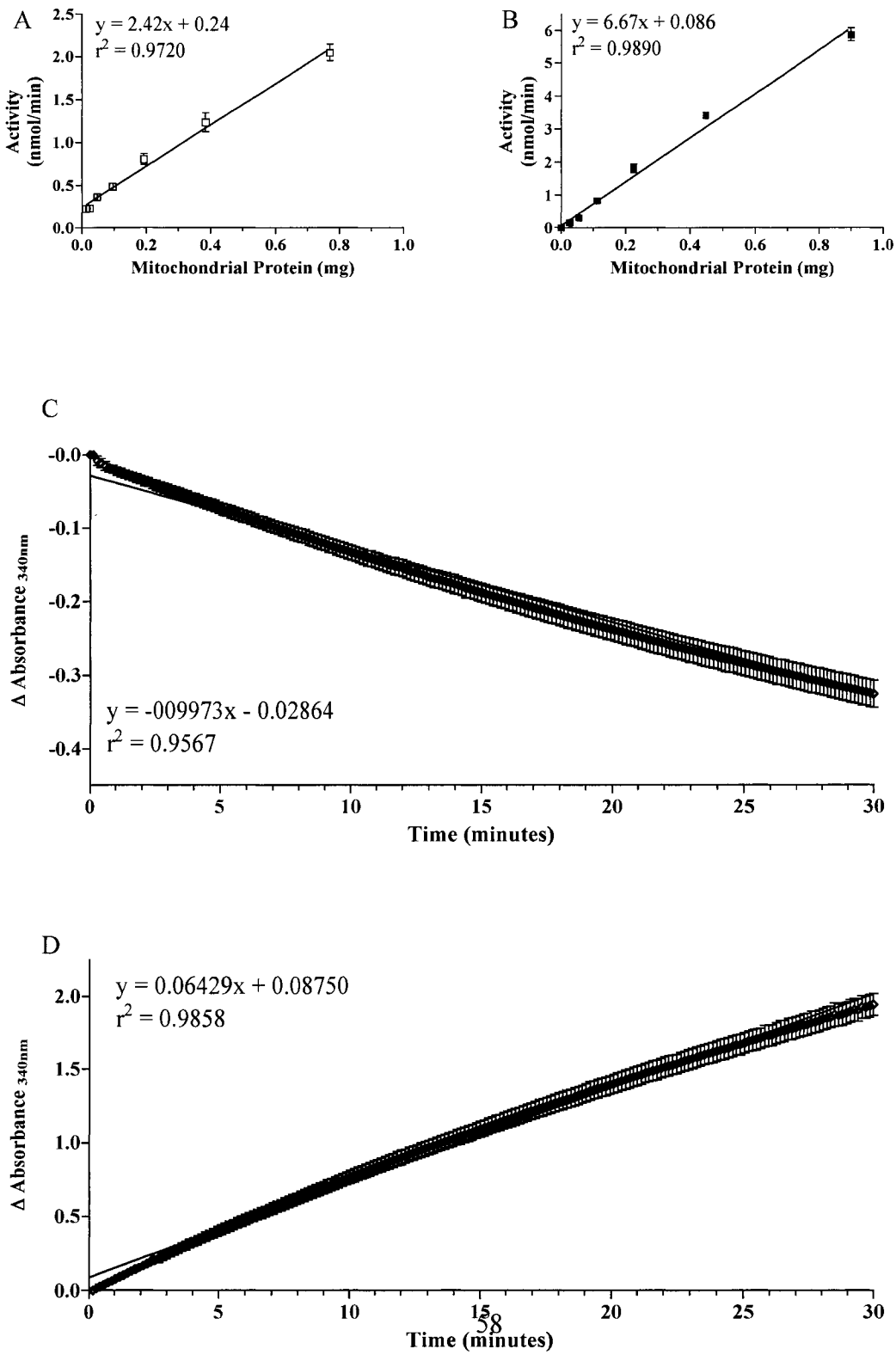
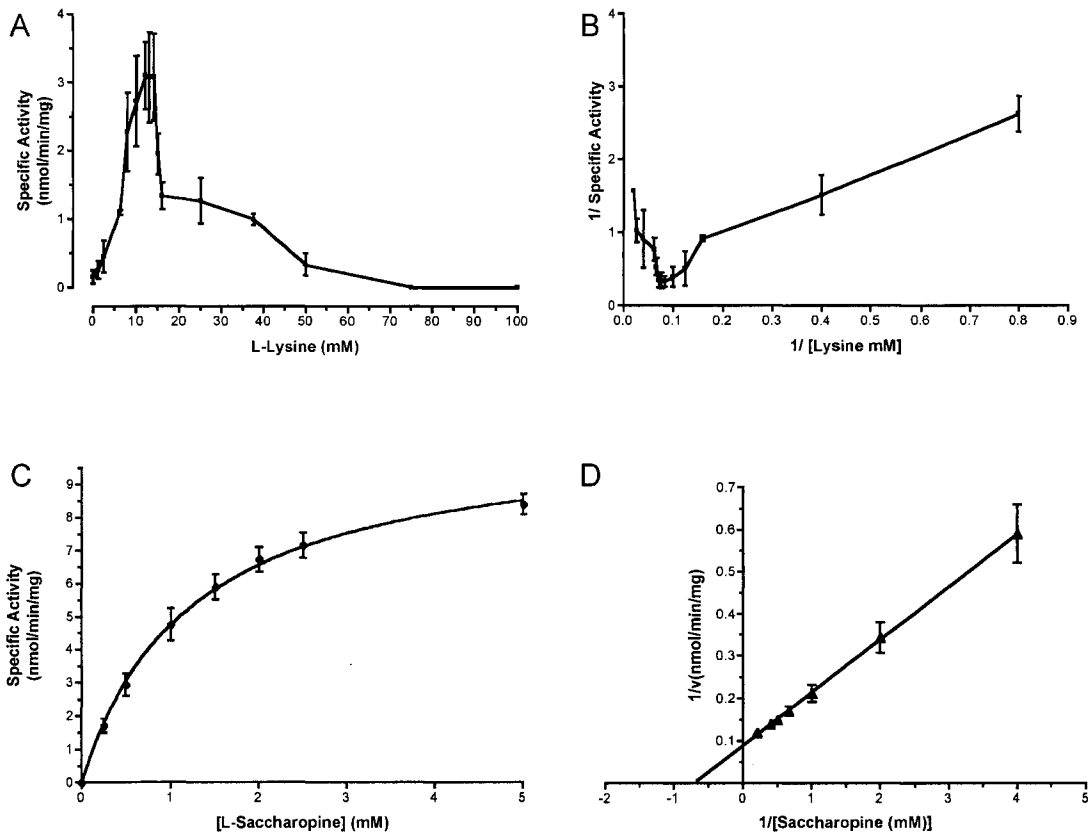


Figure 3.2 Kinetic Analysis of Hepatic LKR and SDH Enzyme Activities



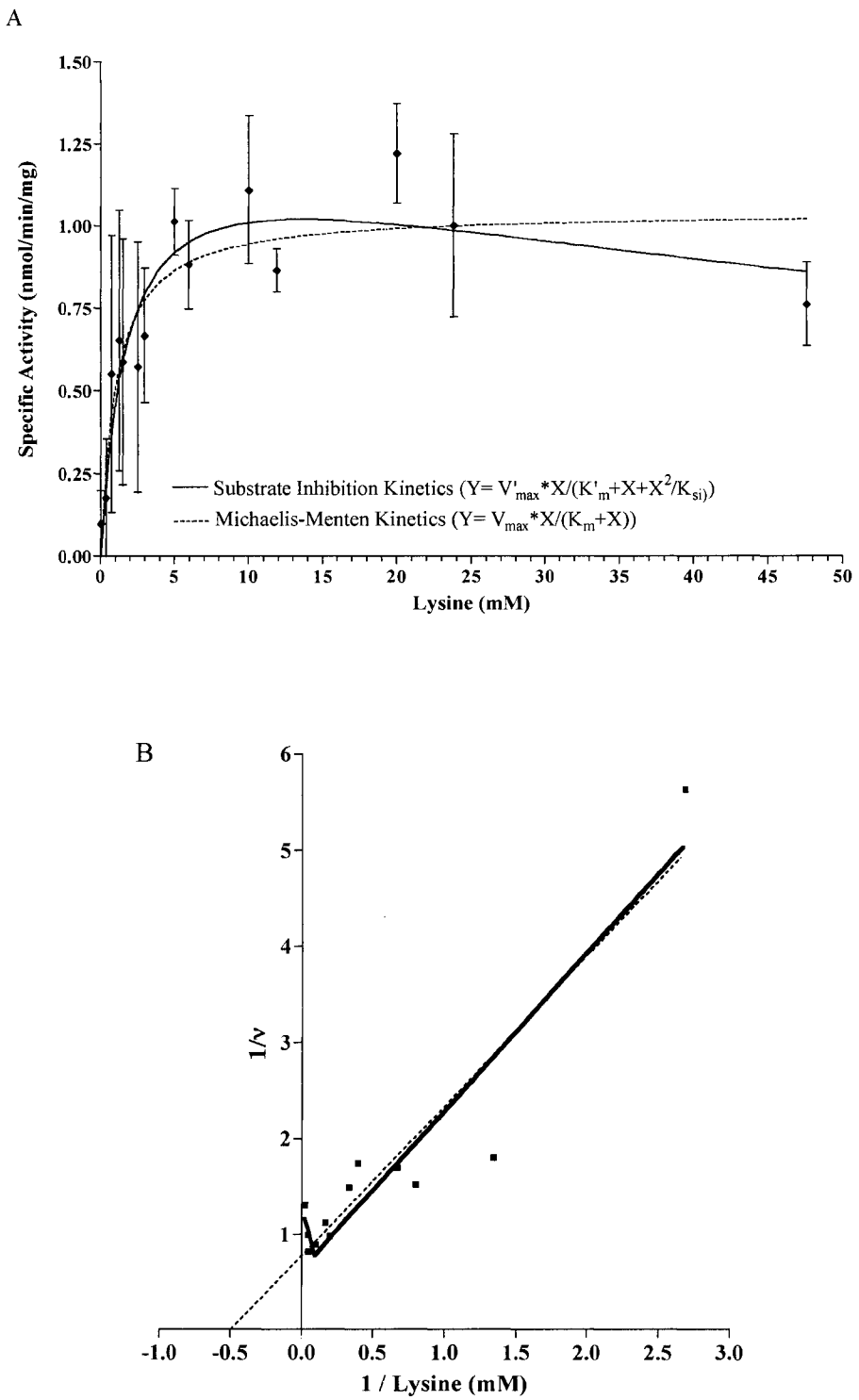
Panels A/B Lysine α-Ketoglutarate Reductase - Kinetic Analysis. Enzyme kinetics of hepatic LKR were investigated using solubilized mitochondria preparations. Panel A shows the Michaelis-Menten kinetics analysis demonstrating substrate inhibition of LKR at lysine concentrations greater than 15 mM. Panel B shows the Lineweaver-Burke manipulation of the Michaelis-Menten analysis also clearly demonstrating substrate inhibition of LKR. The data are presented as the Mean ± SD of 3 separate experiments.

Panels C/D Saccharopine Dehydrogenase - Kinetic Analysis. Enzyme kinetics of hepatic SDH were investigated using solubilized mitochondria preparations. Panel C shows the Michaelis-Menten kinetics analysis demonstrating a Vmax of 10.63 nmol/min and a Km of 1.23 mM. Panel D shows the Lineweaver-Burke manipulation of the Michaelis-Menten analysis. The data are presented as the Mean ± SD of 3 separate experiments.

Following page: Figure 3.3

Mitochondria isolated from (jejuna) intestinal sections were solubilized and LKR activity was measured with increasing lysine concentrations. Data are presented as Mean ± SEM for three separate experiments. Solid lines represent typical kinetic graphical solutions, hatched or dotted lines represent the graphical solution for substrate inhibition. Panel A: Michaelis-Menten compared to substrate inhibition; Panel B: Lineweaver-Burke compared to substrate inhibition.

Figure 3.3 Kinetic Analysis of LKR activity in Mucosal Epithelial Mitochondria



3.4.2 Activity of Enzymes Involved in Lysine Catabolism

Enzyme activities were determined for LKR (Table 3.1) in solubilized mitochondria from pig tissues. The liver had the highest specific LKR activity, followed by mammary gland, kidney, heart, IEC and muscle mitochondria. Analysis of LKR activity in other mitochondrial preparations demonstrated substrate inhibition, although not as dramatic as was seen in the liver samples.

Saccharopine dehydrogenase activity was similarly measured in mitochondria (Table 3.1). Liver activity was highest, followed by SDH activity in kidney mitochondria. Activity of SDH in intestinal epithelial cells mitochondria was below the level of detection of our assay system and considered to be zero. Kidney SDH was ~ 40% of the liver activity.

Table 3.1 Tissue Activities of Lysine Catabolic Enzymes

	Lysine α-Ketoglutarate Reductase	Saccharopine Dehydrogenase
Tissue (*mitochondrial protein*)	**Specific Activity** (*nmol/min/mg*)	
Liver	4.26 ± 1.80 (13)	6.43 ± 1.20 (3)
Intestinal epithelial cell	2.08 ± 0.92 (5)	< detection limits (0.00) (3)
Heart	2.31 ± 0.20 (5)	---
Kidney	2.60 ± 0.49 (5)	2.67 ± 0.03 (3)
Muscle	0.85 ± 0.49 (3)	---
Mammary Gland*	3.29 ± 1.11 (3)	---
Liver*	2.80 ± 0.54 (3)	---

Data are presented as Mean ± SEM (n). Specific activities were calculated using 10 mM L-lysine (LKR) or 3.0 mM L-saccharopine (SDH). *Samples retrieved from lactating gilts.

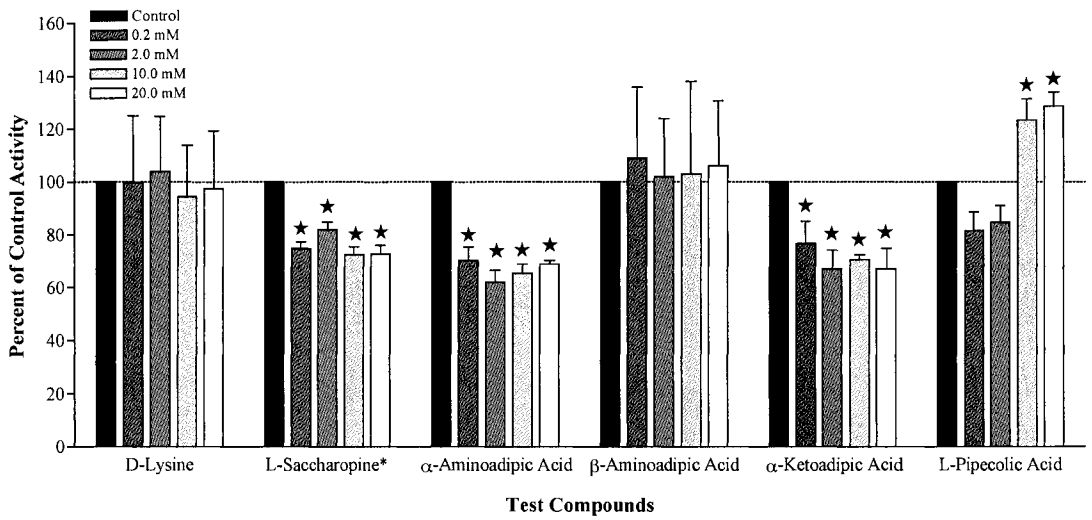
3.4.3 Inhibition of Lysine α-Ketoglutarate Reductase Activity

Solubilized mitochondrial preparations from liver were assayed for LKR activity in the presence of potential regulatory compounds. Increasing concentrations (0.2 mM to 20 mM) of each compound were tested for effect. Appropriate controls (substrate and protein) were used to measure baseline values. Activities of test samples were normalized to controls (100% activity, no compound present); control samples were measured each day and were not significantly different from each other on different days. Control activities were included in the calculation of LKR specific activity (Table 3.1) and may be assumed to be 4.26 ± 1.8 nmol/min/mg.

Catabolites and closely related derivatives of lysine were investigated as potential regulators of hepatic LKR activity (Figure 3.4); L-saccharopine, α-aminoadipic acid and α-ketoadipic acid significantly decreased the activity of LKR by 18% - 38% at all concentrations tested. L-pipecolic acid, an intermediate of the secondary lysine degradation pathway, did not significantly decrease the activity of LKR however, at 10 mM and 20 mM concentrations. L-pipecolic acid increased the activity of LKR by 23% and 28% respectively. D-Lysine was ineffective as an inhibitor of LKR activity showing that the enzyme is stereo-specific.

The degradative pathway of tryptophan intersects with lysine catabolism at the point of α-ketoadipic acid. Therefore the possibility that tryptophan intermediates could influence LKR activity was investigated (Figure 3.5). Neither tryptophan, nor any intermediate tested affected the activity of LKR. L-Kynurenine did however significantly increase LKR activity at 2 mM (20% increase) and 10 mM (55% increase). 3-Hydroxy-

Figure 3.4 Inhibition of Hepatic LKR Activity by Lysine Metabolites



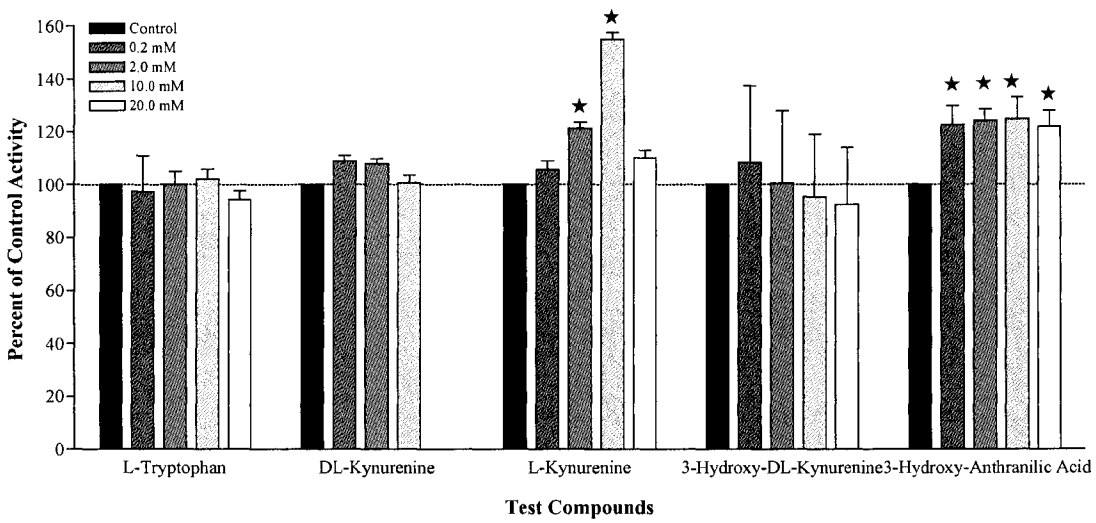
LKR activity was determined in the presence or absence of various potential regulatory compounds. Compounds were added prior to addition of protein. Control tubes had water substituted for compound and were run each day for each animal; data are presented as Percent of Control Activity: Mean ± SEM for n = 3 separate experiments (samples were measured in duplicate). Stars (★) indicate values are significantly different from control ($p<0.05$), as assessed by Repeated Measures ANOVA and Dunnett's Multiple Comparison Test. *The concentrations of saccharopine used were 0.3, 3.0,15 and 30 mM.

-anthranilic acid significantly increased LKR activity by 22%-25% at all concentrations tested.

The effect of various polyamines on LKR activity was measured and is illustrated in Figure 3.6. Polyamines did not decrease the activity of LKR, however, at the higher concentrations of putrescine (20 mM), and both spermidine and spermine (10 mM and 20 mM), LKR activity was significantly increased compared to control. Cadaverine (0.2 mM) significantly increased LKR activity, but higher concentrations did not affect LKR activity.

A selection of other relevant compounds was tested for additional reasons (Figure 3.7). The lysine derivatives 5-hydroxy-L-lysine and acetyl-lysine were both investigated. The hydroxylated lysine significantly decreased LKR activity, however acetyl-lysine was

Figure 3.5 Effects of Tryptophan Metabolites on Hepatic LKR Activity

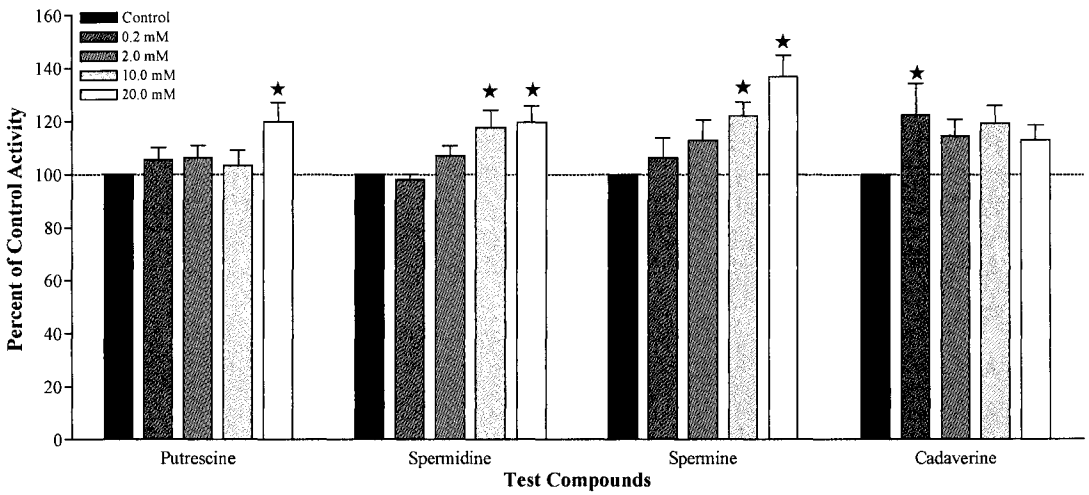


LKR activity was determined in the presence or absence of various potential regulatory compounds. Compounds were added prior to addition of protein. Control tubes had water substituted for compound and were run each day for each animal; data are presented as Percent of Control Activity: Mean ± SEM for n = 3 separate experiments (samples were measured in duplicate). Stars (★) indicate values that are significantly different from control ($p<0.05$), as assessed by Repeated Measures ANOVA and Dunnett's Multiple Comparison Test.

ineffective. ε-Amino-n-caproic acid, a structurally similar analog of metabolites of the pipecolic acid pathway, also did not affect LKR activity. β-Mercaptoethanol, a potent thiol reducing agent, did not significantly affect the enzyme activity. The α-ketoglutarate analog, dimethyl-2-oxoglutarate, dramatically increased LKR activity by more than 1000 times when added at 7.5 mM and 15 mM (Figure 3.7).

LKR activity was sensitive to the influence of particular metals (Figure 3.8). Additions of mercuric and zinc chloride solutions (2.0 mM – 20 mM) significantly inhibited LKR activity by more than 60%. Copper sulfate addition decreased LKR activity by ≥ 50% when provided at 0.2 mM and 20 mM. While activities of LKR appear

Figure 3.6 Effect of Polyamines on Hepatic LKR Activity



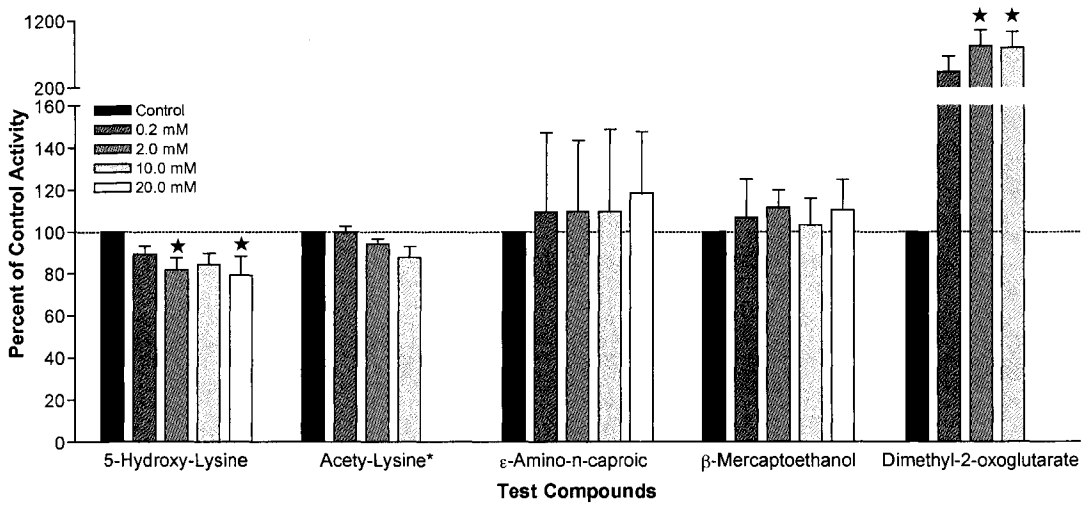
LKR activity was determined in the presence or absence of various potential regulatory compounds. Compounds were added prior to addition of protein. Control tubes had water substituted for compound and were run each day for each animal; data are presented as Percent of Control Activity: Mean ± SEM for n = 3 separate experiments (samples were measured in duplicate). Stars (★) indicate values are significantly different from control ($p<0.05$), as assessed by Repeated Measures ANOVA and Dunnett's Multiple Comparison Test.

to be decreased in the presence of cobalt chloride, the inhibition was not significant, possibly because of the higher variation with $CoCl_2$.

3.4.4 Influence of Selected Compounds on Hepatic Saccharopine Dehydrogenase Activity

SDH activity was measured in the presence of selected compounds that significantly inhibited LKR activity (Figure 3.10). Zinc chloride (2-20mM) inhibited the activity of SDH by ≥ 80%. Neither of the lysine catabolites, α-aminoadipic acid, α-ketoadipic acid, nor 5-hydroxy lysine decreased the activity of SDH. α-Ketoadipic acid significantly increased the activity of SDH ~ 10% at 2.0 mM and 20 mM. L-lysine (not shown) had no effect on SDH activity.

Figure 3.7 Effect of Various Lysine-Related Compounds on Hepatic LKR Activity

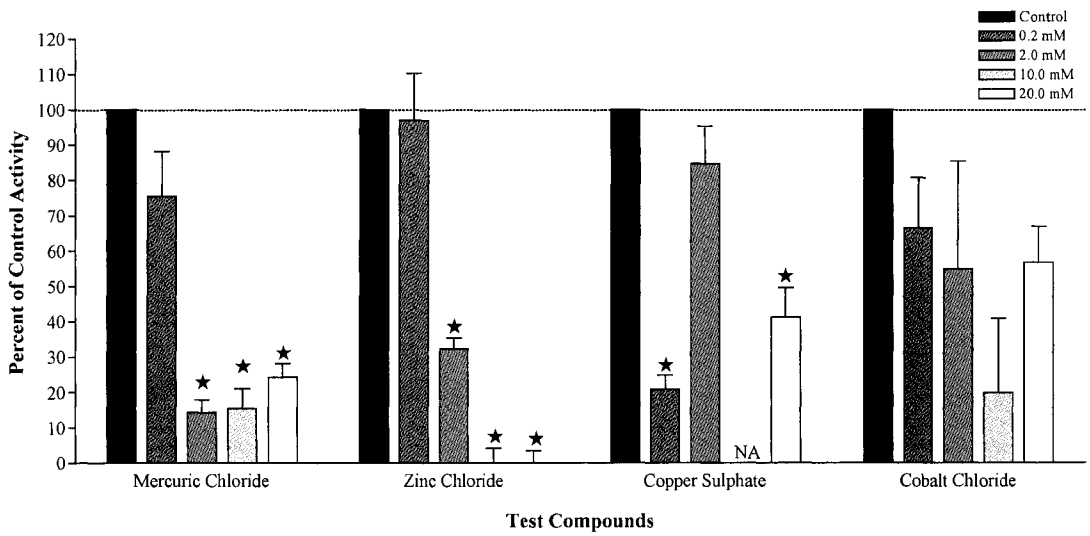


LKR activity was determined in the presence or absence of various potential regulatory compounds. Compounds were added prior to addition of protein. Dimethyl-2-oxoglutarate was added in the following concentrations 3.75 mM, 7.5 mM and 15 mM. Control tubes had water substituted for compound and were run each day for each animal; data are presented as Percent of Control Activity: Mean ± SEM for n = 3 separate experiments (samples were measured in duplicate). Stars (★) indicate values are significantly different from control ($p<0.05$), as assessed by Repeated Measures ANOVA and Dunnett's Multiple Comparison Test.

3.4.5 Lysine Oxidation: Production of $^{14}CO_2$ by Pig Tissues

Freshly isolated mitochondria were incubated with universally labeled or [1]-^{14}C-L-Lysine for 180 minutes. Production of $^{14}CO_2$ was linear between 30 and 240 minutes and up to 60 mg hepatic mitochondrial protein (Figure 3.9). Other experiments were conducted within the linear range of these parameters. Due to demands for mitochondria from mucosal preparations, intact cells, disrupted cells (homogenates) and mitochondria from isolated cells, were compared in oxidation experiments (Figure 3.10). Rates of $^{14}CO_2$ production were not different between mitochondria or intact cells, and both of these preparations produced significantly more $^{14}CO_2$ than the IEC homogenates.

Figure 3.8 Effect of Metals on Hepatic LKR Activity



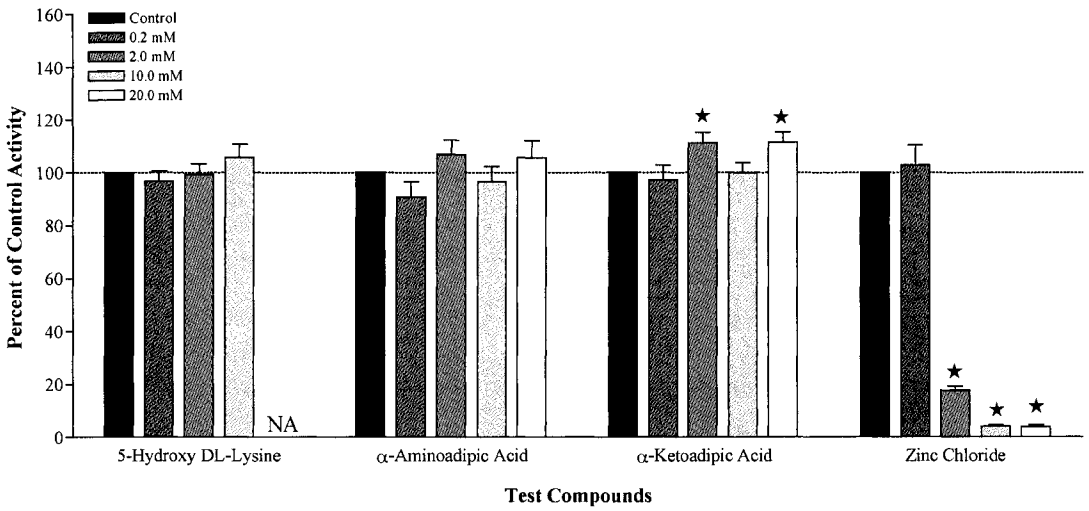
LKR activity was determined in the presence or absence of various metals. Compounds were added prior to addition of protein. Control tubes had water substituted for compound and were run each day for each animal; data are presented as Percent of Control Activity: Mean ± SEM for n = 3 separate experiments (samples were measured in duplicate). Stars (★) indicate values are significantly different from control ($p<0.05$), as assessed by Repeated Measures ANOVA and Dunnett's Multiple Comparison Test. NA: samples were not acceptable for analysis.

Mitochondria isolated from liver, intestinal mucosal cells and muscle were incubated with universally labeled ^{14}C-Lysine and compared (Figure 3.11). Liver mitochondria had significantly higher $^{14}CO_2$ production, compared to muscle and intestinal epithelial cell mitochondria, which did not differ from each other.

3.5 Discussion

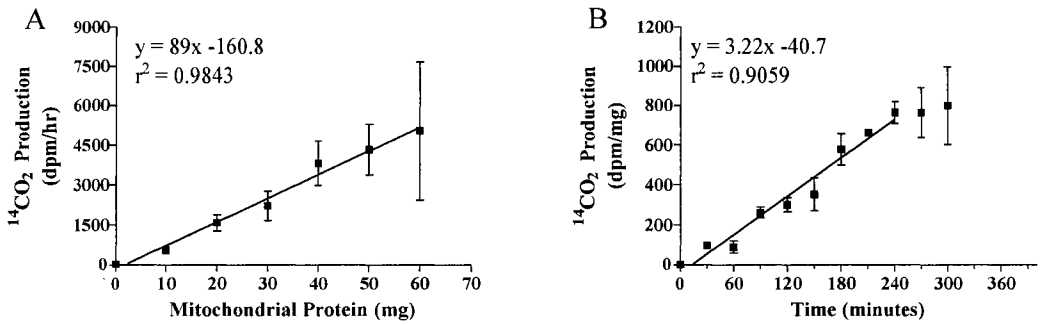
Lysine utilization in the growing animal is a very important consideration for swine producers. Lysine is typically the first limiting amino acid for protein synthesis, in grain based diets for both animals (277, 279) (see also Table 1.1) and humans (157). Therefore, understanding the fate of ingested lysine is critical for any attempts at

Figure 3.9 Effect of Selected Compounds on Hepatic SDH Activity



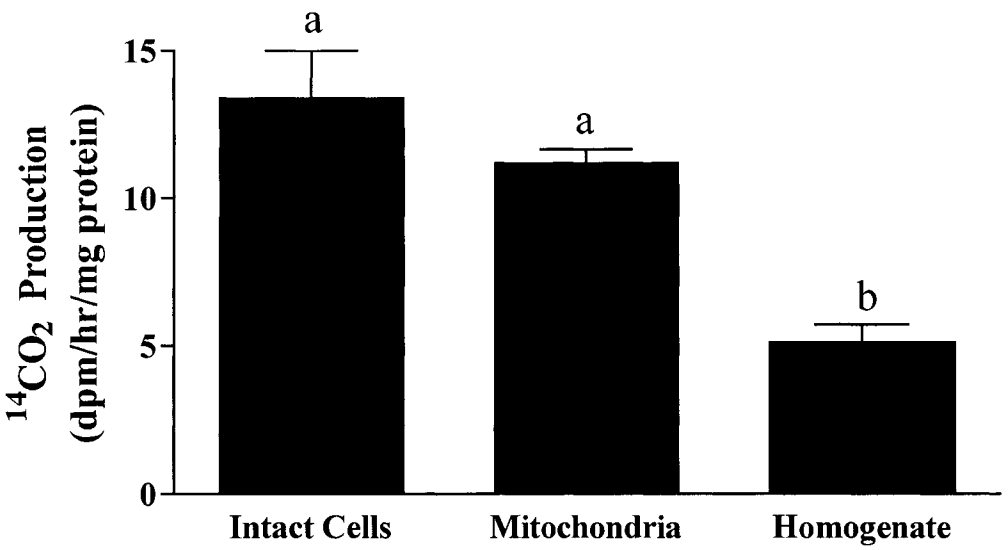
SDH activity was determined in the presence or absence of various potential regulatory compounds. Compounds were added prior to addition of protein. Control tubes had water substituted for compound and were run each day for each animal; data are presented as Percent of Control Activity: Mean ± SEM for n = 3 separate experiments (samples were measured in duplicate). Stars (★) indicate values are significantly different from control ($p<0.05$), as assessed by Repeated Measures ANOVA and Dunnett's Multiple Comparison Test. NA: samples were not acceptable for analysis. NA: samples were not available for analysis.

Figure 3.10 Linearity of $^{14}CO_2$ Production from [1-^{14}C]-Lysine



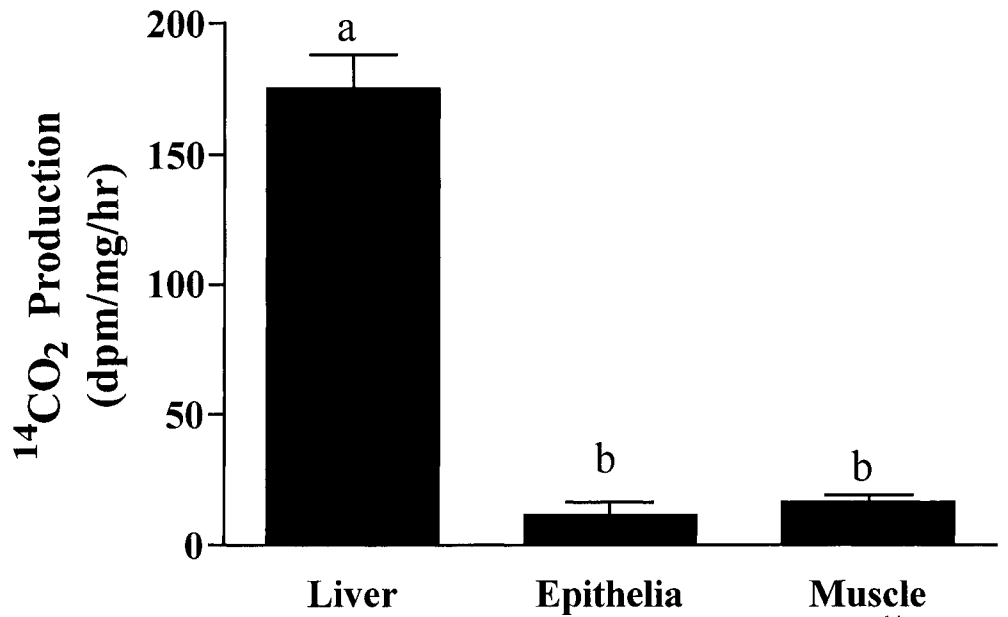
Freshly isolated liver mitochondria (0-60 mg) were incubated with universally labeled ^{14}C-Lysine for up to 5 hours. Data are presented as Mean ± SEM for 3 separate experiments.

Figure 3.11 $^{14}CO_2$ Production in Different Intestinal Epithelial Cell Preparations



Different preparations of isolated mucosal epithelial cells were incubated with universally labeled ^{14}C-L-Lysine and the production of $^{14}CO_2$ was measured. Data are presented as Mean ± SEM for four separate experiments. Bars with different letters indicate significant differences, $P< 0.05$.

Figure 3.12 Oxidation of Lysine by Pig Tissues



Intact mitochondria from liver, IEC and muscle were incubated with U-^{14}C-L-Lysine for 3 hours; $^{14}CO_2$ was collected simultaneously and for one hour after reaction termination in an ethylene glycol monomethyl ether: ethanolamine trap. Radioactivity was quantified by scintillation. Data are presented as Mean ± SEM for 3 separate experiments. Values were corrected for protein and substrate blanks. Different letters indicate significant difference, $P< 0.05$.

improving nutritional status and protein quality of foods. Previous work in our laboratory (313) showed that basal catabolism of lysine accounts for approximately 10-14% of the available dietary lysine. In growing swine , we have therefore investigated the catabolism of lysine in various pig tissues through examination of the activity and regulation of lysine α-ketoglutarate reductase and saccharopine dehydrogenase, the first two enzymes in the lysine catabolic pathway.

3.5.1 Kinetic Analysis of Hepatic Lysine α-Ketoglutarate Reductase and Saccharopine Dehydrogenase

Examination of the kinetic data of the first two enzymes of lysine catabolism revealed that while SDH exhibited classical Michaelis-Menten kinetics, the activity of hepatic LKR was susceptible to substrate inhibition. Substrate concentrations exceeding 15 mM resulted in decreased activity of the enzyme, eventually reaching complete inhibition at lysine concentrations > 75mM. The Lineweaver-Burke manipulation of the kinetic data again suggested substrate inhibition. This result is of particular importance when considering most published research (353, 403) used a concentration of 20-25 mM lysine to examine the activity of LKR; this concentration will cause a more than 50% inhibition based upon our analyses. These previous studies (353, 403) did not include a full kinetic analysis of LKR. To the best of the author's knowledge the present research is the first complete report of LKR and SDH kinetics on pig tissues.

Physiologically, such high concentrations (20-25 mM) at the cellular level are highly unlikely although portal blood may approach millimolar levels in the post-prandial situation (44, 378). In the growing pig fed a lysine-supplemented diet, synthetic lysine, which requires little metabolism to generate the free amino acid form, will be present at

very high concentrations in both the absorptive cells and indeed in the portal blood reaching hepatocytes following a meal (509). Substrate inhibition of LKR will decrease catabolism of lysine at high concentrations and may help conserve the amino acid for other functions, e.g. protein synthesis.

Substrate inhibition is believed to be due to multiple substrate molecules binding simultaneously within the enzyme's active site (223, 260), resulting in decreased turnover of substrate at one of these sites. Mechanistically, it is unclear whether the inhibition is due to steric hindrance by a substrate molecule at one site blocking catalytic action at the primary active site or whether the multiple binding results in a conformational change in the protein that blocks substrate binding at the active site (260, 284, 412). The catalytic event initiating lysine catabolism involves the joining of α-ketoglutarate to lysine, producing saccharopine, mediated by electron transfer from NADPH (155). Collectively, these structures suggest that a large active site is necessary to facilitate the reaction; large enough to allow multiple binding of one substrate. Regardless, the end result is the diminished catabolism of lysine.

Kinetic analysis of LKR in mitochondria isolated from intestinal epithelial cells suggests that LKR degradation of lysine is catalyzed in the classical manner (Figure 3.4). Comparison of Michaelis-Menten to substrate inhibition kinetics reveals a close agreement (r^2 = 0.4923 for Michaelis-Menten versus r^2=0.5149 for substrate inhibition) in the fit of the curves. At physiological concentrations of lysine (~0.2-0.8 mM in plasma (44, 378) and 3-4mM in hepatic mitochondria (34)), it seems more likely that the enzyme follows Michaelis-Menten kinetics in the intestinal epithelia. The presence of positive activity of LKR in these cells strongly supports the hypothesis that lysine catabolism does

occur in these cells via the main degradative pathway. The possibility that LKR may utilize substrate inhibition as a means of regulating catabolism suggests that this pathway is under a complex level of regulation. The metabolic status of the intestine and the animal as a whole will likely affect transport of lysine across the absorptive cells; transport kinetics will likely be tied into the regulation of lysine catabolism in these cells. Blemings et al. (35) showed that as dietary protein content increased, the amount of lysine oxidized by liver mitochondria increased but did not differ from the amount (of lysine) transported into the mitochondria. However, the activities of LKR and SDH under these conditions, predicted a significantly greater amount of flux through the pathway, and hence greater amount of lysine oxidized. Therefore, the oxidation of lysine was limited by the amount of lysine being transported into the mitochondrion. These data suggest that lysine uptake into mitochondria may provide a regulatory limit on the potential catabolism and subsequent oxidation of lysine.

3.5.2 Tissue Activity of α-Lysine Ketoglutarate Reductase

The tissue distribution of LKR was measured in several tissues including liver, kidney, muscle and intestine. The liver has traditionally been considered the primary site of amino acid degradation; our data are in agreement with others (221) suggesting that the liver is the main tissue of lysine catabolism in the pig. Hepatic LKR activity has been considered a function of both energy and nitrogen balance, transforming the amino groups of the primary amino acid structure to glutamate and the remaining carbon backbone to ketones. Different researchers (353, 403) have demonstrated that hepatic LKR enzyme activity and mRNA levels are responsive to the starved state as well as to the glucagon status of the animal, giving credence to lysine catabolism being involved in

energy metabolism and nitrogen balance. A possible role for the presence of LKR in other tissues, notably the kidney and intestine is less well understood. Papes et al (353) suggested that kidney LKR/SDH activities functioned in energy homeostasis, being influenced by starvation in a similar fashion to hepatic LKR. The authors further noted that the cortical localization of SDH in the kidney may further suggest a role for LKR/SDH in the lysine reabsorption process. Forsberg and colleagues (159) presented data demonstrating that the fate of lysine is affected by the acid-base balance (dietary electrolyte balance) of feed. Diets formulated to promote acidosis decreased hepatic lysine oxidation while significantly increasing renal oxidation; conversely alkalogenic diets appeared to elicit the opposite effects in each tissue, although the results were not significant ($p \geq 0.05$). The authors speculated that lysine catabolism in the kidney may be reflective of a role in the maintenance of cellular ion balance, citing evidence from studies of potassium deficiency (343, 404). The fate of intracellular lysine, via protein synthesis or degradation, may then be dependent upon the electrolyte and acid-base balance in the cellular milieu.

The localization of rat liver LKR was shown to be mitochondrial (35). We have seen similar results using pig liver samples; LKR activity was localized to the mitochondrial fraction and no activity was detected in the cytosolic fraction (Appendix 8.4.5.1, Figure 8.6). Higashino et al. (203) detected LKR activity in both mitochondrial and cytosolic fractions of mouse liver extracts. Papes et al. (353) used two computational methods to predict a mitochondrial localization of the mouse liver enzyme after examination of the protein sequence deduced from a cDNA-LKR/SDH clone. They discussed the possibility that the "cytosolic isoform" may be due to activity of the

monofunctional SDH, but also state that their method of protein purification did not permit resolution of this argument. Based on these results, we assumed that LKR in intestinal cells would be similarly/primarily localized to the mitochondrial fraction.

Previous studies (82, 221) led to the general belief that cells of the intestine do not possess lysine catabolic enzymes. However, van Goudoever et al. (468) showed that intestinal oxidation of dietary lysine in pigs may account for ~30% of whole body lysine oxidation. Results from other studies citing intestinal oxidation of essential amino acids (29, 510), along with van Goudoever's data, led us to examine, more closely, the enzymic capability of the intestine to degrade lysine. We attempted to prepare mitochondria from both mucosal scrapings and homogenates of pig intestinal sections following methods cited in the earlier references. These experiments did not result in any useable preparations of mitochondria. Agitation of the mucosal scrapings or the intestinal sections, in normal processing to obtain a mitochondrial isolate, resulted in the formation of a gel. Agitation of goblet cells during physical processing is a stimulator of mucin secretion (333) and the combination of secreted mucins with the cold isolation buffers most likely resulted in the formation of this gel. The formation of such a gel produced an unsuitable environment for the isolation of mitochondria based upon differential centrifugation techniques. To overcome this problem we decided to isolate "*free*" intestinal cells and then prepare mitochondria from these cells using a combination of several methods. These methods allowed us to first successfully isolate intestinal cells, and then to isolate mitochondria from these cells, which could be used to examine LKR activity.

Mitochondria isolated from pig intestinal epithelial cells demonstrated LKR activity that was about 50% of the hepatic activity under our conditions (Table 3.1). To verify these results, solubilized mitochondria were incubated with lysine and the production of saccharopine was determined using HPLC analyses; these results that lysine was being converted to saccharopine (Appendix 8.4.5.2, Table 8.1). These data suggested very strongly that at least the first enzyme in the lysine catabolic pathway was present and functional in intestinal cells. Next we questioned whether the whole pathway was present. To do this, we incubated either intact mitochondria, isolated from intestinal cell preparations or intact intestinal cells, isolated from sections of pig jejunum, with radiolabeled- lysine (U-^{14}C- or 1-^{14}C-Lysine). If the entire lysine catabolic pathway was present in these cells, then the eventual release of the α-carboxyl group of lysine as $^{14}CO_2$ would be evident. The results illustrated in Figures 3.11, 3.12 show that, while the rate of oxidation was significantly lower than the hepatic rate, the production of $^{14}CO_2$ was clearly demonstrated. Given that the predicted mass of intestinal epithelial cells in market weight pigs is ~800-900g` and liver mass is ~1.25-1.5 kg, the intestine must be considered an important contributor to whole-body lysine oxidation.

Ornithine decarboxylase (ODC) can also degrade lysine, generating cadaverine and CO_2 (358). Significant cadaverine production *in vivo* would occur only when ODC activity is high and lysine concentrations substantially exceed those of ornithine. Therefore, a potent inhibitor of ODC, difluormethylornithine (DFMO) (482) was added to mitochondria prepared from intestinal epithelial cells and we observed no significant changes in $^{14}CO_2$ production. It should also be noted that ODC is cytosolic, and there were also no changes in $^{14}CO_2$ production when DFMO was added to whole intestinal

cell homogenates (Appendix 8.4.5.4). Therefore, this provides more evidence that $^{14}CO_2$ production in IEC preparations is due to flux of labeled lysine through the lysine catabolic pathway and not catabolism by some other mechanism.

While $^{14}CO_2$ production by intestinal cell mitochondria did not match the rate of LKR activity demonstrated by intestinal cell mitochondria (compared to hepatic rates, ~ 8% versus ~ 50%), it is important to note that with CO_2 production experiments, five separate enzymes of the lysine catabolic pathway must be present and receiving substrates before the release of the α-carboxyl group from α-ketoadipate is possible; eight steps would be required in the alternate route via pipecolic acid. Transport of radiolabeled lysine into the cell and/or mitochondria must also be considered. It is highly probable that although we have demonstrated $^{14}CO_2$ production from labeled lysine, we may not have optimized the conditions for activity of each enzyme or transporter involved. For example, Kasahara et al. (238), recently elucidated a novel cofactor involved in the lysine catalytic pathway, pyrroloquinoline quinone (PQQ), and have demonstrated that there may be a PQQ requirement as a redox cofactor for the proper functioning of α-aminoadipate semialdehyde dehydrogenase (AAS-dehydrogenase). The regulatory characteristics/requirements of this compound in relation to lysine catabolism have, however, not been elucidated and are indeed being debated (148, 389). The changes that result from tissue processing may have actually depleted the levels of cellular PQQ required for optimal activity of the aminoadipate semialdehyde dehydrogenase. The same may be said for other intermediates. Therefore we may actually be underestimating the oxidative potential of the IEC mitochondria with respect to lysine oxidation. Furthermore, it is possible that regulation of intestinal lysine degradation is different than

hepatic regulation, reflecting the different roles of each tissue, for example in a manner analogous to the tissue-specific regulation of glucokinase activity (286).

In addition to the enzyme and oxidation data, samples were analyzed for the presence of the LKR/SDH protein. The results shown in Figure 8.13 indicate that the protein responsible for the initial steps of lysine degradation is indeed present in cells of the small intestine as well as other tissues. The (apparent) quantity of enzyme protein was much higher in liver and brain. That LKR/SDH is present in these tissues has been suggested in other species, but not previously demonstrated in pigs (220, 353, 354).

The effects of bacterial contamination have also been addressed. Bacterial contamination of intestinal cell preparations will skew any experiments regarding nutrient utilization. First, extensive washing steps were performed in the preparation of mitochondrial fractions and this would act to substantially remove/dilute any bacteria initially associated with the intestinal preparations, such that any metabolism of lysine by contaminating bacteria under the conditions described would not be significant. Furthermore, we have prepared fresh samples of intestinal cells from pig jejunum and then isolated mitochondria from these cells. At different stages of the mitochondrial isolation, samples were taken and examined for gram staining characteristics; mitochondrial samples in the final stage of preparation did not show any bacterial contamination when examined microscopically. These results provide evidence that the LKR activity detected in the intestinal cell mitochondria is due to intestinal cell mitochondria and not to bacterial contamination. Therefore, the capacity of the intestine to degrade lysine via LKR is a real, measurable phenomenon and not an experimental artifact.

3.5.3 Regulation of Hepatic Lysine α-Ketoglutarate Reductase Activity

Understanding the kinetics of LKR *in vitro* provides insight into the mechanism of enzyme activity *in vivo*. Compounds that affect the rate of enzyme activity *in vitro* provide clues about regulation of the enzyme *in vivo*. We investigated the effects of several metabolically and structurally related compounds on the activity of LKR.

Catabolites of lysine, including L-saccharopine, α-aminoadipate, and α-ketoadipate, were shown to inhibit the activity of LKR (Figure 3.5) to various degrees, suggesting that the enzyme may be susceptible to at least some moderate level of feedback regulation *in vivo*. It is worthwhile to consider however, the costs of these catabolites. L-saccharopine and α-ketoadipic acid are not easily cheaply synthesized. Indeed, α-ketoadipic acid is no longer available for commercial purchase (to the author's knowledge). L-α-aminoadipic acid, while easier to access is considered a potent gliotoxin (47, 299, 346). Use of this compound for in vivo experiments may presents ethical issues. For instance, if aminoadipate was added to cereal diets to improve protein deposition, via improved lysine utilization, and then discovered to cause neuronal degeneration and compromise the quality of life of the animals, it is highly unlikely that today's ethically conscious consumer would purchase products derived from these animals. Furthermore, a compound that causes neuronal damage to the animal ingesting it will not be allowed to enter the food chain.

The catabolic pathways of lysine and tryptophan intersect at α-ketoadipate; we hypothesized that intermediates in the tryptophan catabolic pathway would inhibit LKR activity. Most metabolites had no effect, however L-kynurenine and 3-hydroxy anthranilic acid increased the activity of LKR. Interactions between kynurenine and the

lysine catabolites α-ketoadipate and α-aminoadipate have been previously documented (69, 208, 345). These interactions suggest a more complex level of regulation and function in mammalian systems than previously recognized for these two intersecting pathways.

Owing to their similarity to the structure of the functional group of lysine, and the roles of polyamines in various tissues, these short aliphatic but highly positively charged compounds were tested as potential candidate inhibitors. None of the polyamines tested showed any inhibitory qualities. In fact, it appeared that as the polycations increased in size and charge (putrescine to spermine) the relative level of LKR activity increased (Figure 3.7). Polyamine addition, especially putrescine, to pig and poultry diets, has been suggested to have beneficial effects on growth, although it appears that the beneficial effects occur only within particular levels of addition (315, 418, 426). While polyamine addition may stimulate growth, our results provide a mechanism that may help, in part, explain the detrimental effects of excess levels of supplemented polyamines. Addition of the polyamines outside of these ranges was found to be toxic. Cadaverine, the product of ornithine decarboxylase activity on lysine, also significantly increased LKR activity. Polyamine additions to swine diets have affected the activities of various digestive enzymes, but not consistently (142, 185). This is the first evidence that polyamines influence lysine catabolic enzyme activity.

Several metabolically- or structurally-related compounds to lysine were also tested (Figure 3.8). Of these compounds only 5-hydroxy-L-lysine inhibited the activity of LKR. This hydroxylated form of lysine, formed in the post-translational modification of collagen, is an important branch of lysine utilization. Acetyl-lysine has been used as an

alternative lysine source in lysinuric protein intolerance (373), but did not inhibit the activity of LKR. The structural analog of α-ketoglutarate, the co-substrate of lysine in LKR catabolism, significantly increased the activity of LKR. Kamoun and colleagues (235) suggested that mitochondrial lysine degradation might in part be regulated by the availability of α-ketoglutarate in that compartment. They cited treatment of an infant that had pyruvate carboxylase deficiency type B. In this patient, high plasma lysine concentrations were accompanied with low plasma glutamate + glutamine concentrations, in addition to moderate hyperammonaemia. Increased ammonia levels would increase the production of glutamate from glutamine and α-ketoglutarate (via ureagenesis in the liver), resulting in decreased α-ketoglutarate levels in liver mitochondria. Decreased flux through the saccharopine pathway was implied by increased urinary pipecolic acid output. When the infant was treated with α-ketoglutarate, a dramatic (60%) decrease in plasma lysine occurred. In another infant, a defect in α-ketoglutarate dehydrogenase resulted in a dramatic increase in α-ketoglutarate concentration in liver mitochondria. This infant had plasma lysine concentrations of 46±28 μmol/L (mean of 22 determinations) compared with 183±39 μmol/L for a control infant, a 75% difference (235). These results are in agreement with the effect (1000 fold increase) of dimethyl-2-oxoglutarate on hepatic LKR activity (Figure 3.7). These data, taken with the past research suggest that control of LKR activity is sensitive to mitochondrial α-ketoglutarate concentrations.

Finally, the beneficial influence of metals in swine diets has been previously investigated (102, 409). In addition, the importance of metal ions on enzyme activity is widely accepted and has been extensively investigated. However, the effect of common

metals on LKR activity in swine has not previously been described. Dietary supplementation with zinc is often greater than recommended (331), although improvements in growth performance have not been consistently reported (73, 207, 397, 469). Similarly, tissue levels were sometimes increased following zinc supplementation (73, 397), and sometimes no effects were seen (469). Supplementation levels of zinc may account for these differences. Copper, added as Cu sulphate (125 – 250 ppm), has also been shown to improve growth in pigs (13, 103, 135, 207), but no data on tissue levels was found. Our results indicated that metals significantly decreased the activity of LKR in hepatic mitochondria (Figure 3.9). Interestingly, (hepatic) intracellular levels of zinc did not change following supplementation of zinc (sulphate, 80 or 160 ppm) (469). These authors also noted no change in tissue levels of zinc; other studies, which reported increased tissue levels of zinc following supplementation, did not report intracellular levels. Our results do not indicate that addition of metals to swine diets should be stopped, rather these data suggest that supplementation with metals may lead to an additional regulatory consideration if found in sufficient concentrations in organelles high enough to affect enzyme activity.

Our studies also examined the influence of selected compounds on hepatic saccharopine dehydrogenase activity (Figure 3.10). Of the compounds tested, only zinc chloride significantly decreased the activity of SDH. Hydroxylysine was ineffective as was α-aminoadipic acid. α-Ketoadipic acid increased the activity of SDH at higher concentrations. These limited results suggest that SDH may not be susceptible to the same regulatory compounds as is the LKR activity of the bifunctional enzyme.

The inhibition of LKR or SDH, even slightly *in vivo*, may represent an important savings with respect to lysine availability for protein synthesis. However, all of the potential metabolic roles of lysine catabolism and of the various lysine catabolites remains unclear but should not be discounted due to obscurity. The management of dietary lysine utilization with inhibitory compounds, especially for the purpose of improved protein deposition, should also encompass the potential alternate roles of lysine or lysine catabolites.

3.6 Implications and Concluding Remarks

The fate of dietary lysine is paramount to the success of swine and poultry producers. Understanding that the intestine has the necessary enzymology and indeed the capacity to oxidize dietary lysine and that presentation of lysine to the intestine in a non protein-bound form may act to influence catabolism to some degree, indicates that more research is necessary to better understand the role of lysine in the intestine. In this chapter we have shown that tissues other than liver, especially the intestinal epithelial cells and muscle can degrade lysine *in vitro*, this result is supported by various *in vivo* data from other authors. The inter-organ metabolism of lysine represents an important consideration in the question of whole body lysine utilization and degradation. Interactions between tissues during different metabolic situations may significantly alter lysine utilization, but these interactions and communication pathways remain unclear. Manipulation of lysine degradation through regulation of the rate-limiting enzyme(s) by exogenous compounds suggests that addition of some inhibitory component to the diet to decrease the lysine requirement is plausible, but at present, research aimed towards this endpoint has been unsuccessful (471).

4 Effect of Age and Nutritional Transition on Lysine Catabolism in Growing Pigs

4.1 Abstract

Lysine α-ketoglutarate reductase (LKR) is the rate-limiting enzyme in the catabolism of lysine, an indispensable and often limiting amino acid in the diets of growing animals. Amino acid utilization in the growing animal is often coordinated with developmental cues. Mitochondrial LKR and SDH activity in various tissues of the growing pig was measured through several developmental periods associated with nutritional transition. Liver and kidney LKR activity was not different among ages, however, LKR activity in the muscle and heart declined soon after birth. There was no effect of age on SDH activity for any tissue examined. Lysine oxidation to $^{14}CO_2$ was demonstrated in liver, muscle and intestinal epithelial cell (IEC) mitochondria during the first week of life; mitochondria from muscle oxidized significantly more lysine on days 3 and 7 compared to either liver or IEC mitochondria. Substrate inhibition was shown to play a role in different tissues at different ages in the growing pig. A potential role for this type of regulation was described. Several compounds were shown to inhibit hepatic LKR activity, but the potency of these compounds changed during the life of the pig. Enzyme protein localization in liver, IEC, kidney and brain was verified using antibodies. These results demonstrate that lysine degradation in growing pigs occurs in many tissues in addition to liver. These tissues make significant contributions to whole body lysine degradation. Lysine degradation in muscle mitochondria was substantial (47% of liver LKR activity) and unaffected by lysine concentration and by age after birth. Lysine

degradation in the kidney and IEC were 41% and 28%, respectively, of liver activity, and were unaffected by age. These data, taken together, suggest the possibility of tissue-specific isozymes of LKR.

4.2 Introduction

Nutrient intake, developmental cues and environmental stressors are amongst the signals that coordinate amino acid utilization in the developing animal. The transitions from fetal to neonatal nutrition are characterized by the shift from placental feeding to suckling, and at weaning, characterized by major changes in diet composition, physical form, digestibility, and (nutrient) bioavailability. These are often accompanied by major changes in nutrient utilization. For example, the high postnatal rate of protein turnover, which supports the rapid deposition of body protein, is associated with a very efficient utilization of dietary amino acids by the neonate mediated by feeding (253, 456), hormones (53, 115, 116, 340, 444, 474, 475, 500), amino acids (140, 340-342, 445), signal transduction pathways and efficient translation processes (52, 117, 140, 252, 253, 342, 444, 445). However this high rate of protein turnover declines dramatically with development; logarithmically decreasing from birth to the attainment of sexual maturity (377). Lysine, typically the first-limiting amino acid in diets based on cereal grains, controls the rate of protein deposition in growing pigs by acting as the fulcrum between amino acid adequacy (or excess) and limitation. Metabolic availability of lysine for protein synthesis is a result of both lysine intake and the rate of lysine degradation. Lysine degradation, in turn, may be linked to potential neurological and stress-related roles for lysine catabolites (354, 423, 424, 429).

Maturation of enzyme systems, for example activation of the urea cycle enzymes in the piglet small intestine (114, 502, 503), and development of ammonia metabolizing enzyme and acinar systems of the liver (269, 316), occur during the neonatal period. For example, arginine degradation in developing pig enterocytes was shown to be negligible in newborn and suckling pigs but was greatly enhanced in post-weaning pigs (504). Lysine degradation, via the activity of lysine α-ketoglutarate reductase (LKR), in the livers of young rats increased with age while brain LKR activity decreased during the same developmental period (374). The reasons for the changes in lysine degradation in these tissues are unclear.

Recent studies have shown that lysine-degrading enzymes are found in several extra-hepatic tissues of the chicken and pig (289). Under normal feeding conditions, since the utilization of lysine controls protein synthesis in the growing animal, it is important to understand fully the regulation of those enzymes controlling lysine degradation. The objective of this study was to determine the changes in lysine degradation via activity of lysine α-ketoglutarate reductase and saccharopine dehydrogenase in different tissues of the developing pig. Our results demonstrate that 1) liver is a primary tissue of lysine degradation throughout development; 2) lysine degradation in muscle was high immediately after birth but rapidly decreased; 3) intestinal epithelial cells contained LKR, but SDH activity was marginal; 4) different tissues exhibited substrate inhibition kinetics at different ages.

4.3 Materials and Methods

4.3.1 Animals and tissue sampling

All protocols were approved by the Departmental Animal Care committee, and were in accordance with procedures outlined by the Canadian Council on Animal Care. For the developmental study, a total of 50 male Yorkshire piglets (n=35, enzyme studies; n=15, oxidation studies); 5 per age group, at 0, 3, 7, 14, 21, 28 and 84 days of age were obtained from the Swine Research and Technology Centre (SRTC), University of Alberta. Oxidation studies were performed on tissues from piglets at ages 0, 3 and 7 days. All piglets were weighed and Ketamine (2-(2-chlorophenyl1)-(methylamino)-cyclohexanone hydrochloride; 30mg/kg) was injected intramuscularly prior to a lethal intravenous injection of sodium pentobarbital (0.3ml/kg). Pigs at 84 days of age were killed by exsanguination following stunning by the captive bolt method as per standard operating procedures at the SRTC.

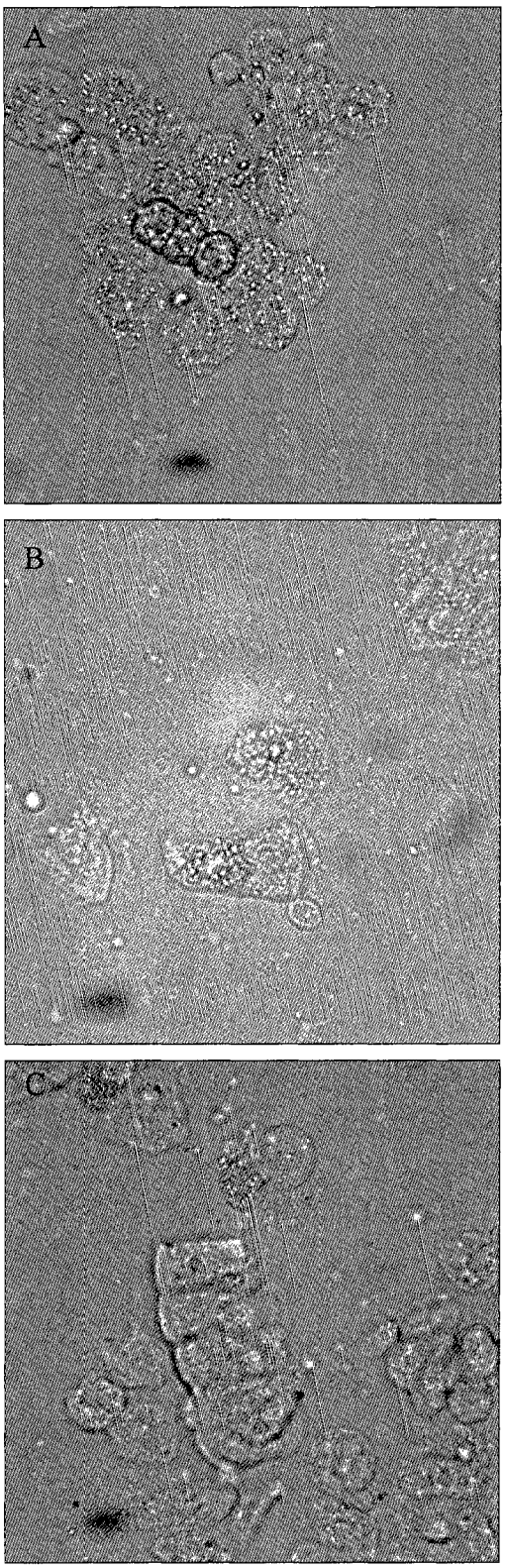
Jejunal sections from the small intestine were excised and stored in cold phosphate buffered saline (PBS) /5mM dithiothreitol (DTT) (pH 7.4). Sections of liver, kidney, skeletal muscle (longissimus dorsi), heart, and brain were obtained and stored in cold 0.3M mannitol /1mM EDTA. For oxidation studies, only liver, muscle and intestinal samples were taken. All samples were transferred to the laboratory on ice within 45 minutes for further processing.

4.3.2 Isolation of intestinal mucosal epithelial cells

Intestinal epithelial cells were prepared using modifications of previously described methods (195, 293, 486). A section of small intestine was obtained and

transported to the laboratory in ice cold phosphate buffered saline with protease inhibitor cocktail added (PBS: 137mM NaCl / 2.7mM KCl / 4.3 mM Na_2HPO_4 / 1.4 mM KH_2PO_4 / 5 mM dithiothreitol (DTT). The section was rinsed (1X) with fresh cold PBS to remove any remnants of excreta and then rinsed (3X) with fresh oxygenated (19:1 O_2:CO_2; Carbogen) Krebs-Henseleit Ca^{2+}-free buffer (121mM NaCl, 4.7mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 25.2mM $NaHCO_3$) containing 20 mM Hepes (pH 7.4), 5 mM EDTA, 20 mM DL-glucose, 2.1 mM lactate and 0.3 mM pyruvate (KHB buffer) / 5 mM DTT. The intestinal section was then filled with pre-warmed (37°C) oxygenated KHB (Ca^{2+}-Free)/DTT, sealed with clamps and immersed in a container filled with the same buffer. This system, with continuous Carbogen gassing, was then shaken for 45 minutes at 37°C with gentle massaging of the sealed intestinal sections at regular intervals. The resulting cell suspensions were filtered through 2 layers of cheesecloth and centrifuged for 3 minutes at 400x *g*, 4°C. Cells were washed 3 times in fresh oxygenated KHB buffer. At this time the cell viability was assessed by Trypan blue exclusion; preparations routinely demonstrated ≥90% viability. See Figure 4.1 and also appendix 8.4.1 for a complete protocol description.

Figure 4.1 Freshly Isolated Porcine Intestinal Epithelial Cells



Freshly isolated intestinal epithelial cells were photographed after removal from the intestine. Panel A; cells from a day 0 piglet intestine (40X); Panel B: cells from a day 3 piglet intestine (40X); Panel C: cells from a day 7 piglet intestine (40X).

4.3.3 Isolation of mitochondria

The re-suspended mucosal epithelial cell pellet [in 250mM sucrose/5mM HEPES/5mM DTT (pH 7.4)] was then transferred to a cold Dounce homogenizer (Wheaton Science Products, Millville, NJ) and the cell suspension disrupted by 8 passes of pestle A followed by 8 passes of pestle B. Mitochondria from the cells were then obtained by differential centrifugation according to previously published methods (293). The final mitochondrial pellet was re-suspended in 0.3M mannitol/2mM DTT.

All other tissues were weighed and then homogenized (VirTis tissue homogenizer, VirTis, Gardiner, NY) (1.0 g tissue / 5 mL of buffer). Mitochondria were then isolated by differential centrifugation (221, 323, 403). Mitochondrial pellets were re-suspended in 0.3 M mannitol. See also appendix 8.4.2 for a complete protocol description.

4.3.4 Lysine Ketoglutarate Reductase Assay

The initial step in lysine degradation was measured according to published methods (403) modified to a 96 well microplate format (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA). Oxidation of NADPH to $NADP^+$ was measured spectrophotometrically at 30°C using an extinction coefficient of 6.22 $M^{-1}cm^{-1}$. The reaction mixture contained 0.125 mM NADPH, 7.5 mM α-ketoglutarate, 100 mM HEPES, pH 7.8 and 300 μg of solubilized mitochondrial protein (0.2% Non-Idet P-40). Lysine concentrations ranged between 0-50 mM for kinetic analyses. A lysine concentration of 10 mM was chosen for the determination of specific activity because the enzyme in adult tissues was demonstrated to exhibit substrate inhibition at concentrations

exceeding 10-15 mM in adult pigs (Chapter 3, Figure 3.3). The reaction was initiated by the addition of NADPH. The assay was checked for linearity with time and protein concentration and optimized for substrate concentrations of lysine with each age group. See appendix 8.4.3 for a complete protocol description.

4.3.5 Saccharopine Dehydrogenase Assay

The activity of saccharopine dehydrogenase was measured in a similar fashion to LKR (403) and adapted to the microplate. The reduction of NAD^+ to NADH was measured in a reaction mixture containing 2.0 mM L-saccharopine, 2.0 mM NAD^+, 50 mM Tris/HCl, pH 9.4 and using an extinction coefficient of 6.22 $M^{-1}cm^{-1}$. Addition of saccharopine was used to start the reaction. Activity of SDH was linear with time and protein concentration at 30°C. See appendix 8.4.4 for a complete protocol description.

4.3.6 Inhibition Assays

For inhibition studies of both enzymes, potential inhibitors were added prior to the addition of protein. Controls were treated in exactly the same manner with the exception of substituting an equal volume of water for inhibitor.

4.3.7 Protein Determination

Protein concentrations of mitochondria and intact mucosal epithelial cells were determined by the Bicinchoninic acid (BCA) technique (Sigma-Aldrich, BCA1).

4.3.8 Lysine oxidation

Intact mucosal epithelial cells, or mitochondria from these cells, liver and muscle mitochondria were used to examine the oxidation of U-^{14}C- lysine to $^{14}CO_2$ in pigs age 0, 3 or 7 days. Assay conditions were based upon previously described methods (403); each data point was performed in duplicate for a minimum of three separate experiments. The oxidation media contained 60 mM KCl, 25 mM $KHCO_3$, 25 mM TEA-HCl (pH 7.6), 10 mM NaCl, 5 mM $MgCl_2$, 5 mM K_2HPO_4, 2 mM ADP, 1 mM EGTA, 5 mM sodium succinate, 5 mM sodium citrate, 3 mM α-ketoglutarate, 0.1 mM malate (sodium salt), 130 mM mannitol, 40 mM sucrose, 0.5 mM lysine and 0.5 μCi U-^{14}C-lysine per reaction. Oxidation media was titrated to pH 7.6 and made fresh each day immediately prior to use. The oxidation was performed in 25ml Erlenmeyer flasks using 2ml of oxidation media and 1ml of protein added to start the reaction. Flasks were sealed with rubber stoppers, which had a center well containing 300μL of CO_2 absorber (ethylene glycol monomethyl ether: ethanolamine, 2:1, v/v).

Oxidation assays were performed for 3 hrs at 30°C in a shaking water bath, at the end of which the reaction was stopped by injecting 1ml of ice cold 20% (v/v) $HClO_4$. The flasks were then allowed to continue shaking in the water bath for an additional 60 min to ensure complete collection and trapping of $^{14}CO_2$. Center wells were then completely transferred to scintillation vials, mixed with 5 ml of Atomlight (Packard BioScience B.V., Groningen, The Netherlands) and the radioactivity counted in a liquid scintillation counter (Beckman LS 5801, Beckman Coulter Canada Inc., Mississauga, ON).

Perchloric acid (20% (v/v), ice cold) was added to flasks prior to protein addition for zero time controls and non-radioactive (cold) lysine controls were performed with

each experiment. The oxidation assay was validated for linearity with time and protein concentration.

4.3.9 Chemicals

L-[1-^{14}C]-lysine (specific activity 54mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc., St. Louis, MO. L-[U-^{14}C]-leucine (Specific activity 58mCi/mmol) was purchased from Amersham Biosciences Corp., Piscataway, NJ. All other laboratory chemicals were purchased from Sigma unless specified otherwise.

4.3.10 Statistical Analyses

Data are presented as means ± SEM. One-way ANOVA (SAS/STAT version 8.01, SAS institute, Cary, NC) was performed on the specific activity of enzyme rates employing PROC GLM procedure with differences assessed by least square means procedure. Data were fitted to non-linear regression, Michaelis-Menten or substrate inhibition, or Lineweaver-Burke regression equations using Prism 4 for Macintosh, version 4.0b. Effect of pig age and inhibitor concentration were analyzed by repeated measures-ANOVA and differences from control were assessed using Dunnett's Multiple Comparison Test. Data was considered significant at $P < 0.05$.

4.4 Results

4.4.1 Analysis of Lysine α-Ketoglutarate Reductase and Saccharopine Dehydrogenase Activities

Both enzyme assays were linear for 30 minutes and routinely used 200 – 300 μg of protein, which was within the linear range for each tissue assayed. Assays were routinely terminated after 15 – 25 minutes (Appendix 8.4.5.4, Figure 8.9).

The specific activity of lysine α-ketoglutarate reductase in pig tissues is described in Table 4.1 and graphed in Figure 4.2. Liver LKR activity was approximately 2 nmol/min/mg throughout the experimental period, the only significant time effect was seen between days 14 and 21. Liver mitochondria had the highest LKR activity of any tissue at any time point with the exception of muscle on Day 0, but this difference was not significant. Muscle LKR activity significantly declined after Day 0; and remained relatively constant throughout the remainder of the experimental period. Average muscle LKR activity between 3 and 84 days of age was 0.87 nmol/min/mg, which was about 47% of liver activity. No other tissue demonstrated significant changes in LKR activities with age. Kidney LKR activity was significantly higher than heart and brain on day 0, but the only other difference measured was on day 3 for brain. On average, kidney LKR activity was 41% of liver or 0.77 nmol/min/mg. Mitochondria isolated from intestinal epithelial cells had LKR activities ranging from 0.15–0.83 nmol/min/mg with a mean activity of 0.53 nmol/min/mg or 28% of liver activity. LKR activity in the heart was typically the lowest measured, except on day 14, throughout the experiment. Brain LKR activity was measured in total brain homogenates in samples from the first week of life only; activities were low and significantly lower than liver on each day.

Saccharopine dehydrogenase (SDH) activity (Table 4.2) was measured only during the first week of life due to inability to source and purchase sufficient saccharopine for additional metabolic studies. SDH activity did not significantly change

for any tissue during these first seven days of life, therefore mean activity and percent of liver activity were calculated (Table 4.2). Liver SDH activity was significantly greater than any other tissue activity on either day with the exception of day 0 brain SDH activity. Brain SDH activity on day 3 was significantly less than liver activity but significantly greater than all other tissue activities on that day. Mean brain SDH activity was 1.50 nmol/min/mg or 32% of liver. SDH activity in kidney, IEC, muscle and heart mitochondria were similar on each day, accounting for 13%, 2.7%, 4.1% and 4.3 % of liver activity respectively.

Enzyme kinetics were measured using lysine concentrations ranging from 0-50 mM and fitted to the Michaelis-Menten (MM) and/or substrate inhibition (SI) equations: Michaelis-Menten: $Y=V_{max}\bullet X/(K_m + X)$; Substrate Inhibition: $Y=V_{max}\bullet X/(K_m+X+X^2/D)$; where V_{max} is the maximal velocity at saturation; K_m, or the Michaelis constant, is the substrate concentration required to reach half-maximal velocity ($V_{max}/2$); and D is the dissociation or inhibition constant. The graphical solutions to the kinetic analyses of LKR in different tissues from birth through to 84 days of age are depicted in Figures 4.3 to Figure 4.9.

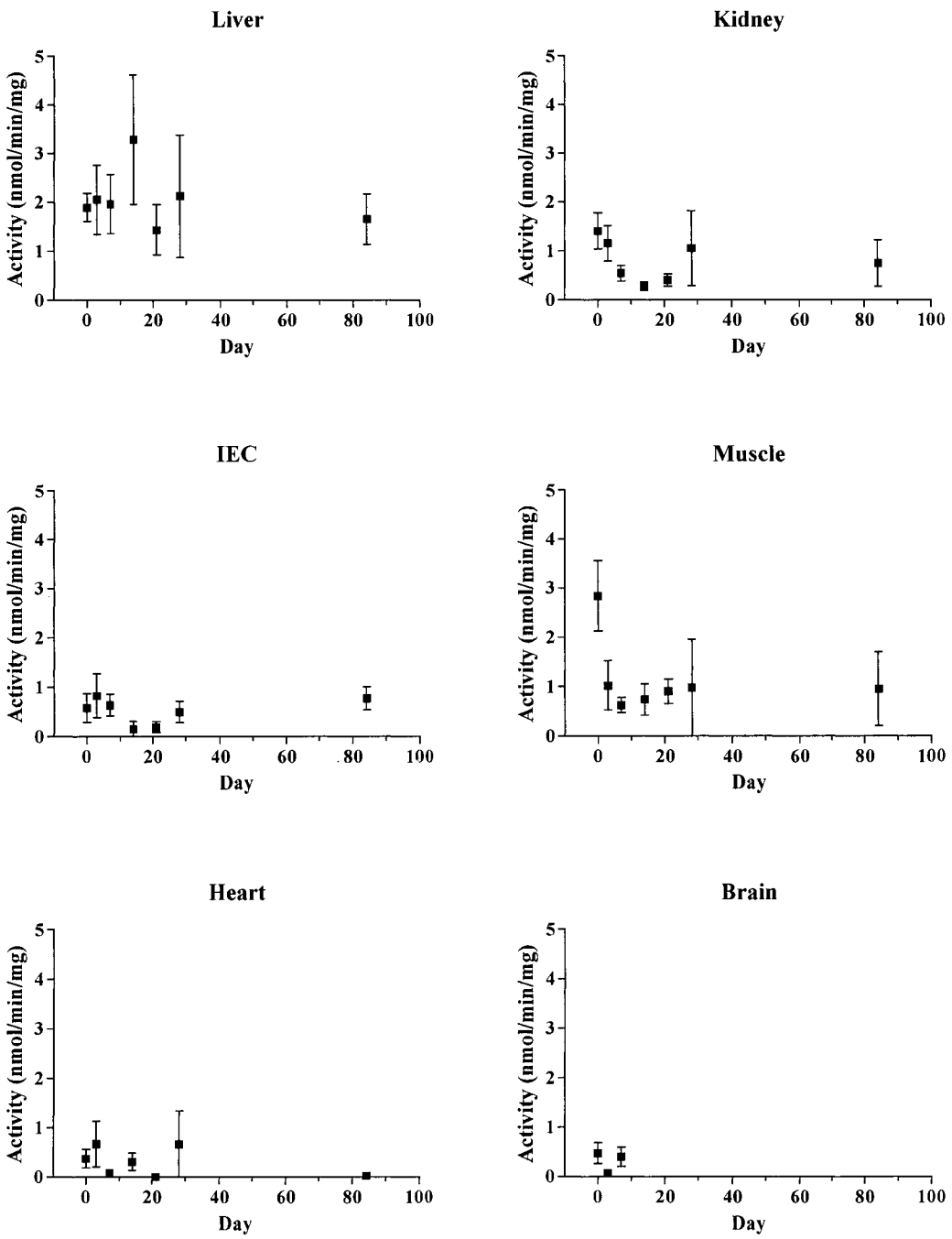
Most tissues exhibited characteristics consistent with both substrate inhibition as well as Michaelis-Menten kinetic models. Some data, for example day 3 brain, did not fit either model. Using data from both analyses, the Michaelis constants were solved for each tissue (Table 4.3). Liver and kidney had the most stable Michaelis constants throughout the experimental period, while some tissues demonstrated negative values, reflecting the low activities and high variability with some of the data.

Table 4.1 Tissue Activity of Lysine α-Ketoglutarate Reductase in Developing Pigs

	Day 0[5]		3 Days		7 Days		14 Days		21 Days		28 Days		84 Days		Mean Activity[6]		Percent of Liver
Tissue	Activity[1]	SEM	Activity	SEM	Activity	SEM	Activity	SEM	Activity	SEM	Activity	SEM	Activity	SEM	Activity	SEM	
Liver	$1.90^{ab;AB}$	0.28	$2.06^{a;A,B}$	0.71	$1.97^{a;A,B}$	0.60	$3.29^{a;A}$	1.33	$1.44^{a;B}$	0.51	$2.13^{a;A,B}$	1.25	$1.66^{a;B}$	0.51	1.86	0.11	100%
Kidney	$1.42^{a,c}$	0.37	$1.16^{a,b}$	0.36	0.55^{b}	0.16	0.28^{b}	0.09	$0.41^{a,b}$	0.12	$1.06^{a,b}$	0.76	$0.47^{a,b}$	0.10	0.77	0.17	41%
IEC[2]	$0.59^{c,d}$	0.29	$0.83^{b,c}$	0.45	0.64^{b}	0.22	0.15^{b}	0.15	$0.20^{a,b}$	0.11	0.50^{b}	0.22	$0.78^{a,b}$	0.23	0.53	0.10	28%
Muscle	$2.85^{b;A}$	0.71	$1.03^{b;B}$	0.50	$0.63^{b;B}$	0.15	$0.74^{b;B}$	0.32	$0.91^{a;B}$	0.20	$0.98^{a,b;B}$	0.98	$0.96^{a,b;B}$	0.75	0.87	0.06	47%
Heart	$0.38^{d,e;AB}$	0.19	$0.68^{b;A}$	0.36	$0.08^{b;B}$	0.07	$0.31^{b;A,B}$	0.18	$0.00^{b;B}$	0.00	$0.00^{b;B}$	0.00	$0.03^{b;B}$	0.03	NC[7]	---	NC
Brain[3,4]	$0.48^{c,e;A}$	0.21	$0.069^{c;B}$	0.062	$0.41^{b;A,B}$	0.19	NA[7]	---	NA	---	NA	---	NA	---	NC	---	NC

[1]Values are presented as specific activities; (nmol/min/mg mitochondria protein);
[2]IEC: Intestinal epithelial cell mitochondria;
[3]Brain tissue represents 10% homogenate of half of the brain (either left or right hemispheres of the cerebral cortex and 1/2 of the cerebellum cut along the midsagittal plane and severed at the brain stem);
[4]Brain tissue was collected only on days 0, 3 and 7.
[5]Days 0, 3 and 7; n = 5; Days 14, 21, 28 and 84, n = 4.
Data with different letter superscripts are significantly different; within column represents differences among tissues (lowercase script), within rows represents differences among different ages within the same tissue (uppercase script).
[6]Where age effect was not significant a mean value for all other measurements was calculated.
[7]NA – not available. NC – not calculated.

Figure 4.2 Tissue Activity of Lysine α-Ketoglutarate Reductase in Developing Pigs



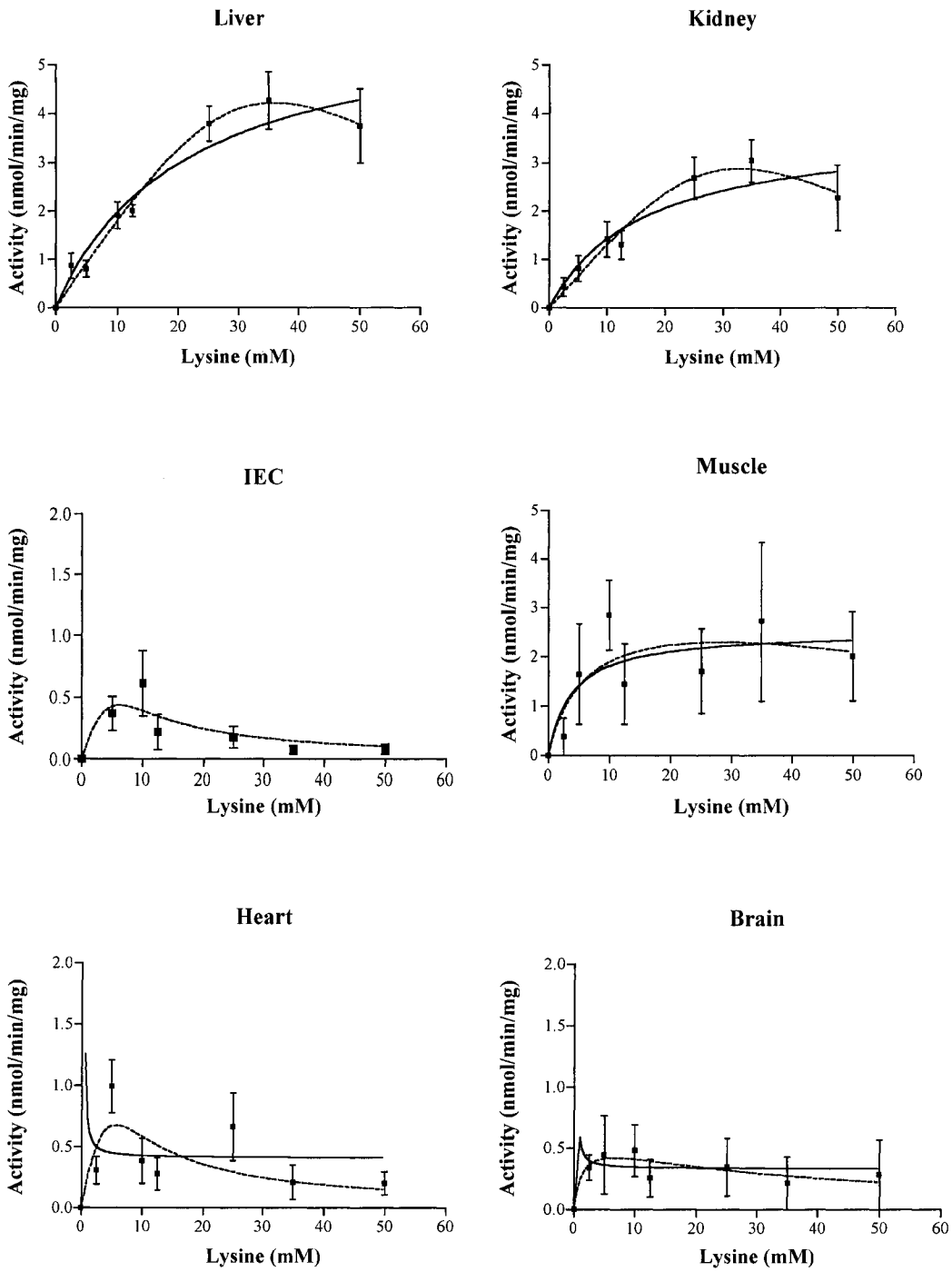
Graphical representation of data presented in Table 4.1.

Table 4.2 Activity of Saccharopine Dehydrogenase in Developing Pig Tissues

Tissue	0 Days[1]		3 Days		7 Days		Mean Activity[5]		% of Liver
	Activity	SEM	Activity	SEM	Activity	SEM	Activity	SEM	
Liver	3.07^{a}	0.89	4.64^{a}	1.21	4.68^{a}	0.80	4.66	0.55	100
Kidney	0.92^{b}	0.38	0.26^{b}	0.22	0.66^{b}	0.25	0.61	0.19	13.1
IEC[2]	$0.13^{b,c}$	0.09	ND[6]	---	ND	---	0.13	0.09	2.7
Muscle	$0.19^{b,c}$	0.19	0.14^{b}	0.08	0.24^{b}	0.16	0.19	0.030	4.1
Heart	$0.32^{b,c}$	0.15	0.19^{b}	0.06	0.09^{b}	0.07	0.20	0.067	4.3
Brain[3,4]	$1.06^{a,b}$	0.42	1.99^{c}	0.57	1.46^{b}	0.46	1.50	0.27	32.0

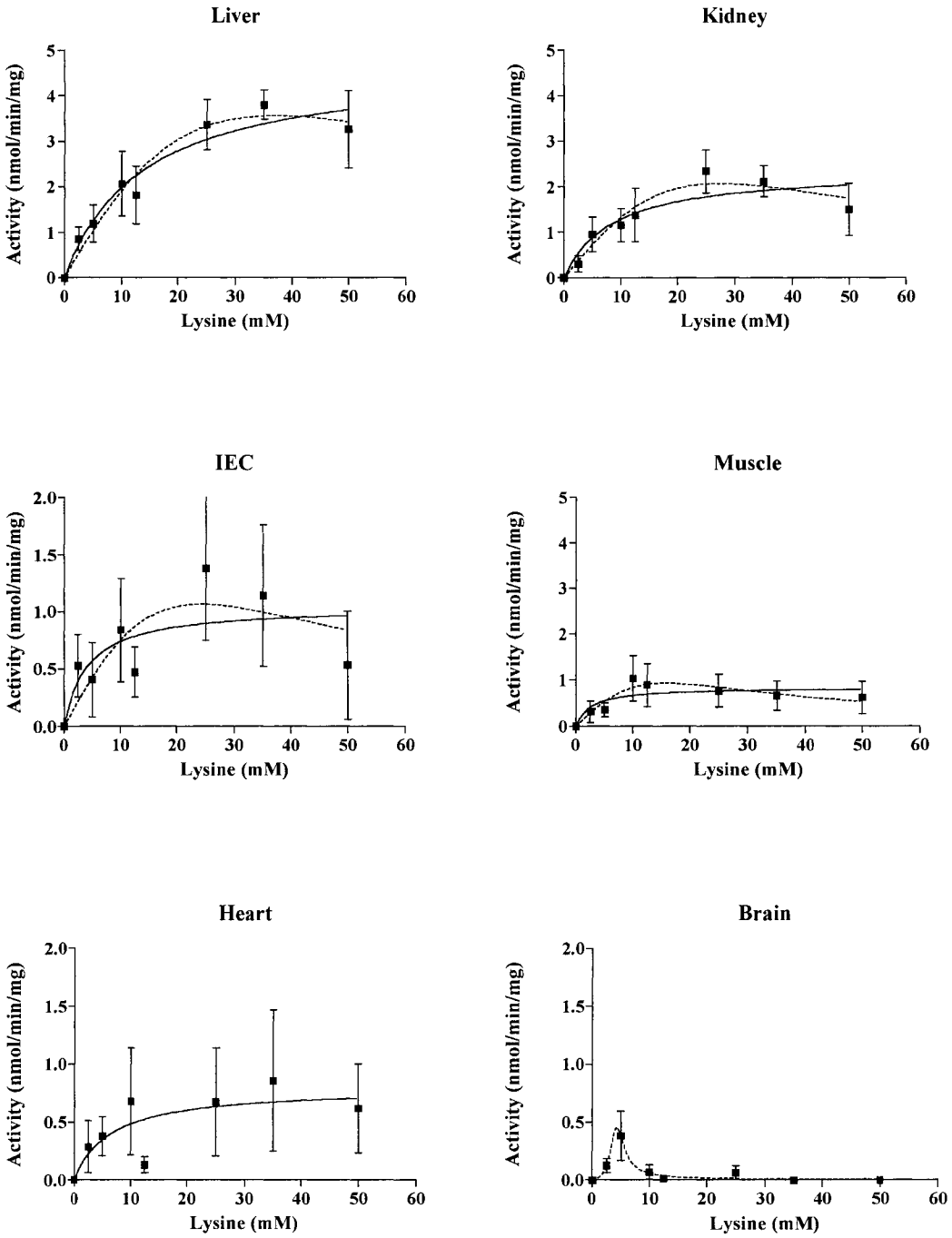
[1]Values are presented as specific activities; (nmol/min/mg mitochondria protein) for n=5 per tissue;
[2]IEC: Intestinal epithelial cell mitochondria;
[3]Brain tissue represents whole homogenate of half of the brain (either left or right hemispheres of the cerebral cortex and 1/2 of the cerebellum cut along the midsagittal plane and severed at the brain stem);
[4]Brain tissue was collected only on days 0, 3 and 7;
[5]IEC values from day 0 were used as the Mean calculation.
[6]ND: not detected.
Data with different letter superscripts are significantly different ($p<0.05$); within column represents differences among tissues (lowercase script), there were no differences among different ages within the same tissue (uppercase script).

Figure 4.3 Day 0 Enzyme Kinetics of LKR in Pig Tissues



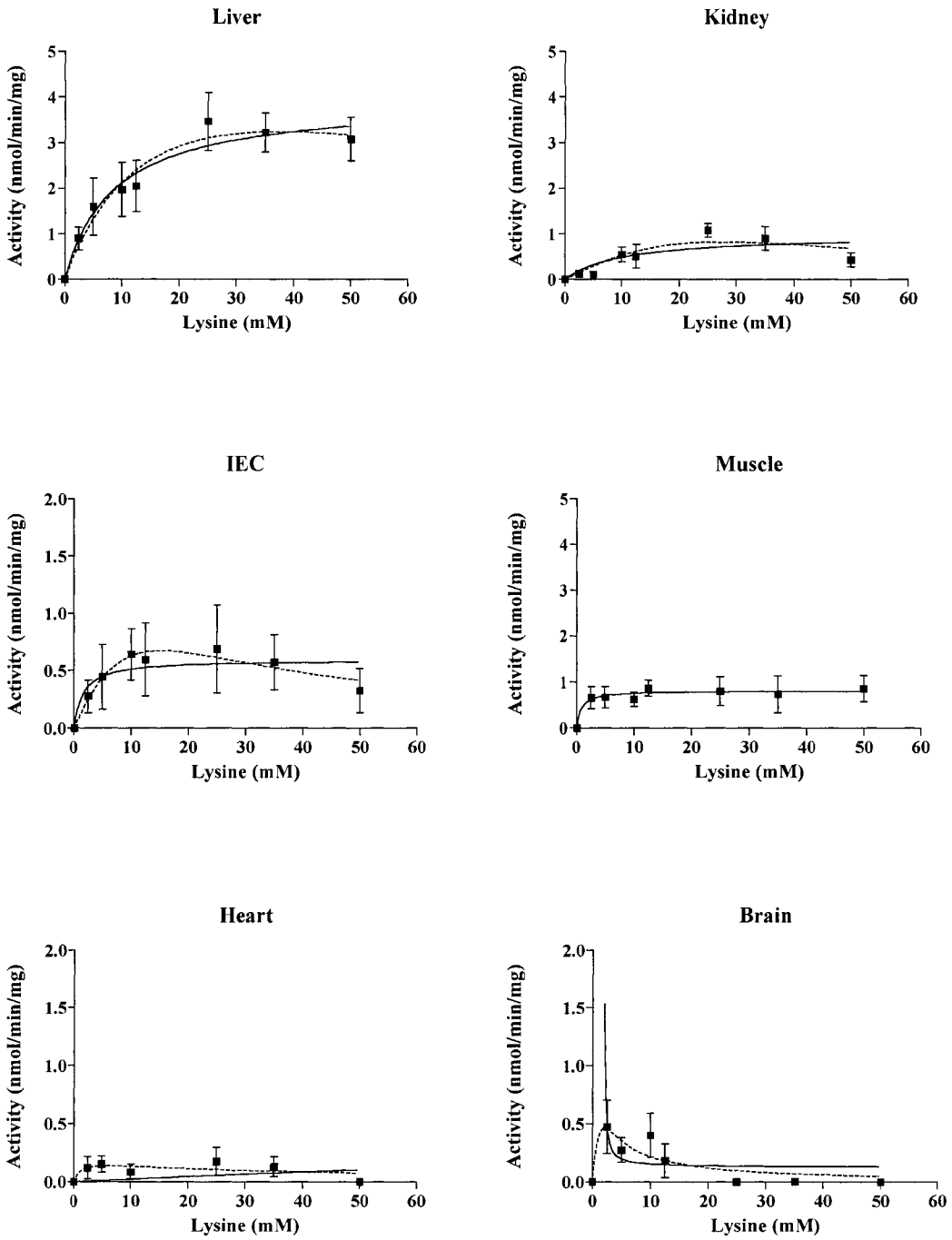
LKR enzyme kinetics in different pig tissues assayed using L-lysine concentrations ranging from 0 to 50 mM (n=5). Data represent Mean ± SEM and are fitted to Michaelis-Menten (solid lines) and substrate inhibition (dashed lines) non-linear regression equations. Note differences in Y-axes.

Figure 4.4 Day 3 Enzyme Kinetics of LKR in Pig Tissues



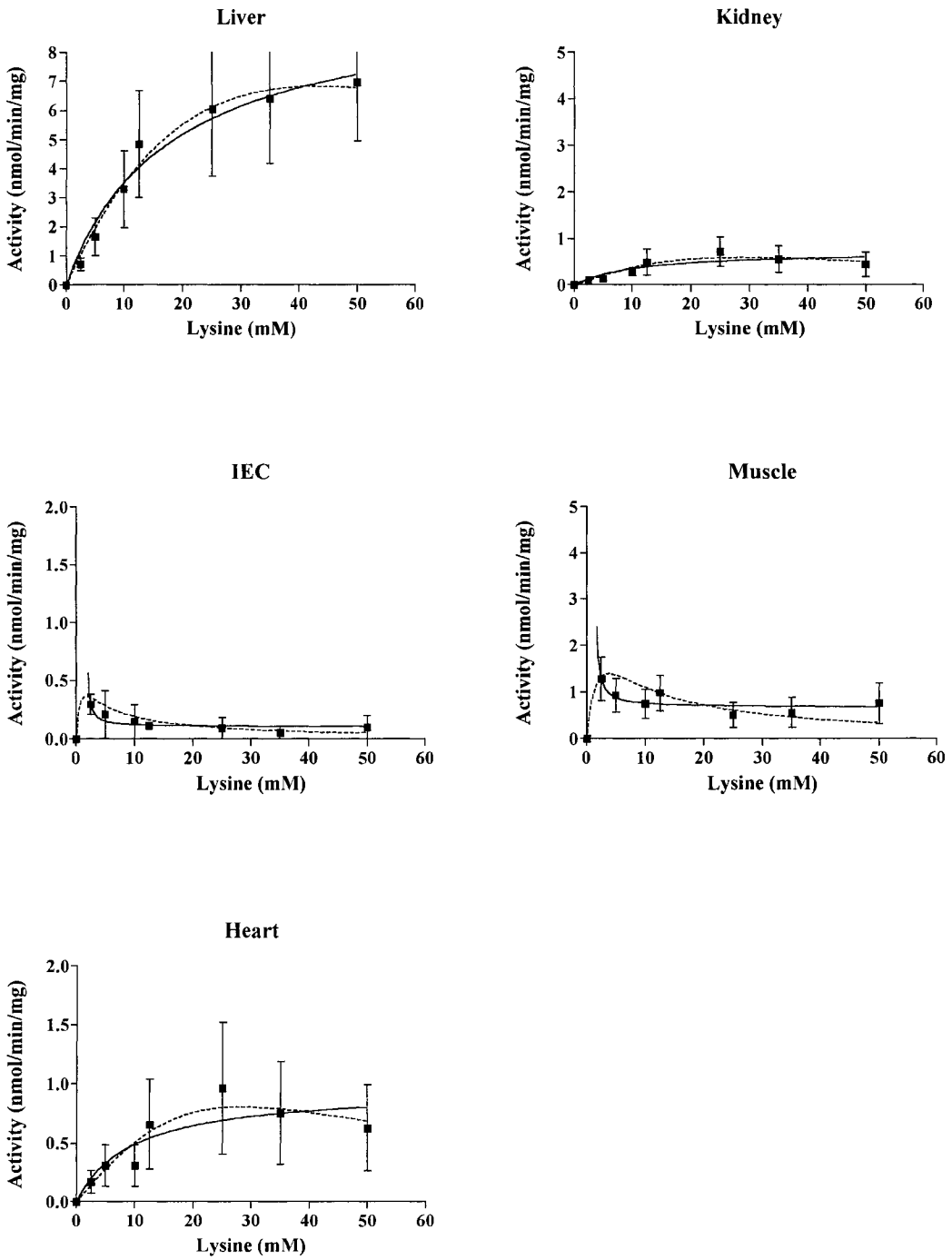
LKR enzyme kinetics in different pig tissues assayed using L-lysine concentrations ranging from 0 to 50 mM (n=5). Data represent Mean ± SEM and are fitted to Michaelis-Menten (solid lines) and substrate inhibition (dashed lines) non-linear regression equations. Note differences in Y-axes.

Figure 4.5 Day 7 Enzyme Kinetics of LKR in Pig Tissues



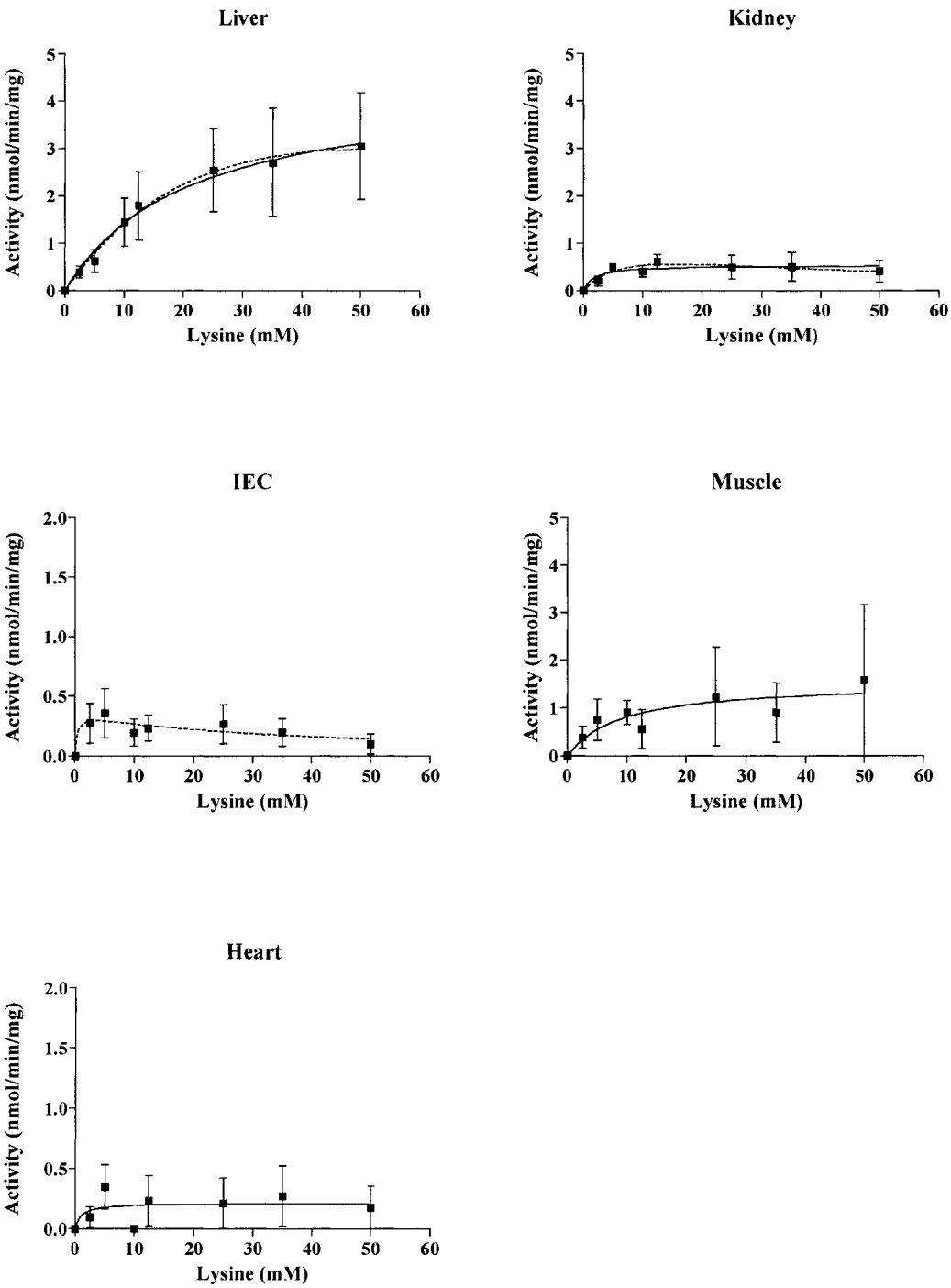
LKR enzyme kinetics in different pig tissues assayed using L-lysine concentrations ranging from 0 to 50 mM (n=5). Data represent Mean ± SEM and are fitted to Michaelis-Menten (solid lines) and substrate inhibition (dashed lines) non-linear regression equations. Note differences in Y-axes.

Figure 4.6 Day 14 Enzyme Kinetics of LKR in Pig Tissues



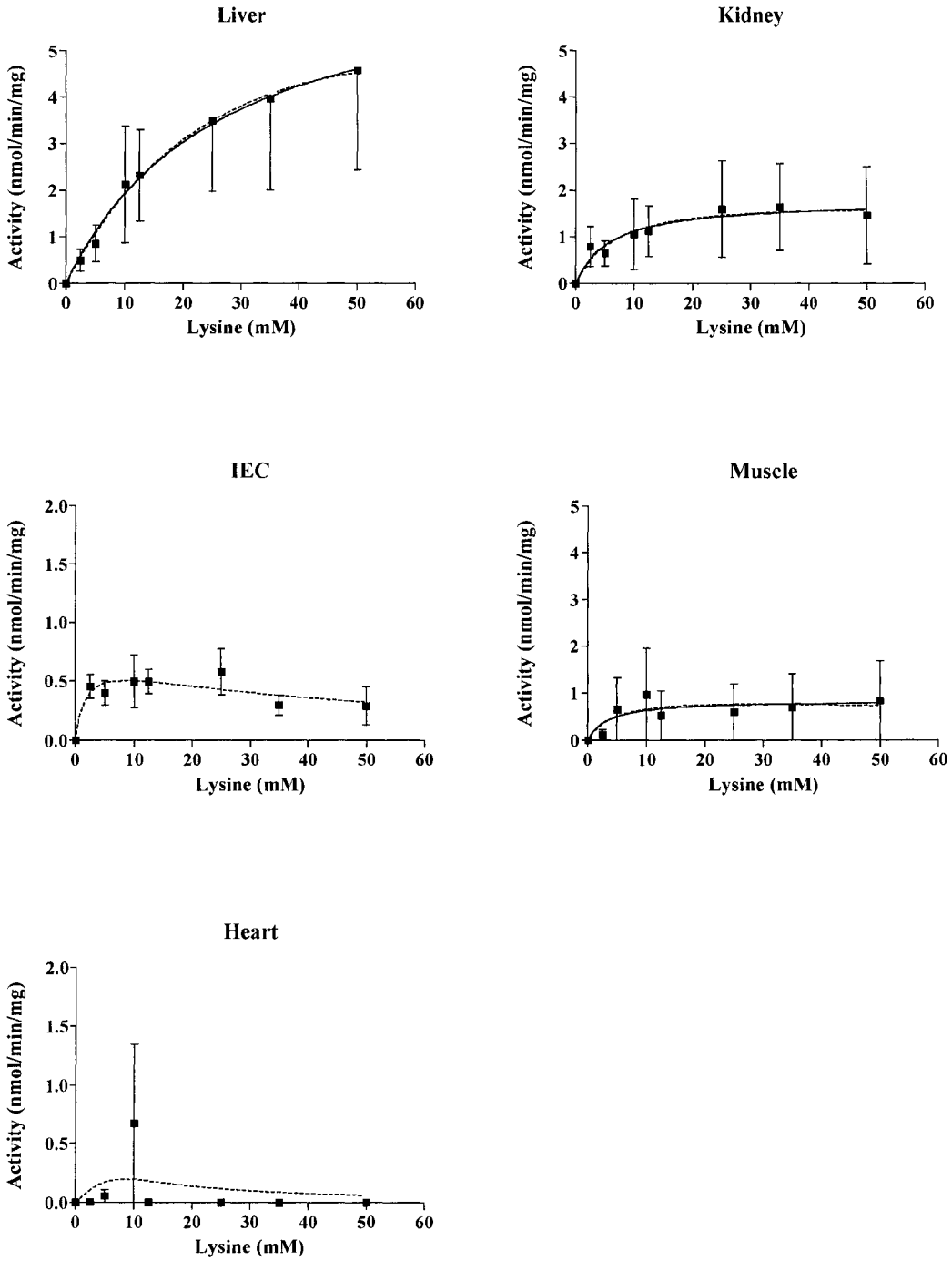
LKR enzyme kinetics was investigated in different pig tissues using L-lysine concentrations ranging from 0 to 50 mM. Data represent Mean ± SEM and are fitted to Michaelis-Menten (solid lines) and substrate inhibition (dashed lines) non-linear regression equations. Note differences in Y-axes.

Figure 4.7 Day 21 Enzyme Kinetics of LKR in Pig Tissues



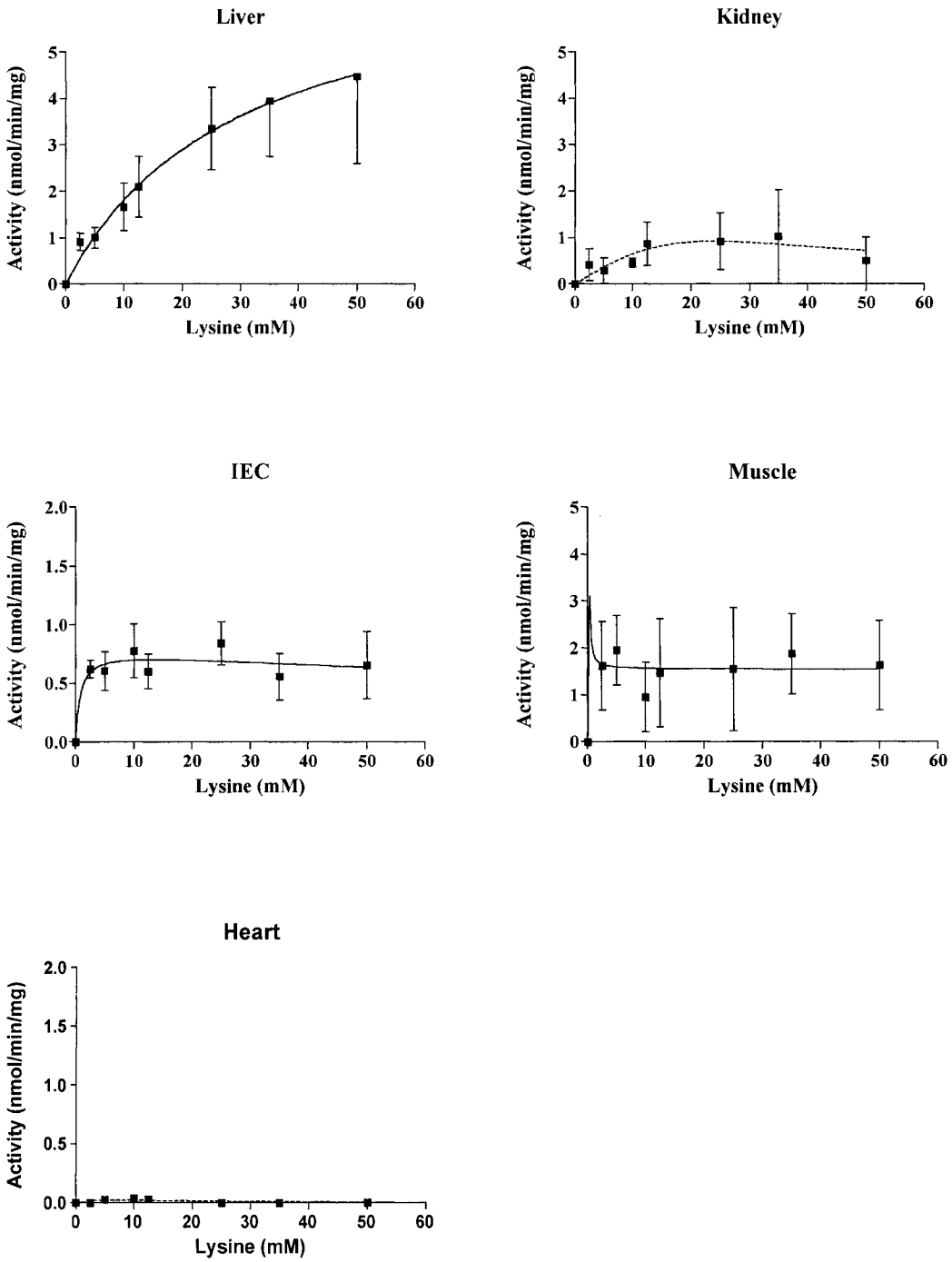
LKR enzyme kinetics was investigated in different pig tissues using L-lysine concentrations ranging from 0 to 50 mM. Data represent Mean ± SEM and are fitted to Michaelis-Menten (solid lines) and substrate inhibition (dashed lines) non-linear regression equations. Note differences in Y-axes.

Figure 4.8 Day 28 Enzyme Kinetics of LKR in Pig Tissues



LKR enzyme kinetics was investigated in different pig tissues using L-lysine concentrations ranging from 0 to 50 mM. Data represent Mean ± SEM and are fitted to Michaelis-Menten (solid lines) and substrate inhibition (dashed lines) non-linear regression equations. Note differences in Y-axes.

Figure 4.9 Day 84 Enzyme Kinetics of LKR in Pig Tissues



LKR enzyme kinetics was investigated in different pig tissues using L-lysine concentrations ranging from 0 to 50 mM. Data represent Mean ± SEM and are fitted to Michaelis-Menten (solid lines) and substrate inhibition (dashed lines) non-linear regression equations. Note differences in Y-axes.

Table 4.3 Effect of Age on Tissue Michaelis-Menten Constants (Km) of Lysine α-Ketoglutarate Reductase in Pigs

	0 Day			3 Days			7 Days			14 Days			21 Days			28 Days			84 Days		
Tissue	MM[1]	SI[2]	LB[3]	MM	SI	LB	MM	SI	LB	MM	SI	LB	MM	SI	LB	MM	SI	LB	MM	SI	LB
Liver	20.88	496.7	10.73	13.74	130.8	9.26	8.56	20.10	7.96	17.67	59.82	423	20.71	46.08	35.42	26.17	37.878	55.16	29.70	31.37	32.65
Kidney	16.17	258.0	21.18	8.32	$6.2x10^6$	34.04	10.02	$2.3x10^7$	28.92	10.93	$9.4x10^6$	29.59	1.68	7.68	6.33	5.73	7.15	13.20	5.34	$2.0x10^6$	37.09
IEC[4]	0.54	$9.8x10^6$	18.88	4.11	$3.4x10^6$	14.86	1.57	27.94	5.54	-3.04	$7.7x10^4$	---	-0.83	0.39	2.22	-0.25	1.71	2.99	-1.03	DNC	2.86
Muscle	3.81	5.47	33.74	2.47	$1.3x10^6$	8.36	0.73	DNC[4]	0.61	-1.33	DNC[5]	---	1.53	DNC	5.32	3.31	4.68	30.52	-0.17	DNC	10.98
Heart	-0.45	$1.7x10^5$	2.58	$6.4x10^3$	DNC	5.74	-0.86	-2.00	1.23	9.36	$5.2x10^6$	13.67	0.95	DNC	4.90	-2.50	DNC	---	$8.6x10^{-2}$	$1.2x10^6$	0.92
Brain[6,7]	-0.44	3.24	1.65	$1.7x10^{17}$	DNC	---	-1.85	3.11	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

[1]MM: Michaelis-Menten derived K_m given in mM;
[2]SI: Substrate inhibition derived K_m given in mM;
[3]LB: Lineweaver-Burke derived K_m given in mM;
[4]IEC: Intestinal epithelial cell mitochondria;
[5]DNC: the data did not converge to the equation for the non-linear equation;
[6]Brain tissue was collected only on days 0, 3 and 7; NA Not available.
[7]Brain tissue represents whole homogenate of half of the brain (either left or right hemispheres of the cerebral cortex and 1/2 of the cerebellum cut along the midsagittal plane and severed at the brain stem).

4.4.2 Inhibition of Hepatic LKR in Developing Pigs

Hepatic LKR was previously shown to be sensitive to a variety of compounds including catabolic intermediates such as saccharopine and α-aminoadipate (Chapter 3). In the current experiments, some of the more successful inhibitory compounds were investigated to determine their effectiveness at different ages of the pig.

Saccharopine significantly decreased LKR activity (20% – 25%) only on days 3 and 7, and only at the higher concentrations tested (10 and 20 mM) (Table 4.4). Conversely, on days 14 to 28 the addition of low concentrations of saccharopine significantly increased the activity of hepatic LKR by 50 to 300%, whereas at high concentrations LKR activity was not different from control. The level of inhibition tended to increase with age until day 14 and then decreased (Figure 4.10).

Aminoadipate addition had little significant effect on LKR activity (Table 4.5). However, the pattern of response was similar to saccharopine; within a given concentration of aminoadipate, LKR activity increased to a peak at day 14 and then declined (Figure 4.10). Within ages, increasing aminoadipate concentration had no significant effect on LKR activity except for 20 mM aminoadipate on day 21.

The addition of α-ketoadipate significantly decreased LKR activity on days 0 and 3 (Table 4.6) by 12% - 22%. However, the percent of inhibition did not increase with α-ketoadipate concentration. The effects of α-ketoadipate were limited to this early period; no significant effects were seen after 3 days of age. On day 14, it appeared as if the ketoadipate was stimulating LKR activity. Again, similar to the responses seen with saccharopine and aminoadipate, within a given concentration of ketoadipate, LKR

activity increased until day 14 and then declined (Figure 4.10). Within ages, there was no effect of increasing ketoadipate concentration.

Pipecolic acid, an intermediate in the overflow pathway of lysine catabolism significantly decreased LKR activity on day 0 by 15% - 18%. No other significant effects of pipecolic acid were measured (Table 4.7). However, the same pattern of increasing LKR activity, which peaked on day 14 and then decreased, again as with saccharopine, aminoadipate and ketoadipate, was observed (Figure 4.10).

Spermine, at 20 mM on day 7 decreased LKR activity by 25%, but significantly increased the activity by ~60% – 240% on days 14 and 21 (Table 4.8). Again the same trend with age was observed; regardless of spermine concentration, LKR activity increased, peaked on d14 and then decreased again in response to spermine addition (Figure 4.10). On day 7, LKR activity decreased in response to increasing concentration of spermine, but this trend was reversed on day 14. No effect of spermine concentration was observed on the other experimental days.

Kynurenine, a metabolite of tryptophan degradation, completely abolished the activity of LKR at 20 mM on each of days 0, 3 and 7. In addition, 10 mM kynurenine on these days either abolished LKR activity or decreased the activity by more than 80% (Table 4.9). Homocitrulline, a lysine with urea attached to the ε-amino group, had no significant effect on LKR activity (Table 4.10).

Table 4.4 Inhibition of Hepatic LKR at Different Ages by Saccharopine

	Concentration of Inhibitor[1]							
	0.20 mM		2.0 mM		10.0 mM		20.0 mM	
Age	*% Activity of Control*[2]							
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
Day 0	81.0^{a}	7.2	110.2^{acd}	13.5	115.8^{acd}	3.1	116.1^{a}	11.7
Day 3	94.8^{a}	5.3	115.3^{ac}	11.6	84.7^{ac}	6.5	77.4^{*bc}	11.8
Day 7	98.5^{a}	4.5	102.7^{ab}	10.8	78.0^{*a}	5.7	73.5^{*c}	6.7
Day 14	317.7^{*b}	14.0	259.3^{*b}	12.8	148.5^{b}	14.8	139.5^{ad}	14.7
Day 21	184.2^{*c}	19.5	174.1^{*d}	23.0	117.3^{bc}	6.6	111.4^{ab}	15.8
Day 28	150.0^{c}	8.7	180.7^{d}	22.4	117.5^{bc}	8.7	107.6^{abc}	8.7
Day 84	152.6^{*c}	20.3	142.6^{*d}	10.8	143.4^{*bd}	13.6	166.8^{*d}	15.3

[1]LKR activity was determined in the presence of various potential inhibitory compounds. Compounds were added prior to addition of protein. Control tubes had water substituted for inhibitory compound and were run each day for each animal; data are presented as Percent of Control Activity for n ≥3 separate experiments (samples were measured in duplicate). Control activities were used to normalize treated samples; control values are those listed in Table 4.1.

[2]Values with * indicate activities were significantly different from control ($p<0.05$), as assessed by Repeated Measures ANOVA and Dunnett's Multiple Comparison Test. Values with different letters were significantly different among ages within a column (compound concentration) as assessed by Two-way ANOVA and Bonferroni post tests.

Table 4.5 Inhibition of Hepatic LKR at Different Ages by Aminoadipate

	Concentration of Inhibitor[1]							
	0.20 mM		2.0 mM		10.0 mM		20.0 mM	
Age	*% Activity of Control*[2]							
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
Day 0	92.7^{ab}	4.0	95.4^{ab}	5.6	106.2^{a}	5.0	98.0^{a}	10.6
Day 3	93.0^{a}	4.4	92.5^{a}	3.7	101.0^{a}	4.4	112.3^{ab}	5.0
Day 7	120.6^{ab}	8.2	105.4^{ab}	7.1	112.7^{a}	11.5	123.2^{ac}	14.2
Day 14	164.6^{b}	9.8	124.2^{b}	15.9	156.5^{b}	13.7	143.6^{bc}	11.2
Day 21	113.6^{a}	5.8	108.9^{ab}	5.3	115.8^{ab}	8.0	144.3^{*c}	28.5
Day 28	125.8^{ab}	16.5	117.4^{ab}	16.8	112.5^{a}	10.9	113.2^{abc}	10.2
Day 84	92.6^{a}	9.0	96.9^{ab}	1.8	88.3^{a}	6.8	97.6^{a}	5.0

[1]LKR activity was determined in the presence of various potential inhibitory compounds. Compounds were added prior to addition of protein. Control tubes had water substituted for inhibitory compound and were run each day for each animal; data are presented as Percent of Control Activity for n ≥3 separate experiments (samples were measured in duplicate). Control activities were used to normalize treated samples; control values are those listed in Table 4.1.

[2]Values with * indicate activities were significantly different from control ($p<0.05$), as assessed by Repeated Measures ANOVA and Dunnett's Multiple Comparison Test. Values with different letters were significantly different among ages within a column (compound concentration) as assessed by Two-way ANOVA and Bonferroni post tests.

Table 4.6 Inhibition of Hepatic LKR at Different Ages by α-Ketoadipate

	Concentration of Inhibitor[1]							
	0.20 mM		2.0 mM		10.0 mM		20.0 mM	
Age	*% Activity of Control*[2]							
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
Day 0	86.5[a]	5.4	78.3[*a]	7.7	82.1[*a]	4.4	81.6[*a]	3.5
Day 3	83.8[*a]	4.2	87.5[*ab]	4.2	83.7[*a]	3.5	88.1[*a]	6.1
Day 7	101.5[a]	9.2	90.7[ab]	10.9	138.3[b]	18.7	104.8[a]	13.9
Day 14	142.1[b]	6.8	122.1[c]	3.4	124.3[b]	13.2	140.0[b]	14.5
Day 21	104.9[ac]	4.6	99.2[ac]	7.6	109.7[ab]	11.6	104.6[a]	9.4
Day 28	135.2[bc]	24.4	115.9[bc]	7.7	123.9[b]	15.3	106.8[ab]	8.8
Day 84	91.7[a]	6.3	91.6[ac]	6.1	92.6[ab]	9.0	108.3[ab]	17.6

[1]LKR activity was determined in the presence of various potential inhibitory compounds. Compounds were added prior to addition of protein. Control tubes had water substituted for inhibitory compound and were run each day for each animal; data are presented as Percent of Control Activity for n ≥3 separate experiments (samples were measured in duplicate). Control activities were used to normalize treated samples; control values are those listed in Table 4.1.

[2]Values with * indicate activities were significantly different from control ($p<0.05$), as assessed by Repeated Measures ANOVA and Dunnett's Multiple Comparison Test. Values with different letters were significantly different among ages within a column (compound concentration) as assessed by Two-way ANOVA and Bonferroni post tests.

Table 4.7 Inhibition of Hepatic LKR at Different Ages by Pipecolic Acid

	Concentration of Inhibitor[1]							
	0.20 mM		2.0 mM		10.0 mM		20.0 mM	
Age	*% Activity of Control*[2]							
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
Day 0	81.9*[a]	4.6	84.4[*a]	0.9	87.2[a]	3.7	82.6[*a]	4.9
Day 3	90.0[a]	6.1	91.6[ab]	9.0	87.9[ac]	7.2	95.1[ab]	6.8
Day 7	101.3[a]	10.8	81.3[a]	5.2	94.6[ac]	12.6	90.7[ab]	8.5
Day 14	130.4[b]	12.8	114.8[b]	6.2	123.9[b]	7.7	109.9[b]	7.8
Day 21	108.1[ab]	7.5	94.0[ab]	7.3	114.2[bc]	6.0	108.3[ab]	8.0
Day 28	109.3[ab]	1.9	102.4[ab]	11.8	102.6[ab]	18.1	99.5[ab]	8.0
Day 84	110.3[ab]	13.1	90.7[ab]	7.1	89.8[ac]	6.1	86.5[ab]	6.7

[1]LKR activity was determined in the presence of various potential inhibitory compounds. Compounds were added prior to addition of protein. Control tubes had water substituted for inhibitory compound and were run each day for each animal; data are presented as Percent of Control Activity for n ≥3 separate experiments (samples were measured in duplicate). Control activities were used to normalize treated samples; control values are those listed in Table 4.1.

[2]Values with * indicate activities were significantly different from control ($p<0.05$), as assessed by Repeated Measures ANOVA and Dunnett's Multiple Comparison Test. Values with different letters were significantly different among ages within a column (compound concentration) as assessed by Two-way ANOVA and Bonferroni post tests.

Table 4.8 Inhibition of Hepatic LKR at Different Ages by Spermine

	Concentration of Inhibitor[1]							
	0.20 mM		2.0 mM		10.0 mM		20.0 mM	
Age	*% Activity of Control*[2]							
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
Day 0	101.4^{a}	2.1	114.3^{abc}	4.6	116.2^{ac}	10.2	109.2^{ac}	15.8
Day 3	102.1^{a}	6.1	99.8^{ac}	9.2	124.6^{ac}	15.4	116.7^{acd}	13.0
Day 7	106.9^{ab}	6.7	88.1^{ac}	7.8	93.8^{ac}	6.6	75.0^{*a}	9.0
Day 14	163.9^{*bc}	15.7	166.0^{*b}	13.3	225.8^{*b}	22.8	238.5^{*b}	17.4
Day 21	105.5^{a}	5.4	121.7^{abc}	16.7	164.2^{*c}	18.0	160.4^{*cd}	21.1
Day 28	117.0^{c}	4.8	133.5^{ab}	12.3	144.3^{c}	7.6	145.1^{d}	7.3
Day 84	95.0^{abc}	6.3	89.9^{c}	8.8	78.1^{ad}	6.5	84.2^{a}	13.3

[1]LKR activity was determined in the presence of various potential inhibitory compounds. Compounds were added prior to addition of protein. Control tubes had water substituted for inhibitory compound and were run each day for each animal; data are presented as Percent of Control Activity for n ≥3 separate experiments (samples were measured in duplicate). Control activities were used to normalize treated samples; control values are those listed in Table 4.1.

[2]Values with * indicate activities were significantly different from control ($p<0.05$), as assessed by Repeated Measures ANOVA and Dunnett's Multiple Comparison Test. Values with different letters were significantly different among ages within a column (compound concentration) as assessed by Two-way ANOVA and Bonferroni post tests.

Table 4.9 Inhibition of Hepatic LKR at Different Ages by Kynurenine

	Concentration of Inhibitor[1]							
	0.20 mM		2.0 mM		10.0 mM		20.0 mM	
Age	*% Activity of Control*[2]							
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
Day 0	82.5	2.8	92.6	11.2	0.0^{*}	0.0	0*	0
Day 3	93.8	4.3	94.2	4.8	19.9^{*}	19.9	0*	0
Day 7	102.1	8.4	110.5	5.0	16.6^{*}	16.6	0*	0

[1]LKR activity was determined in the presence of various potential inhibitory compounds. Compounds were added prior to addition of protein. Control tubes had water substituted for inhibitory compound and were run each day for each animal; data are presented as Percent of Control Activity for n ≥3 separate experiments (samples were measured in duplicate). Control activities were used to normalize treated samples; control values are those listed in Table 4.1.

[2]Values with * indicate activities were significantly different from control ($p<0.05$), as assessed by Repeated Measures ANOVA and Dunnett's Multiple Comparison Test. Values with different letters were significantly different among ages within a column (compound concentration) as assessed by Two-way ANOVA and Bonferroni post tests – no significant differences were observed..

Table 4.10 Inhibition of Hepatic LKR at Different Ages by Homocitrulline

	Concentration of Inhibitor[1]							
	0.20 mM		2.0 mM		10.0 mM		20.0 mM	
Age	*% Activity of Control*[2]							
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
Day 21	106.2	2.2	95.9	7.8	113.4	10.3	107.0	15.0
Day 28	105.6	11.3	105.4	11.3	100.9	11.3	100.5	6.8
Day 84	100.8	8.9	88.0	2.6	88.2	7.0	90.1	6.8

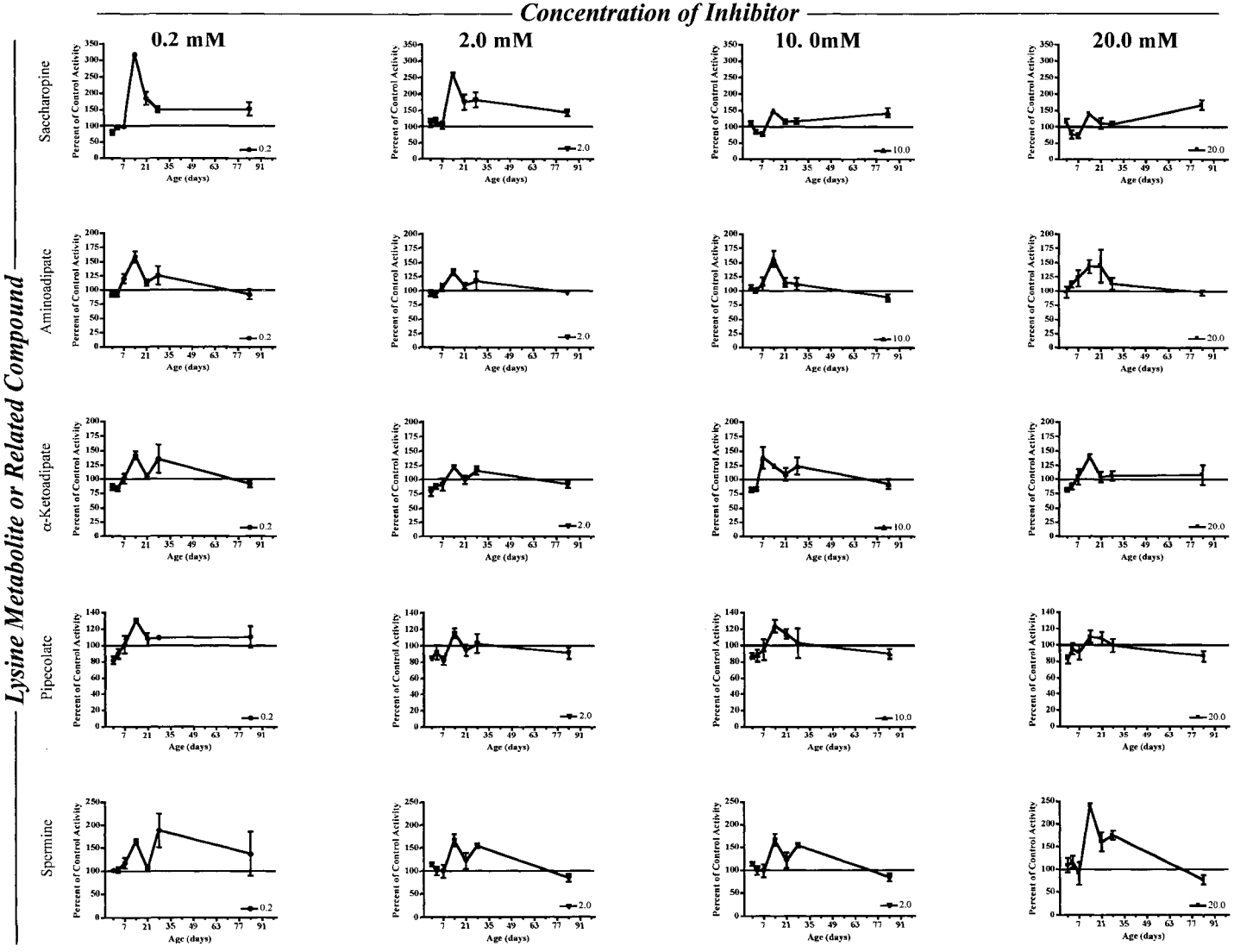
[1]LKR activity was determined in the presence of various potential inhibitory compounds. Compounds were added prior to addition of protein. Control tubes had water substituted for inhibitory compound and were run each day for each animal; data are presented as Percent of Control Activity for n ≥3 separate experiments (samples were measured in duplicate). Control activities were used to normalize treated samples; control values are those listed in Table 4.1.
[2]Values with * indicate activities were significantly different from control ($p<0.05$), as assessed by Repeated Measures ANOVA and Dunnett's Multiple Comparison Test. Values with different letters were significantly different among ages within a column (compound concentration) as assessed by Two-way ANOVA and Bonferroni post tests – no significant differences were observed..

4.4.3 Production of $^{14}CO_2$ by developing pig tissues

To determine whether the complete pathway for lysine catabolism was present in the tissues of the developing pig, the production of $^{14}CO_2$ from universally labeled or 1-^{14}C-lysine was measured in mitochondria isolated from liver, muscle and the intestinal epithelial cells. Due to the demands for IEC protein and the limited quantities available per animal[A], we earlier determined that intact IEC could be used to measure oxidation of lysine, as well as the oxidation of the branched chain amino acids and ketoisocaproic acid (Chapter 3, section 3.4.5, Figure 3.12, (138)). Production of $^{14}CO_2$ from lysine by liver or IEC did not change significantly during the first 7 days, however the production of $^{14}CO_2$

[A] Experiments were conducted to measure $^{14}CO_2$ production from labeled lysine, valine, leucine, isoleucine and ketoisocaproic acid. To meet the demands, it was more economical to utilize whole intact IEC, rather than utilize more animals for this procedure. The numbers of animals required would have would have doubled (at least), with no increase in the experimental n.

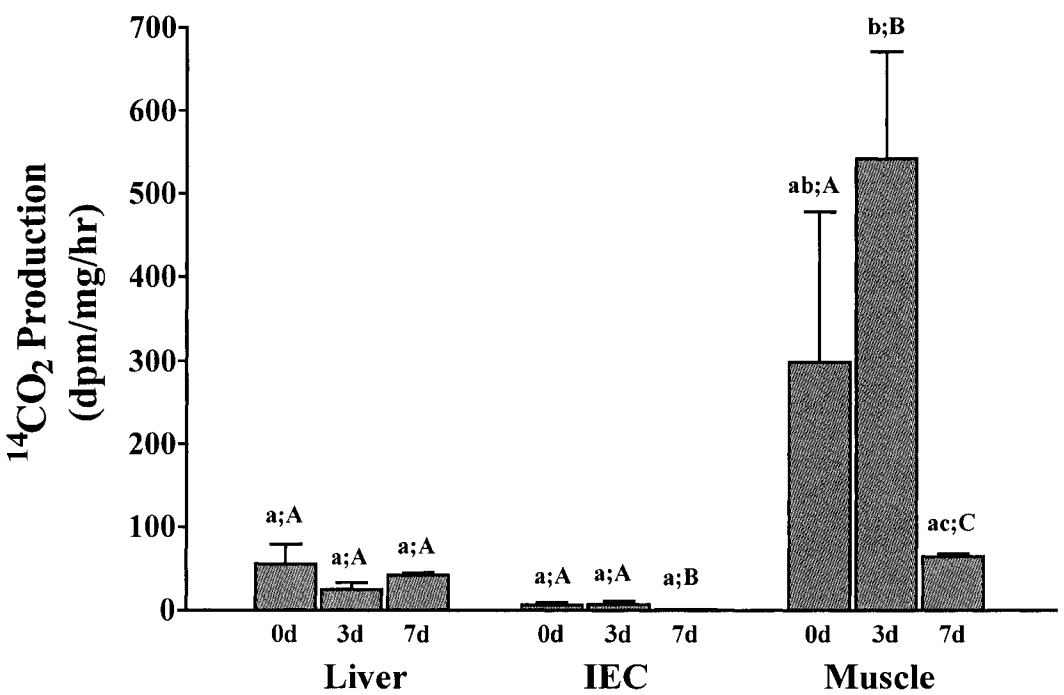
Figure 4.10 Effect of Age and Inhibitor Concentration on LKR Inhibition



Preceding page; Graph summarizing data from Tables 4.4 - 4.8 to illustrate trend seen with increasing age of growing pig. Dashed line represents the level of control LKR activity.

from muscle mitochondria was significantly lower after 7 days, compared to day 3 data, but was not different from day 0 $^{14}CO_2$ production (Figure 4.9). Comparing between tissues, $^{14}CO_2$ production was the same on day 0, but on day 3 oxidation of lysine by muscle mitochondria was significantly greater than IEC, but the same as liver. On day 7 all tissues had $^{14}CO_2$ production rates that were significantly different.

Figure 4.11 Recovery of $^{14}CO_2$ from ^{14}C-Lysine in Young Pig Tissues



Intact mitochondria from liver and muscle preparations, and intact IEC were incubated with universally labeled ^{14}C-L-Lysine for 3 hours; $^{14}CO_2$ was collected simultaneously and post-reaction termination in ethylene glycol monomethyl ether: ethanolamine. Radioactivity was quantified by scintillation. Data are presented as Mean ± SEM for 5 separate experiments. Values are corrected for protein and substrate blanks. Different uppercase letters indicate significant differences among tissues on the same day; different lowercase letters indicate significant differences within tissues among ages; $P< 0.05$.

4.5 Discussion

There is a readily adaptive metabolic milieu in developing tissues and organ systems that allow the growing animal to adjust to major transitions in nutritional supply, form and availability. The efficient utilization of nutrients, especially essential amino acids like lysine, is necessary for survival because these parameters largely determine protein synthesis in growing animals. To this end, we have investigated the catabolism of lysine in various pig tissues at different ages through examination of lysine α-ketoglutarate reductase and saccharopine dehydrogenase, the first two enzymes in the lysine catabolic pathway.

The nutritional transitions that occur in the first weeks of life are extremely significant and the dietary environment of these periods can have lasting effects on the health and growth (potential) of the animal. The initial suckling period includes colostrum delivery to the newborn animal to supply immunoglobulins and a rich supply of nutrients. These not only sustain life but also set the stage for intestinal growth and development (50, 274, 366, 372, 395, 433, 515, 516) that in turn directly impacts whole body metabolic status. Indeed, one of the more contentious issues in the global pig industry is the most suitable duration for suckling, ranging from 10 – 28 days or even longer in some cases (55, 334, 476). Additional areas of this debate are the merits of creep feeding; the addition of solid grain diets for neonatal piglet *ad libitum* consumption while suckling. This transition from suckling to weaning can shift the profile of amino acids entering the intestine and portal blood supply (101, 158). This shift may lead to acute metabolic shortages in the concentrations of amino acids critical for protein

synthesis during this period. Stoll et al (438) reported extensive catabolism of threonine and BCAA by the small intestinal mucosa of milk-fed piglets. Following these reports, Flynn and co-workers (158) demonstrated that plasma glutamine concentrations, along with alanine, threonine and the BCAA concentrations decreased in piglets from one day of age to day 21. One explanation to account for the decrease of these amino acids in plasma was increased catabolism of these amino acids by the small intestine and other tissues. In addition, if amino acids are required or utilized for roles other than protein synthesis, then abrupt deficiencies may have longer-term consequences, especially if these amino acids are acting as signals for other metabolic activities. For example, arginine, and its immediate precursors, ornithine and citrulline, were markedly decreased in 7- to 21-day old pigs compared with 1- to 3-day old piglets (158). This decrease indicated a deficiency of arginine for hepatic ammonia detoxification, and as a substrate for nitric oxide synthase. Guan et al (192) have recently shown that in TPN-fed neonatal piglets, the GLP-2 stimulation of intestinal blood flow and glucose utilization was dependent upon nitric oxide. Therefore, decreasing the nitric oxide signal via lowered arginine levels is expected to decrease the GLP-2 signals that stimulate intestinal growth (129) and thus lead to a diminished intestinal capability. Decreased intestinal capacity in the neonate will negatively impact the growth and hence performance of the animal. Thus understanding the roles of the amino acids and their catabolites will help more clearly define the impact of acute deficiencies or excesses of these compounds on cellular, tissue and whole body metabolism.

4.5.1 Tissue Distribution and Age Effects on LKR and SDH activities

In these current experiments we questioned whether lysine catabolism during the major nutritional transitions in the young, growing pig was altered to match nutritional changes associated with birth, suckling, and weaning. The activity of the rate-limiting enzyme, lysine α-ketoglutarate reductase was detected in each of the tissues assayed (Table 4.1, Figure 4.2). Regarding development, little previous data was available on the activity of the lysine catabolic enzymes. Two studies in rats showed a significant rise in hepatic LKR activity during the initial stages of development, with a concomitant decline in brain activity (374, 411). Another rat study (440) showed that brain, heart and kidney α-ketoadipate dehydrogenase (α-KADH) activity showed a continuous rise (7, 8 and 29 fold, respectively) from day –5 to day 28. Liver α-KADH activity did not increase until day 10, and a 7-fold increase was noted by day 28. No other data in the literature were found regarding age effects on other lysine catabolic enzymes.

In the present experiment, unlike the rat (374, 411), hepatic LKR activity in the pig did not change in the first weeks of life and was stable at ~2 nmol/min/mg. This contrast may be a species difference (320) reflecting differences in nutritional requirements or tissue ontogeny of rats and pigs during similar periods of growth and development. However, LKR activity in the kidney appeared to decrease during the first 3 weeks after birth (from ~1.5 to 0.3-0.4 nmol/min/mg) and then stabilize at ~ 0.5 nmol/min/mg. As shown in Chapter 3, kidney LKR activity was ~2.5 nmol/min/mg in market weight (110 kg) pigs, thus an increase in kidney LKR from d84 to ~120-140 days of age occurs. Examining Figure 4.2, it appears that kidney LKR activity was, unlike liver LKR, influenced by developmental cues. During the first days after birth, kidney

function increases significantly (211). While experiments have shown that dietary electrolyte balance can influence lysine metabolism (159, 356), experiments describing an interaction between LKR activity or lysine degradation in the kidney coinciding with renal functional development have not been reported. This question is worthy of further research.

Muscle LKR activity was highest on day 0, and then decreased significantly on day 3, at which point the activity stabilized to ~1nmol/min/mg for the remainder of the experimental period. Although not significant, muscle activity was the highest amongst the different tissues analyzed on day 0. Our $^{14}CO_2$ production data also showed significant lysine oxidation during the first week of life (Figure 4.9). The high rate of muscle LKR activity and lysine oxidation early in the neonatal period may be due in part to the demand for a large increase of substrate flux through the TCA cycle, so that ATP synthesis matches the increased ATP demands of muscle tissue, because of the increase in physical activity. During the first day after birth, physical activity accounts for 46% of the piglet's time, of which 37% is spent suckling (306). Le Dividich (273) showed that the energy costs (measured as heat production) of standing activity (amounting to 0.159 kJ kg^{-1}BW min^{-1}) in the 1-day-old piglet were proportional to standing time. These authors calculated that during the first day of life, spontaneous physical activity could contribute to 30% of the total heat production. This contribution was increased if suckling activity was taken into account. Thus the energy cost of physical activity is a major factor in energy metabolism in the newborn piglet.

The main energy stores available at and soon after birth are colostrum, since suckling starts within 20-30 minutes after birth, and body energy stores (30, 202, 273).

The main body energy stores are body protein (72.1%) followed by glycogen (18.1%) and fat (9.6%) (30, 139). Benevenga and colleagues (30) calculated that it would take 10.7 hours to consume all of the body fat or 20.3 hours to consume all of the stored carbohydrate (glycogen). Although, calculations by these authors (30) and others (273) show that only 3.4% - 6.8% of the heat produced by a newborn piglet is derived from body protein, it is unlikely that fat and glycogen stores would be consumed sequentially or totally. Thus some body protein must be catabolized to meet the fuel demands of the piglet. In support of this hypothesis: while neonates have a high rate of protein gain, facilitated by a high ribosome content in the skeletal muscle (151), several studies have suggested that newborns demonstrate a resistance to depressing the rate of protein breakdown (367, 368), but that the ability to reduce proteolysis in response to parenteral nutrition increases with development (121). This resistance to depress protein breakdown is most likely because the dynamic remodeling of muscle protein requires constant amino acid availability at the cellular level. Other studies have further shown that neonatal whole-body proteolysis reduction was primarily regulated by the availability of free amino acids in the intracellular pool in an insulin-independent manner (367, 368). Furthermore, neither glucose nor lipid could reduce the rate of proteolysis in neonates as well as amino acids. More recently, work by Thivierge and colleagues (456) showed that although feeding decreased whole-body protein breakdown, hind limb (specifically the longissimus dorsi muscle) proteolysis was increased by ~33% in response to feeding. Therefore, a sustained high rate of muscle protein breakdown early in the life of the piglet will insure that a large pool of intracellular amino acids is always available. A high rate of protein degradation will also buffer the changes that incoming amino acids from the

diet have on amino acid concentrations in the intracellular pool, i.e. larger diet changes are required to change plasma and intracellular amino acid concentrations – more uniform amino acid ratios will provide sustainable protein synthesis. That the major fate of these amino acids is de novo protein synthesis is not argued; however, it may be that a small proportion of these amino acids may be used, or must be used, to meet the demands for energy in the muscle. Evidence showing that protein breakdown is not reduced when glucose or lipids, the main energy fuel substrates, were provided to the neonate (122) supports this argument.

Immediately after birth, an increase in plasma glucagon and a fall in plasma insulin (382) provide the appropriate hormonal environment for gluconeogenesis in neonatal piglets. Along with substantial activity of the gluconeogenic enzymes (35% - 105% of adult values), the hormonal environment enables the newborn piglet to meet its glucose demands during the first hours following parturition provided there is ample supply of substrate (202). Lysine, at physiological concentrations, has been shown to stimulate gluconeogenesis from lactate (93, 94, 264), presumably by increasing the intracellular concentrations of glutamate and aspartate and thereby increasing the rate of cytoplasmic NADH reoxidation (94). Lysine may therefore have an important, but previously undervalued, role in postnatal energy homeostasis.

Therefore, the metabolic environment of the newborn piglet facilitates protein catabolism, which would release lysine from body protein. Once released, this lysine would then be available for stimulation of gluconeogenesis and-or degradation via LKR to provide energy.

Newborn piglets also experience a sudden 15-20°C drop in their thermal environment (202). Having no brown adipose tissue (460), very low glycogen storage, being virtually hairless and devoid of subcutaneous fat (336), the piglet must use muscular shivering thermogenesis to maintain body temperature (32). Although a warm microenvironment is usually provided in the farrowing pen, the pig tends to ignore it on the first day (336). To accommodate the energy demands of thermogenesis, the oxidative capacity of muscle is higher during the first days of extra-uterine life compared to subsequently (32). Additionally, mitochondrial protein mass in muscle was shown to increase by 49% (in longissimus dorsi) during the first 5 days post-partum, (400) to support the demand for increased oxidative capacity. Because the newborn pig lacks energy storage as fat and glycogen, amino acids are a major fuel source in the early postnatal period. The rate of lysine oxidation to CO_2 by muscle was 3-10 times higher than liver oxidation for day 0 and 3, respectively (Figure 4.10) indicating that lysine (and possibly other amino acids) are important energy sources for muscle in the early postnatal period.

Lysine degradation merges with fatty acid oxidation at glutaryl-CoA dehydrogenase, which catalyzes the flavin-dependent oxidation of glutaryl-CoA to crotonyl-CoA and CO_2. Suda and colleagues (440) showed that liver activity of α-KADH, which converts α-ketoadipate to glutaryl-CoA, was low immediately after birth and suggested that this was due to increased hepatic lipid oxidation rates during this period (15, 66, 161), in response to the high fat, low carbohydrate content of milk (131) which requires CoA. Thus CoA would not be available for the catalytic action of α-KADH, hence leading to repression or inhibition of the liver enzyme. During the suckling

period, Suda and colleagues (440) suggested that the conversion of α-ketoadipate to glutaryl CoA would be carried out by the kidney and heart. However, the young pig, unlike the rat and other mammals, does not have a high rate of hepatic β-oxidation (3, 359). Carnitine palmitoyltransferase I (CPTI) is essential in regulating flux of fatty acids through β-oxidation (300, 335, 344, 399), but data from neonatal pigs has shown that while CPTI activity doubles between birth and 24 hours (33) to a rate similar to that of a 24-day-old pig, fatty acid oxidation is only one third of the rate seen in the 24-day-old pig. The cause for this discrepancy in neonatal pigs has been linked to the sensitivity of hepatic CPTIα to the inhibitory effects of malonyl-CoA (335). However, the other isoform, CPT1β, expressed in pig muscle and heart (335) has a low K_M for carnitine (197 μM) and a low sensitivity for malonyl-CoA inhibition (IC_{50} = 906 nM) (380). As noted by Peffer and colleagues (357), although fatty acids serve as the principal substrate for hepatic oxidative metabolism during the late suckling period (178), the low capacity of the neonatal pig for hepatic β-oxidation may actually indicate impaired nutrient handling. The muscle CPT1β isoform functions to effectively circumvent the hepatic deficiency. Therefore, in the newborn pig, the muscle is a very oxidative tissue that is critical to thermogenesis. Although increased β-oxidation has not been investigated as to whether it increases lysine degradation, the increased oxidative capacity of muscle and muscle mitochondria during this period may contribute to the high rate of lysine degradation in the muscle tissue.

Lysine catabolism in the brain during development and through life may be an important source of glutamate, an excitatory neurotransmitter. As described by Papes et al. (354), the sources of nitrogen and carbon for the de novo synthesis of glutamate in the

brain have remained elusive. The main precursor for the carbon skeleton comes primarily from glucose (414), while the BCAA have been suggested as potential donors of amino groups for the synthesis of approximately 30-50% of the glutamate produced within the brain. However, the enzyme BCAT is restricted to the astrocyte (232, 236, 511-513) and therefore sources for glutamate synthesis in the neuron are presently unknown. In addition, the amino acids that contribute the remaining 50-70% of the amino groups needed for glutamate synthesis in the brain have not been identified. LKR/SDH enzyme protein and activity has been localized to neurons in several regions in the brain (354) and lysine could thus be the source of amino groups for the de novo synthesized glutamate in neurons.

During brain maturation and immediately after birth, when most cellular communications are being established and refined in the central nervous system, glutamate-mediated neurotransmission is essential (225, 226, 308, 479). Thus if lysine degradation via the saccharopine pathway was associated with or necessary for glutamate production in the central nervous system, a deficiency in LKR or SDH activity would be highly detrimental. The fact that mental retardations and neuromotor control problems have been associated with genetic disorders of lysine catabolism (64, 106, 174, 176, 256, 292, 392, 497) provides strong indirect evidence for this hypothesis. Additionally catabolites of lysine (chiefly α-aminoadipate) have been linked with the modulation of glial metabolism via decreased kynurenic acid production. Kynurenate is a broad-spectrum competitive antagonist of all ionotropic excitatory amino acid receptor (439), and more specifically antagonizing the N-*methyl*–D-*aspartate* (NMDA) receptor (355). Therefore lysine catabolism in the brain may indirectly enhance the responses of

excitatory amino acid receptors (184, 462). Whether these mechanisms serve a developmental role is unknown, however a potential mechanism that could have development implications is discussed in section 4.5.2.

In the developing intestine, LKR activity appeared to decrease after the first week until post-weaning when the activity began to increase. Lysine utilization by the neonatal pig intestine has been documented by several studies (44, 435, 438, 468). Presumably much of this lysine utilization is directed towards protein synthesis, however Stoll and colleagues (438) showed that significant lysine catabolism occurred in the mucosal cells. Thus we predicted that activity of the rate-limiting enzyme for lysine catabolism would be found. Intestinal degradation of other indispensable amino acids, including lysine, has been described previously (29, 466, 468). A functional role for lysine degradation in the intestine is presently unknown, however its oxidation to provide energy in the gut has been demonstrated (466, 468).

The production of lysine catabolites in IEC may also serve a to modulate neuromuscular development of the enteric nervous system similar to the potential roles identified for tryptophan and BCAAs. Van Der Schoor and colleagues (465) suggested that a potential role for tryptophan is utilization for serotonin synthesis, because of the high innervation of the intestine (307). Furthermore, the BCAA catabolic enzyme, BCAT has similarly been localized to cells in the stomach and intestine (446) which were closely associated with enteric ganglia. Thus a neurological role for lysine degradation in both the central and peripheral nervous systems is a possibility.

Saccharopine dehydrogenase activity did not differ with piglet age in any tissue assayed (Table 4.2). With the exception of brain SDH activity on day 0, liver

mitochondria had significantly greater SDH activities of any tissue assayed on any day. Brain activity was maintained between 31-43% of liver activity during the first week of extra-uterine life, again suggesting an important role for lysine degradation in the developing brain. In contrast to the LKR data, SDH activities in muscle were considerably lower than liver activities. Similarly, SDH activity in IEC was low on day 0 and below the level of detection on days 3 and 7. These results suggest that there is a tissue-specific level of regulation for SDH activity.

The oxidation data (Figure 4.9) shows that the entire lysine catabolic pathway was present in all tissues examined. It is possible that activation of SDH activity in muscle and intestine is more tightly controlled than in liver or kidney. For example, if both monofunctional SDH and bifunctional LKR/SDH activities are present in the liver and kidney (353), but only bifunctional LKR/SDH activity is present in the intestine and muscle, then activation of the enzyme by increased levels of lysine and calcium (Figure 1.6, as described in the model proposed by Galili (169, 518)) would be increasingly important. If the optimum signals were not presented to SDH, or to any of the enzymes in the pathway, then substrate processing by the enzyme and flux through the whole pathway may be underestimated. While we optimized the conditions of temperature, substrate concentration, protein concentration, and pH, our SDH assay included neither calcium nor lysine. Therefore, our studies could not clarify this mechanism.

Examination of the literature reveals limited investigation of saccharopine dehydrogenase activity in different tissues: liver (35, 145, 147, 320, 353, 403), kidney (353) and human placenta (154). One investigation that did describe SDH tissue distribution was based upon a single (human) sample that employed cell free

homogenates isolated from frozen tissue collected post-mortem; activity was detected in liver, kidney and trace in lung homogenates (221). The lack of SDH investigations may in part be due to the difficulty of synthesizing (or obtaining) adequate quantities of saccharopine for metabolic studies (415); throughout the current research period saccharopine was frequently unavailable from the chemical supplier.

4.5.2 Kinetic Analysis of LKR in Developing Pig Tissues

The data presented in Table 4.3 describes the predicted Michaelis constants (K_m) for the various tissues during the experimental period. For each tissue, values have been predicted using classical Michaelis-Menten parameters and using substrate inhibition kinetics. Lineweaver-Burke transformations were performed to provide an additional estimate of the Michaelis constants. Most of the constants are in the millimolar range, resonant of the role of LKR as a catabolic enzyme of an essential amino acid.

Chapter 3 (Section 3.5.1, Figures 3.3,3.4) presented data from the adult pig demonstrating substrate inhibition of hepatic and IEC lysine α-ketoglutarate reductase by lysine. Following upon these experiments the kinetics of LKR activity was investigated in selected tissues of the developing pig (Figures 4.3 – 4.9). The activity of liver LKR in growing pigs seemed to have the same kinetic response and maximal activity (~2 nmol/min/mg using 10 mM lysine) from d0-d84, rising only to ~4 nmol/min/mg later in life (~140 days, Chapter 3). Liver LKR activity, which clearly demonstrated substrate inhibition in the adult animal, was not similarly affected in the developing pig. On days 0 and 3, the data appear to support substrate inhibition, but the argument for Michaelis–Menten kinetics would appear just as valid. If we consider that LKR followed Michaelis-Menten kinetics from d0-d84, and 25 mM lysine to be saturating, then the

same developmental trend was seen at 10 mM lysine. The mean activity of LKR was ~3.5 nmol/min/mg throughout the experimental period. Thus liver LKR activity, following either kinetic mechanism, appeared to have no developmental influences; this is more clearly illustrated in Figure 4.2.

LKR activity in the kidney, however, appeared to be more susceptible than liver to substrate inhibition but only from birth to weaning and only at concentrations exceeding 25 mM lysine. At this concentration, kidney LKR activity appeared to be maximal at every age, although still less than liver LKR activity. If, as in the case of the liver, we consider kidney LKR to follow Michaelis-Menten kinetics and 25 mM lysine was saturating, the same developmental trend is seen (Figure 4.2) at 10 mM lysine.

IEC LKR kinetics displayed a substrate-inhibited kinetic mechanism throughout development. LKR activity in the brain also appeared to be dramatically susceptible to substrate inhibition, especially on days 3 and 7. Muscle and heart LKR kinetics were more difficult to describe, as the data were more highly variable.

The kinetic mechanism of substrate inhibition was described in Chapter 3, section 3.5.1. In brief, substrate inhibition is believed to be due to multiple substrate molecules simultaneously binding within the active site, resulting in decreased turnover of a substrate at one of these sites (223, 260). In the case of LKR, the end result is the diminished catabolism of lysine.

In the regulation of amino acid catabolism, the tetrahydrobiopterin-dependent amino acid hydroxylases are an interesting group of enzymes to consider. For example, using the synthetic pterin cofactor, 6,7-dimethyltetrahydrobiopterin (DMPH), phenylalanine hydroxylase exhibits classical Michaelis – Menten kinetics (241).

However, in the presence of the naturally occurring pterin, tetrahydrobiopterin (BH_4), the enzyme gave a sigmoidal response (242). In the presence of BH_4 and a potent activator of the enzyme, lysolecithin, the enzyme was substrate inhibited by increasing concentrations of phenylalanine (152). Tryptophan hydroxylase similarly was susceptible to substrate inhibition when the naturally occurring BH_4 replaced $DMPH_4$ (163). The hydroxylation of tryptophan is the initial step in the synthesis of serotonin whereas (tryptophan) indoleamine-2,3-dioxygenase activity results in the formation of formyl-kynurenine, preceding the formation of kynurenine. High levels of cellular tryptophan will inhibit the hydroxylase action via substrate inhibition, and increase the synthesis of kynurenine (which also results in competition for cellular uptake of tryptophan). Kynurenine can be transaminated to kynurenic acid, a neuroprotective excitatory amino acid receptor antagonist, by α-aminoadipate aminotransferase.

This step links the catabolism of tryptophan with the catabolism of lysine because aminoadipate competes with kynurenine for transamination, thus decreasing kynurenate production (505). Decreasing kynurenate synthesis from kynurenine may then effectively enhance the responses of excitatory amino acid receptors (462). Conversely, the presence of increased aminoadipate concentrations (pharmacological) have been shown to be both neurotoxic and gliotoxic (45, 47, 346). McBean (299) showed that the L-isomer of aminoadipate could exert its metabolic effects (inhibition of 1. the plasma-membrane glutamate transporter [K_i of 192 μM] and 2. glutamine synthetase activity [K_i of 109 μM]) at physiological concentrations of α-aminoadipate inside the glial cell. Transport systems in astrocytes may act in concert to limit aminoadipate accumulation and prevent detrimental effects in these cells, while permitting concentrations of the lysine catabolites

to effectively limit kynurenate production (462). A substrate inhibition mechanism utilized by LKR in the brain may then effectively limit lysine degradation. Thus this would limit production of both glutamate and aminoadipate to concentrations that would modulate levels of kynurenine and kynurenic acid. In this scenario, the inhibition of tryptophan hydroxylase by increased levels of tryptophan and inhibition of LKR by increased levels of lysine provides two ways of modulating excitatory amino acid responses in the brain. The inhibition of LKR by kynurenine (Table 4.10) supports these ideas. Whether this scenario is limited to the central nervous system is unclear. However, the enzymes for kynurenine transformation to kynurenate have been documented in various tissues including brain, liver, heart, kidney, intestine (19, 49, 96, 309, 317, 338) as have the enzymes for lysine catabolism (182, 289, 297, 345, 392, 440, 441). As discussed earlier, it has been suggested that tryptophan metabolism in the enteric nervous system may facilitate serotonin synthesis (48, 173, 307, 394, 402, 465), providing a means of modulating appetite (407). Lysine has also been shown to affect serotonin metabolism in the intestine (422) providing indirect evidence that the pathways are linked via feedback. Whether the intertwined activities of these pathways influence developmental changes remains unclear, but appears to be a strong possibility.

4.5.3 Inhibition of Hepatic LKR Activity at Different Ages

In a previous experiment (Chapter 3), several compounds were investigated for inhibitory potential on hepatic LKR activities in adult pigs (sections 3.4.3, 3.5.3). Several compounds inhibited LKR activity including the catabolites of lysine, L-saccharopine, L-

α-aminoadipate and L-α-ketoadipate. The current studies investigated the possibility that the inhibitory potential of these compounds was subject to developmental influence.

The data presented in Tables 4.4 - 4.10 show that the activity of hepatic LKR varies in susceptibility to inhibition at different stages of growth. The immediate catabolites of lysine, saccharopine and aminoadipate, appeared to be relatively ineffective at decreasing the activity of the enzyme. Inhibition by saccharopine during the first week was only observed at the highest concentrations of the catabolite, and later, saccharopine actually increased LKR activity. On day 14 the stimulation of LKR activity by saccharopine was greatest at all concentrations except 20 mM and on day 84 LKR activity was increased by similar amounts at all saccharopine concentrations. L-α-aminoadipate had no effects on LKR activity, except on day 21, when the addition of 20 mM aminoadipate increased LKR activity by 40%. These results contrast to the adult data (Figure 3.5) that showed significant inhibition of LKR activity by both these compounds, suggesting perhaps a feedback mechanism was an important regulator of LKR activity. The lack of inhibition caused by either saccharopine or aminoadipate (at physiological concentrations of 0.2 mM) on LKR activity in the current experiments indicates that a catabolite-driven feedback-mechanism does not regulate LKR activity during the first stages of life, suckling and weaning, in the growing pig. During rapid postnatal growth, catabolites of lysine may have significant metabolic functions that are not as crucial in the adult pig at maintenance. Thus lysine degradation may be necessary in some tissues to facilitate production of these catabolites at critical times during development.

During the first week of life, L-pipecolic acid was a more potent inhibitor of LKR activity than either saccharopine or aminoadipate, especially on day zero. On days three

and seven, LKR activity was decreased by pipecolic acid but not significantly. Early in life, this secondary lysine pathway in liver may play a more prominent regulatory role on LKR activity. This is especially true if specific concentrations of lysine catabolites (saccharopine, aminoadipate) are required during development; for example, catabolite concentrations exceeding an effective range could have detrimental effects as suggested by the gliotoxicity of aminoadipate (45). If a saccharopine or aminoadipate driven feedback-mechanism were absent during the early growth phase of the pig, then excesses of lysine would have to be controlled by pipecolic acid or another metabolite in that pathway. Pipecolic acid inhibition of LKR would provide a mechanism to slow production of saccharopine and aminoadipate if lysine concentrations were excessively high, thus maintaining an effective but not excessive concentration of lysine catabolites.

The inhibition of LKR by α-ketoadipate was significant only during the first three days of life (Table 4.6). Interestingly, L-kynurenine was even more potent during the first week of life. In fact, kynurenine, at supra-physiological concentrations (10 and 20 mM), completely abolished LKR activity (Table 4.9). These results are more evidence supporting of the hypothesis that lysine and tryptophan degradation are regulated in concert to maintain a balance of particular catabolites, as described above. The influence of ketoadipate appeared to become less pronounced after the first week, suggesting that the first week postpartum may represent a period in which the catabolites of lysine and tryptophan may have key developmental influence. In fact, past research in rats and non-human primates has shown that just prior to birth, tryptophan, kynurenine and kynurenic acid concentrations in whole brain tissue were increased (26); these concentrations decreased to adult levels by one day after birth. In developing human fetuses, the

concentration of tryptophan and metabolites were significantly higher in the umbilical vein compared to maternal circulation; tryptophan concentration in neonatal plasma decreased significantly after birth whereas the concentration of kynurenine was not changed (137). Beal and colleagues suggested that high levels of kynurenic acid prior to birth inhibit neurite branching and development of excitatory synapses, which then develop rapidly in parallel with the decrease in kynurenic acid levels (26). These data further suggest that the production of lysine and tryptophan catabolites by peripheral tissues such as liver and muscle would have to be regulated, especially early in development.

The possibility that increasing tryptophan levels in the diet would increase lysine utilization has been attempted in pigs with little success. Experiments under the guidance of David Baker (84, 132, 133, 136) have shown that when tryptophan was supplied in excess, weight gain, feed intake and gain:feed ratio all decreased significantly. Thus, although L-tryptophan does not appear to be unsafe for pigs (84), decreased performance of animals suggests that more is definitely not better after early development is complete.

Spermine, a highly positively charged polyamine, increased the activity of LKR in adult animals, and was chosen to see if these same effects were maintained in a developmental time course. In contrast to the earlier data from adult animals (Figure 3.7), spermine (20 mM) actually inhibited LKR activity on day seven. Additionally, on day 84, spermine lowered the activity of LKR, but not significantly. However, on days 14 and 21 spermine increased the activity of LKR quite significantly suggesting that spermine may exert inhibitory effects but only at very specific periods while at other times being a stimulatory compound. The influence of spermine on LKR activity in tissues that have

rapid turnover, like the IEC, would be of considerable interest. Our results suggest that addition of spermine, or other polyamines (Chapter 3) to pig diets to stimulate gut growth would increase the degradation of lysine at weaning. This could potentially exacerbate the level of stress already present in the pig at this period of growth.

Homocitrulline, chosen because of its close structural relationship to lysine, and reported similarity of metabolism (141, 212, 239, 240, 373), did not elicit any significant inhibitory effects on LKR activity.

The patterns in LKR activity with age and concentration of inhibitors clearly indicate that regulation of LKR activity at birth and the early post-natal period is significantly different than other ages. The consistency of higher stimulation of LKR activity at day 14 implies a shift in LKR regulation compared to the earlier post-natal period. In addition the consistent lack of significant changes in LKR activity on days 28 and 84, regardless of inhibitor concentration suggests a third pattern of LKR regulation develops post weaning and is maintained thereafter.

In total, the inhibition data suggest that LKR activity was more susceptible to inhibition during the first week of life, and again later (day 84). The first week may, as described above, represent a period where the regulated concentrations of particular metabolites of lysine degradation are critical to the normal neurological development of the animal.

4.5.4 Production of $^{14}CO_2$ by Different Pig Tissues

The activity of LKR represents the initial step in lysine degradation, however the production of $^{14}CO_2$ from 1-^{14}C-lysine represents release of the α-carboxyl moiety from

α-ketoadipate in the synthesis of glutaryl-CoA and thus the flux of lysine through the entire degradative pathway. The rate of oxidation of lysine by liver mitochondria was similar on days 0, 3 and 7; similar results were seen with isolated intestinal epithelial cells (figure 4.9). This lack of change during the first week is in agreement with the enzymatic data for liver and IEC (Table 4.1, 4.2). Although more variable than the rate observed in liver mitochondria, lysine oxidation by muscle mitochondria peaked on day 3 and then decreased by day 7. Furthermore, the oxidation rate in muscle was significantly greater (3 –10 times) than either liver or IEC on days 3 and 7. The high rate of lysine oxidation in muscle may, as discussed earlier, be a result of muscle turnover supplementing energy demands of the body for either physical activity and/or thermogenesis.

Considered together, these results suggest that lysine oxidation during the first week of life appears to be regulated at the tissue level, perhaps reflective of the specific demands of each tissue.

4.5.5 Detection of LKR/SDH Protein

The detection of LKR/SDH protein in tissues of pig provides additional evidence in support of the idea that the enzymic machinery required for lysine degradation, specifically LKR/SDH enzyme protein, is present in extra-hepatic tissues (Figure 8.13). Demonstrated presence of enzymic protein, in conjunction with measured activity, in different tissues further illustrates that tissue specific roles of lysine catabolism may be acting in concert to regulate whole body utilization of this essential amino acid. In particular, the detection of LKR/SDH protein in the IEC supports data by others that the

gut is utilizing a significant proportion of dietary lysine for something more than just protein synthesis.

4.6 Implications

These studies have several important implications. First, LKR activity in some extra-hepatic tissues appeared to be controlled, in part, by developmental cues. Early in life, LKR activity in muscle and kidney, is high relative to liver, and is differentially regulated; this may be to provide an alternate energy source, or some other function in the young growing pig. When one considers the protein / amino acid nutrition of the neonate, either pig or human, the essential amino acids like leucine, tryptophan, and now potentially lysine, must be considered to have roles other than as simple substrates for protein accretion.

These data show that intestinal cells and muscle tissue have substantial capacity to degrade lysine. Because these two tissues constitute the two largest metabolic masses in the body, even a low rate of lysine degradation will account for significant lysine utilization and hence may explain the apparently high basal rate of lysine catabolism (313). Thus, when considering lysine nutrition, both of these tissues need to be included as major components of lysine catabolism, as well as utilization for protein synthesis.

The activity of LKR appears to be prone to substrate inhibition. This kinetic mechanism may function to regulate the production of common metabolites of the lysine and tryptophan catabolic pathways. This balance may further modulate the balance of compounds known to influence signaling pathways in both the central and peripheral

nervous systems. This modulation could serve a developmental role in addition to a common homeostatic role.

The activity of LKR was subject to inhibition by different compounds through the developmental period and the level of sensitivity appeared to change with age. Thus lysine degradation may be controlled by several different feedback mechanisms, and the relative influence of each may change with age. This control may also be tissue coordinated. The differences among tissues and ages imply that there may be several isozymes of LKR, which are expressed in different tissues at different ages.

5 Effect of Dietary Lysine Intake on Lysine α-Ketoglutarate Reductase in Broiler Chickens

5.1 Abstract

Lysine α-ketoglutarate reductase (LKR) is the rate-limiting enzyme in the catabolism of lysine, an indispensable and often limiting amino acid in the diets of broilers. The response of broiler poults to changes in dietary lysine intake has been documented in the past, but the effects of dietary lysine intake on LKR activity in present strains of broilers have not been documented. Mitochondrial LKR activity was measured in various tissues of broiler poults fed 0.8% – 1.6% dietary lysine for 10 days. Birds fed 0.8% lysine weighed significantly less and had a significantly lower average daily gain of lysine than birds fed 1.4% lysine. Plasma lysine and lysine catabolites from birds fed the 0.8% diet were higher on days 3 and 6 compared to controls (1.2% diet), but similar or lower than birds fed excess (1.4% and 1.6%) lysine. Mitochondrial LKR activity in the liver followed Michaelis –Menten kinetics, but kidney and intestinal epithelium (IEC) LKR activity was substrate inhibited by lysine concentrations above 25 mM. There was no significant effect of dietary lysine intake on LKR activity in any tissue examined. However, susceptibility of LKR activity to inhibition by aminoadipate and ketoadipate was more pronounced at the dietary extremes of deficiency and excess. Thus LKR may be regulated by different mechanisms when lysine is deficient, adequate or in excess of requirements. The results suggest that LKR activity, hence the capacity for lysine degradation is not necessarily proportional to dietary intake and may therefore be a necessary and indispensable process. The complex level of regulation for degrading

lysine indicated that a more diverse role for either lysine or one of its catabolites in metabolism.

5.2 Introduction

Amino acid nutrition is a central research focus of many animal production systems; lysine is of particular importance because dietary grains, the primary ingredients in both swine and poultry feeds, tend to be limiting in lysine (330). Consequently, synthetic lysine is normally supplemented to fulfill the requirements for optimal growth. Much of the lysine research in swine and poultry has focused on balancing dietary lysine with the other essential amino acids to obtain a dietary amino acid profile optimal for protein synthesis (9, 17, 18, 127, 206, 519). As a result, the effects of a dietary lysine intake on the growth and performance in swine and poultry have been thoroughly investigated. However, the cellular regulation of the enzyme pathways controlling lysine utilization and degradation are not well documented (105, 275, 454, 455), despite the potential for significant impact on efficiency of lysine utilization.

Lysine α-ketoglutarate reductase (LKR; EC 1.5.1.8) and saccharopine dehydrogenase (SDH; EC 1.5.1.9) catalyze the initial reactions in the primary metabolic route of lysine degradation in mammals and birds (Figure 1) (108). However, relatively little is known about the activity, tissue distribution and regulation of these enzymes in growing meat animals and birds. The bulk of the research completed on the LKR to date has focused on rats, which due to their omnivorous lifestyle rarely encounter lysine as a limiting amino acid in their diet (160, 321, 337, 484). Chapter 4 is the first comprehensive report on the activity of the enzyme in swine.

LKR activity in poultry has been examined but mainly concentrated on determining enzyme activity in relation to an induced antagonism between lysine and arginine (484, 485). More recently, it has been demonstrated that LKR in poultry is distributed across a wide range of extra hepatic tissues and can account for significant catabolism of lysine (289). These results clearly indicate that the previous level of knowledge regarding lysine degradation in poultry was incomplete – the capacity for tissues like the muscle and intestine to significantly catabolize lysine was not appreciated and thus was ignored from a nutritional perspective.

Previous research on dietary interactions of amino acids have illustrated that the composition of the diet can dramatically affect the activity of amino acid-degrading enzymes (67, 112, 231, 484, 485) and thus the growth of the bird. For example, in poultry high concentrations of dietary lysine antagonize arginine transport and also induce kidney arginase, resulting in a negative impact on the growth of the bird (14, 230, 231, 234, 343). Effects of different levels of dietary lysine have been reported many times, but to the best of the author's knowledge none have focused on the activity of the lysine-degrading enzymes. Therefore, the objective of the current studies was to investigate the relationship between levels of dietary lysine (from limiting to excess) on the activity of the rate-limiting enzyme for lysine degradation, lysine α-ketoglutarate reductase, in selected tissues of the growing broiler chick.

5.3 Materials and Methods

5.3.1 Birds

Fifty male Ross 308 broiler chicks (Aviagen Inc., Huntsville, Ala;1 day post hatch) were collected from a commercial broiler breeder farm (Lilydale Hatchery,

Edmonton, Alberta, Canada). Birds were raised at the Alberta Poultry Research Centre, University of Alberta under conditions similar to commercial broiler production practices. Specht metabolic cages (285) were used to house birds in groups of ten, to monitor individual growth. Feed and water was provided *ad libitum* and an 8h:16h lighting schedule was used. A commercial mash diet, formulated to meet NRC recommendations for broiler starters (330), was fed until 10 days post-hatch to allow for normal development of the chicks. At 10 days post-hatch the birds were matched for weight and randomly assigned to one of five test diets (n = 10 per diet). All protocols were approved by the departmental Animal Care committee, and were in accordance with procedures outlined by the Canadian Council on Animal Care.

5.3.2 Test Diets

Diets contained 0.8%, 1.0%, 1.2%, 1.4% or 1.6% (w/w) dietary lysine and were formulated to be isocaloric and isonitrogenous by substitution with glutamate and cornstarch. Diet lysine concentrations were chosen to provide a range above and below the industry standard of 1.2% dietary lysine. Diets 1.0% and 0.8%, were considered to be moderately low and low in dietary lysine respectively; diets 1.4% and 1.6%, were considered to be moderately high and high in dietary lysine respectively. Table 5.1 contains a description of diet composition, the analyzed diets are presented in Table 8.2. Test diets were fed for 10 days to allow time for changes in enzyme protein concentration to occur.

Table 5.1 Dietary Formulations for Poultry Dietary Lysine Experiments

Lysine Diet Formulations	0.8%	1.0%	1.2%	1.4%	1.6%
Ingredient (% (w/w))					
Wheat	14.4	14.4	14.4	14.4	14.4
Barley	5	5	5	5	5
Wheat Shorts	10	10	10	10	10
Corn Gluten Meal	12	12	12	12	12
Canola Meal	5	5	5	5	5
Soybean Meal	5	5	5	5	5
Feather Meal	3	3	3	3	3
Corn Distillers Grain	5	5	5	5	5
Corn Oil	10	10	10	10	10
Vitamin/Mineral Premix[1]	1	1	1	1	1
Limestone	2.1	2.1	2.1	2.1	2.1
Dical Phos	0.6	0.6	0.6	0.6	0.6
Salt	0.5	0.5	0.5	0.5	0.5
Amino Acid Mixture[2]	2	2	2	2	2
Corn Starch	21.14	21.17	21.17	21.17	21.17
Phenylalanine	0.16	0.16	0.16	0.16	0.16
Glutamate	2.57	2.16	1.76	1.36	0.96
Lysine Hcl	0.31	0.56	0.81	1.06	1.31
Calculated Analysis:					
ME (kcal/kg)	3408	3408	3408	3407	3407
CP %	24.7	24.7	24.7	24.7	24.7
Ca %	1.01	1.01	1.01	1.01	1.01
Available P %	0.46	0.46	0.46	0.46	0.46
Lysine (calculated) %	0.8	1	1.2	1.4	1.6
Lysine (analyzed)	0.82±0.15	1.07±0.08	1.24±0.07	1.35±0.10	1.53±0.03

[1]The vitamin mineral premix provided the following (per kg of feed): vitamin A, 10,000 IU; vitamin D_3, 2500 IU; vitamin K, 2.0 mg; pantothenic acid, 14 mg; riboflavin, 5.0 mg; folacin, 0.80 mg; biotin, 1.8 mg; niacin, 65 mg; thiamine, 2.0 mg; pyridoxine, 4.0 mg; vitamin B_{12}, 0.015 mg; vitamin E, 35 IU; Mn, 70 mg; Cu, 8.5 mg; Zn, 80 mg; Se, 0.10 mg; Fe, 100 mg; choline, 1000 mg.

[2]The amino acid mixture provided the following (per kg of feed): methionine, 0.26; histidine, 0.15; threonine, 0.375; arginine, 0.375; tyrosine, 0.225; isoleucine, 0.225; valine, 0.15.

5.3.3 Blood Sample Collection

During the experimental period, 1 ml blood samples were collected every three days from the brachial vein into heparinized vacuum tubes. Blood samples were centrifuged at 1500 g for 10 min at 4°C to isolate plasma. Plasma was removed and stored at -20°C until analyzed. Plasma amino acids were separated using a pre-column σ--phthaldialdehyde derivatization method (406) and the concentration of amino acids and the lysine catabolites saccharopine and α-aminoadipate were quantified. Ethanolamine and β-amino-n-butyric acid were used as internal standards. L-saccharopine and α-aminoadipate were added to the amino acid standard profile (Sigma #AAS18) for direct comparison.

Individual bird weights were recorded every three days. At the end of the trial period (day 20 post-hatch) birds were killed by cervical dislocation. Liver and kidney tissues were extracted and placed in ice-cold 0.3M mannitol/1mm EDTA buffer; intestinal sections were stored in cold PBS containing 5mM dithiothreitol (DTT), pH 7.4.

5.3.4 Isolation of intestinal mucosal epithelial cells

Intestinal epithelial cells were prepared using modifications of previously described methods (195, 293, 486). A section of small intestine was obtained and transported to the laboratory in ice cold phosphate buffered saline with protease inhibitor cocktail added (PBS: 137mM NaCl / 2.7mM KCl / 4.3 mM Na_2HPO_4 / 1.4 mM KH_2PO_4 / 5 mM dithiothreitol (DTT). The section was rinsed (1X) with fresh cold PBS to remove any remnants of excreta and then rinsed (3X) with fresh oxygenated (19:1 O_2:CO_2; Carbogen) Krebs-Henseleit Ca^{2+}-Free buffer (121mM NaCl, 4.7mM KCl, 1.2mM

$MgSO_4$, 1.2mM KH_2PO_4, 25.2mM $NaHCO_3$) containing 20 mM Hepes (pH 7.4), 5 mM EDTA, 20 mM DL-glucose, 2.1 mM lactate and 0.3 mM pyruvate (KHB buffer) / 5 mM DTT. The intestinal section was then filled with pre-warmed (37°C) oxygenated KHB (Ca^{2+}-free)/DTT, sealed with clamps and immersed in a container filled with the same buffer. This system, with continuous Carbogen gassing, was then shaken for 45 minutes at 37°C with gentle massaging of the sealed intestinal sections at regular intervals. The resulting cell suspensions were filtered through 2 layers of cheesecloth and centrifuged for 3 minutes at 400x *g*, 4°C. Cells were washed 3 times in fresh oxygenated KHB buffer and finally resuspended in 250mM sucrose/5mM HEPES/5mM DTT (pH 7.4).

5.3.5 Isolation of mitochondria

The re-suspended mucosal epithelial cell pellet [in 250mM sucrose/5mM HEPES/5mM DTT (pH 7.4)] was then transferred to a cold Dounce homogenizer (Wheaton Science Products, Millville, NJ) and the cell suspension disrupted by 8 passes of pestle A followed by 8 passes of pestle B. Mitochondria from the cells were then obtained by differential centrifugation according to previously published methods (293). The final mitochondrial pellet was re-suspended in 0.3M mannitol/2mM DTT. Mitochondrial preparations from IEC were determined to be free of microbial contamination in previous experiments (Chapter 3).

Liver and kidney samples were weighed and then homogenized (VirTis tissue homogenizer, VirTis, Gardiner, NY) (1.0 g tissue / 5 mL of buffer). Mitochondria were then isolated by differential centrifugation (221, 323, 403). Mitochondrial pellets were re-suspended in mannitol (0.3M). See appendix 8.4.2 for additional details.

5.3.6 Lysine Ketoglutarate Reductase Assay

The initial step in lysine degradation was measured according to published methods (403) as modified to a 96 well microplate format (Spectramax 190, Molecular Devices Corp., Sunnyvale, CA) by Pink et al (365) (see Chapter 3). Oxidation of NADPH to $NADP^+$ was measured spectrophotometrically at 30°C using an extinction coefficient of 6.22 $M^{-1}cm^{-1}$. The reaction mixture contained 0.125 mM NADPH, 10 mM L-lysine, 7.5 mM α-ketoglutarate, 100 mM HEPES, pH 7.8 and 300 μg of solubilized mitochondrial protein (0.2% (v/v) Non-Idet P-40). The reaction commenced with the addition of NADPH. The assay was linear with time and protein and optimized for substrate concentrations of lysine and α-ketoglutarate. See also appendix 8.4.3 for additional details.

5.3.7 Inhibition Assays

For inhibition studies, potential inhibitors were added prior to the addition of protein. All inhibitors were added to a final concentration of 5 mM. Controls were treated in exactly the same manner with the exception of substituting an equal volume of water for inhibitor.

5.3.8 Protein Determination

Protein concentrations of mitochondria and intact mucosal epithelial cells were determined by the Bicinchoninic acid (BCA) technique (Sigma-Aldrich, BCA1).

5.3.9 Chemicals

All laboratory chemicals were purchased from Sigma unless specified otherwise.

5.3.10 Statistical Analyses

Data are presented as Mean±SEM. One-way ANOVA (SAS/STAT version 8.01, SAS institute, Cary, NC) was performed employing PROC GLM procedure with the specific activity of enzyme rates assessed by least square means procedure. Specific analyses are mentioned in legends. Data was considered significant at $P < 0.05$.

5.4 Results

5.4.1 Performance

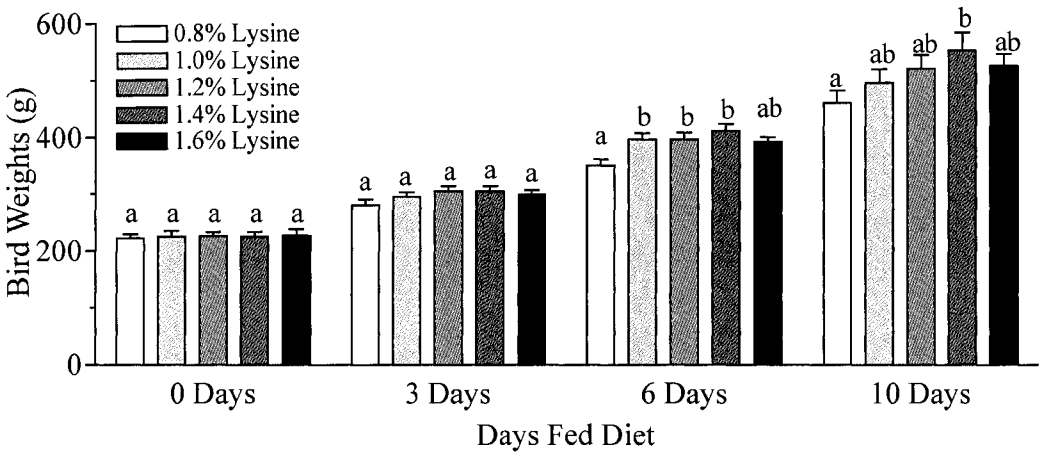
Broiler chicks were matched for weight at the beginning of the experiment and fed one of five lysine diets. All birds had positive weight gain during the experimental period. After 6 days of receiving different lysine diets, birds fed the limiting (0.8%) lysine diet weighed significantly less than birds fed the moderately low (1.0%), adequate (1.2%) and moderately high (1.4%) lysine diets (Figure 5.1). As lysine content in the diets increased, average daily gain increased and plateaued at 1.4% lysine. Birds fed the 1.4% lysine diet had a significantly greater total weight gain and ADG than birds fed the low (08%) lysine diet (Figure 5.2). No other performance differences were noted.

5.4.2 Plasma Amino Acids

During the course of the experiment, plasma amino acids were quantified by HPLC to detect changes due to the effects of the different lysine contents of the diets, particularly for saccharopine and aminoadipate (Complete data shown in Appendix 8.5.2, Tables 8.3

– 8.7). Table 5.2 and Figure 5.3 show the response of selected amino acids to varied dietary lysine. Compared with day 0, plasma threonine concentrations were significantly increased on day 3 in all diet treatments and remained elevated for the

Figure 5.1 Growth of Broilers Fed Different Levels of Dietary Lysine



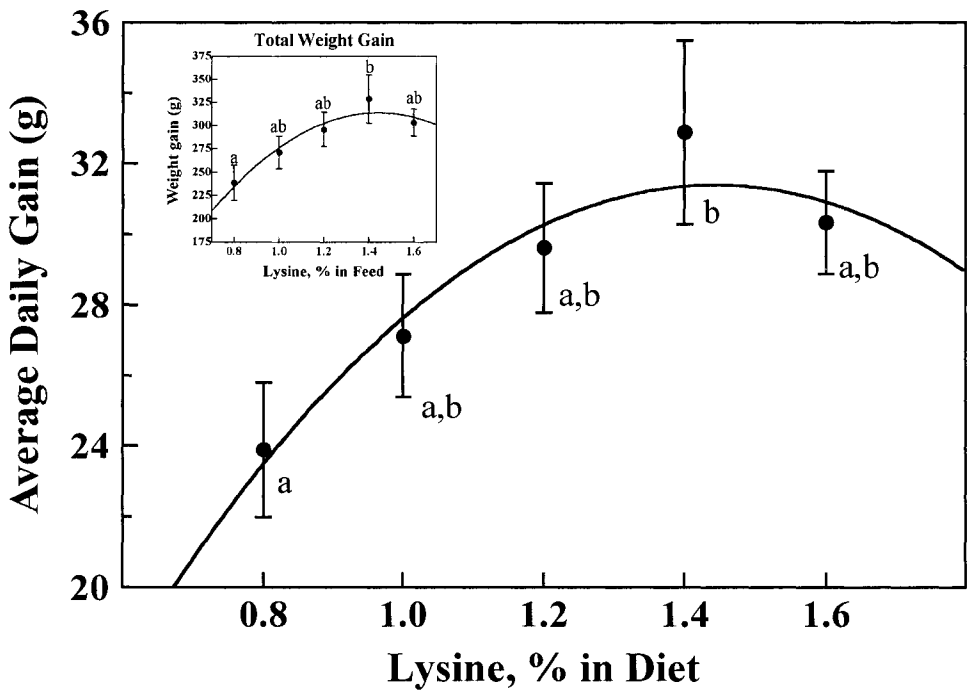
Growth data for broilers allowed ad libitum access to one of 5 diets with different levels of dietary lysine 0.8% - 1.6% for ten days. Birds (n= 10/diet treatment) were 10 days post hatch at start of feeding and were matched for weight at the beginning on day 0. Data are presented as Mean ± SEM with increasing dietary lysine represented as increasing depth of shading. All groups of birds showed positive linear growth (increased weight gain) from day 0 onwards (linear regression, $p<0.001$ for each group of birds); bars with different letters indicate significant differences (repeated measures ANOVA, $P<0.05$) among diets.

duration of the experiment. With the exception of birds fed the 1.2% lysine diet, plasma lysine was significantly higher for day three compared to day 0 concentrations. Lysine concentrations in plasma decreased by day 10 in birds fed 0.8%, 1.4% and 1.6% lysine. Birds fed the 1.2% lysine diet had no changes in plasma lysine concentration over time.

By day 10, the plasma lysine concentrations of birds fed 0.8% and 1.2% lysine were similar to day 0 values, in contrast to birds fed the 1.0%, 1.4% or 1.6% lysine diets that had higher levels of lysine.

Arginine concentrations were not different among days within each dietary lysine level. However, birds fed the 0.8% lysine diet had significantly higher arginine concentrations, compared to all other diets on each day (Table 5.2). Birds fed 1.0% and

Figure 5.2 Average Daily Gain of Broilers Fed Different Levels of Dietary Lysine



Average daily gain data for 10d old broilers allowed ad libitum access to one of 5 diets with different levels of dietary lysine 0.8% - 1.6% for ten days. Birds (n= 10/diet treatment) were 10 days post hatch at start of feeding and were matched for weight at the beginning on day 0. Data are presented as Mean ± SEM and fitted to a second order polynomial equation. Insert graph shows the weight gain of birds fed increasing levels of lysine. Data with different letters are significantly different, $p<0.05$.

1.2% lysine showed no changes in plasma arginine on days 0 or 3. However by day 6, birds fed 1.2%-1.6% lysine had significantly lower plasma arginine concentrations. On day 10, birds fed the adequate (1.2%) lysine diet showed higher concentrations of arginine compared to the 1.0%, 1.4% and 1.6% lysine diets.

The plasma concentrations of saccharopine and aminoadipate, both catabolites of lysine, were measured as an indicator of *in vivo* activity of the lysine pathway (Table 5.2). Plasma saccharopine in the birds fed the 0.8% and 1.0% diets increased significantly until day 6, after which the concentrations dropped. There was no change over time in the birds fed the 1.2% lysine diet. Birds fed moderately high lysine (1.4%) had a more gradual rise in plasma saccharopine, but again the concentrations decreased after day 6. In contrast, plasma saccharopine did not increase in birds fed the highest concentration of lysine (1.6%), rather the levels were maintained until day 6, after which time the concentration of saccharopine decreased.

Plasma concentrations of aminoadipate were significantly increased by day 3 in all groups, except the 1.6% lysine group. Birds fed the 1.6% lysine showed a decline in plasma aminoadipate from day 0 onwards, similar to the plasma saccharopine concentrations for this group. In contrast, aminoadipate concentrations in all other groups generally increased with time and then began to drop. Birds fed 1.2% lysine were the exception; aminoadipate concentration was maintained after the initial rise observed on day 3.

The initial concentrations of both plasma saccharopine and aminoadipate were similar across diet treatments. However by day 3, the birds consuming 0.8% lysine had the highest saccharopine concentrations when compared to all other groups. The same

effect was seen on day 6 for plasma aminoadipate. After feeding the diets for 10 days, the plasma concentrations for both saccharopine and aminoadipate were similar across dietary treatments.

Plasma concentrations of saccharopine and aminoadipate were correlated with the plasma lysine concentrations. No relationship between plasma lysine and saccharopine was defined ($p>0.05$). However, plasma aminoadipate was positively correlated with the plasma lysine concentrations (Figure 5.4A). When plasma concentrations of saccharopine were compared to plasma aminoadipate concentrations, a positive correlation was found. Figure 5.4B).

5.4.3 Tissue Weights

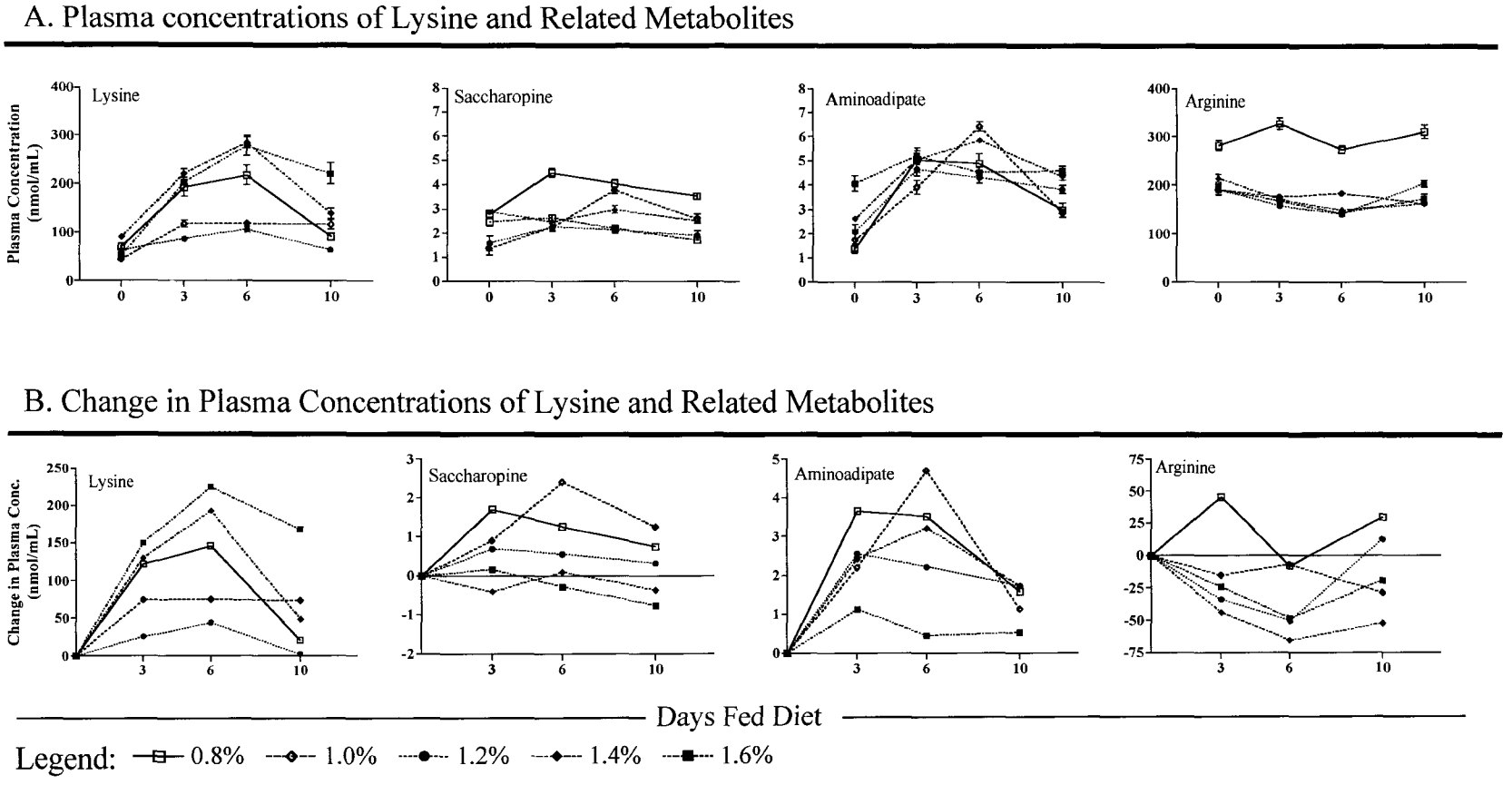
The liver weights of birds fed deficient lysine diets, 0.8% and 1.0%, were significantly less than for other groups. However this effect was not significant when liver weights were expressed as a percent of body weight. The level of lysine in the diet did not affect any other tissue, either by weight or % BW (Table 5.3).

Table 5.2 Effect of Days Fed Varying Dietary Lysine on Concentration of Plasma Lysine and Related Metabolites

Day	0.8%	1.0%	1.2%	1.4%	1.6%
		Selected Plasma Amino Acids (nmol/mL)			
		Lysine			
0	71 ± 14^{Aa}	44 ± 5^{Aa}	63 ± 12^{a}	91 ± 14^{Aa}	53 ± 8^{Aa}
3	193 ± 64^{Ba}	119 ± 24^{Bb}	88 ± 15^{b}	222 ± 28^{Ba}	204 ± 60^{Ba}
6	218 ± 57^{Ba}	119 ± 16^{Bb}	107 ± 20^{b}	285 ± 35^{Cc}	278 ± 69^{Cc}
10	92 ± 23^{Aab}	117 ± 30^{Bac}	65 ± 10^{b}	141 ± 33^{Ac}	222 ± 73^{Bd}
		Saccharopine			
	0.8%	**1.0%**	**1.2%**	**1.4%**	**1.6%**
0	3.7 ± 0.9^{Aa}	2.4 ± 0.8^{Aa}	3.4 ± 0.3^{Aa}	3.0 ± 0.1^{Aa}	3.8 ± 0.7^{Aa}
3	5.4 ± 0.7^{Ba}	3.5 ± 0.3^{Bb}	3.6 ± 0.1^{Ab}	3.4 ± 0.2^{Ab}	3.5 ± 0.1^{Ab}
6	4.8 ± 0.3^{Ca}	4.1 ± 0.3^{Cab}	3.5 ± 0.2^{Ab}	3.9 ± 0.2^{Bab}	3.4 ± 0.1^{Ab}
10	3.9 ± 0.6^{Aa}	2.9 ± 0.2^{Aa}	3.5 ± 0.4^{Aa}	3.2 ± 0.3^{Aa}	2.7 ± 0.3^{Ba}
		Aminoadipic Acid			
	0.8%	**1.0%**	**1.2%**	**1.4%**	**1.6%**
0	2.2 ± 1.0^{Aa}	3.8 ± 0.3^{Aa}	2.5 ± 0.5^{Aa}	3.2 ± 0.1^{Aa}	6.5 ± 1.4^{Aa}
3	8.5 ± 1.8^{Ba}	5.5 ± 0.5^{ABab}	5.6 ± 1.0^{Bab}	6.6 ± 0.6^{Bab}	4.7 ± 1.0^{Bb}
6	10.0 ± 2.1^{Ca}	6.3 ± 0.4^{Bb}	4.7 ± 1.0^{Bb}	6.5 ± 0.2^{Bb}	3.9 ± 0.9^{Bb}
10	5.5 ± 0.6^{Da}	3.7 ± 0.5^{Ca}	4.7 ± 0.6^{Ba}	4.6 ± 0.5^{Ca}	3.6 ± 0.5^{Ca}
		Arginine			
	0.8%	**1.0%**	**1.2%**	**1.4%**	**1.6%**
0	281 ± 29^{a}	191 ± 24^{b}	192 ± 25^{b}	215 ± 22^{b}	192 ± 11^{b}
3	327 ± 40^{a}	176 ± 13^{b}	158 ± 18^{b}	171 ± 10^{b}	168 ± 22^{b}
6	273 ± 22^{a}	184 ± 13^{b}	141 ± 18^{c}	149 ± 8^{c}	143 ± 16^{c}
10	311 ± 44^{a}	162 ± 11^{b}	204 ± 24^{c}	162 ± 11^{b}	173 ± 33^{b}

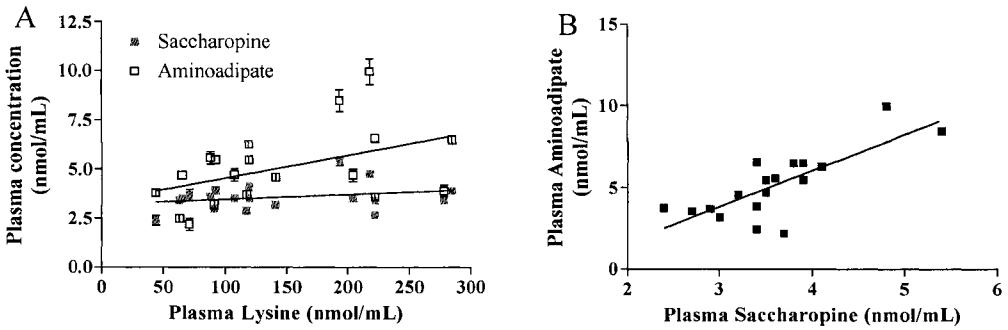
[1]Data are presented as Mean±SEM for n = 5 –10 birds. Samples were analyzed in duplicate and significant differences among ($p<0.05$) are noted with different letters; uppercase letters represent differences within diet, among days; lowercase letters represent differences among diets on same day. Birds received a commercial broiler starter diet for 10d before the experiment.

Figure 5.3 Effect of Days Fed Varying Dietary Lysine on Concentrations of Plasma Lysine and Related Metabolites



Data presented as Mean±SEM for n=5-10 birds per diet. Data are taken directly from Table 5.X and same statistical results apply
Panel A: Absolute plasma concentrations of lysine and related metabolites.
Panel B: Change in plasma concentrations from Day 0 values.

Figure 5.4 Relationship between Plasma Lysine, Saccharopine and Aminoadipate



Pearson correlation was used to examine the relationship between plasma lysine, saccharopine and aminoadipate. Data are presented as Mean±SEM for replicate samples. Panel A: Significant Pearson correlation (0.4514; y = 0.01173x +3.367) was determined for plasma aminoadipate ($p<0.05$); Panel B: Significant Pearson correlation (0.7689; y = 2.206x – 2.768) was noted, $p<0.05$.

Table 5.3 Effect of Dietary Lysine Level on (Wet) Tissue Weights (g)

		Percent Lysine in Diet				
		0.8%	1.0%	1.2%	1.4%	1.6%
Tissue[1]						
Liver	Weight	12.9±0.7[a]	13.1±0.5[a]	15.4±0.8[ab]	15.4±0.8[ab]	15.9±0.6[b]
	% BW[2]	2.7±0.1	2.8±0.2	2.9±0.1	2.9±0.1	3.0±0.1
Kidney	Weight	4.0±0.3	4.4±0.3	4.8±0.4	5.0±0.4	4.9±0.3
	% BW	0.82±0.03	0.90±0.05	0.88±0.05	0.92±0.05	0.93±0.05
Intestine	Weight	17.3±0.9	18.2±1.3	16.5±1.4	18.1±1.0	17.6±0.9
	% BW	3.7±0.3	3.0±0.6	2.9±0.5	3.4±0.2	3.0±0.4

[1]Data are presented as Mean±SEM for 10 birds. Values with different letter superscripts are significantly different ($P<0.05$), ANOVA x Tukeys.
[2]BW: bodyweight.

5.4.4 Lysine α-Ketoglutarate Reductase Activity

Enzyme kinetics were measured using lysine concentrations ranging from 0-100 mM and fitted to the Michaelis-Menten (MM) and/or substrate inhibition (SI) equations:

Michaelis-Menten: $Y=V_{Max}\cdot X/(K_M + X)$; Substrate Inhibition: $Y=V_{Max}\cdot X/(K_M+X+X^2/D)$; where V_{max} is the maximal velocity at saturation; K_m, or the Michaelis constant, is the substrate concentration required to reach half-maximal velocity ($V_{max}/2$); and D is the dissociation or inhibition constant. The graphical solutions to the kinetic analyses of LKR in different chicken tissues can be seen in Figure 5.4. The liver isoform appeared to follow Michaelis Menten kinetics and showed a Michaelis constant of between 0.62 mM to 2.85 mM. The kinetic parameters (V_{Max}, K_M) for the hepatic analyses were derived from the Lineweaver-Burke substrate curves and are presented in Table 5.6. In contrast to the liver results, kidney and IEC analyses of LKR activity showed that the enzyme exhibited substrate inhibition, particularly at lysine concentrations greater than 10-15 mM (Figure 5.3, dashed lines).

The specific activity of LKR was calculated using analyses that had either 10 mM lysine or 25 mM lysine (Figures 5.7, 5.8). The 10 mM lysine concentration was chosen because of the evident substrate inhibition seen in the kidney and IEC results. Because liver LKR activity appeared to follow Michaelis-Menten kinetics a second analysis was used for this tissue assuming 25 mM lysine was saturating. No significant differences in LKR activity were seen among any dietary levels for liver, kidney or IEC (Figure 5.7), or in the liver when using 25 mM values (Figure 5.8).

Following page:

LKR enzyme kinetics was investigated in different chicken tissues using L-lysine concentrations ranging from 0 to 100 mM. Data represent Mean ± SEM (n=10; samples were assayed in triplicate) and are fitted to Michaelis-Menten (solid lines) and substrate inhibition (dashed lines) non-linear regression equations. Liver LKR activity did not exhibit substrate inhibition; therefore no dashed lines are shown.

Figure 5.5 Kinetic Analysis of LKR Activity in Different Tissues of Broilers Fed Different Lysine Diets

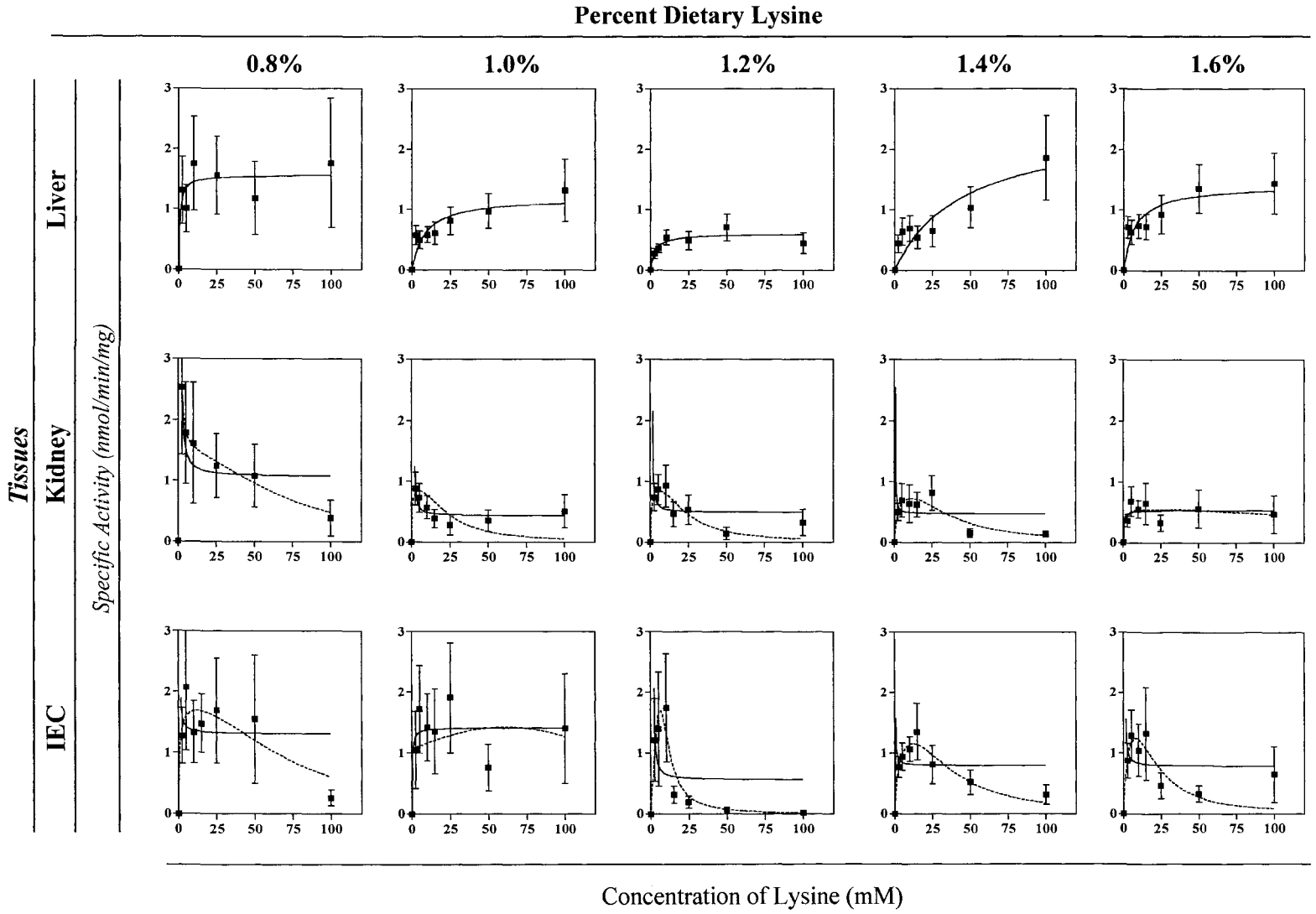
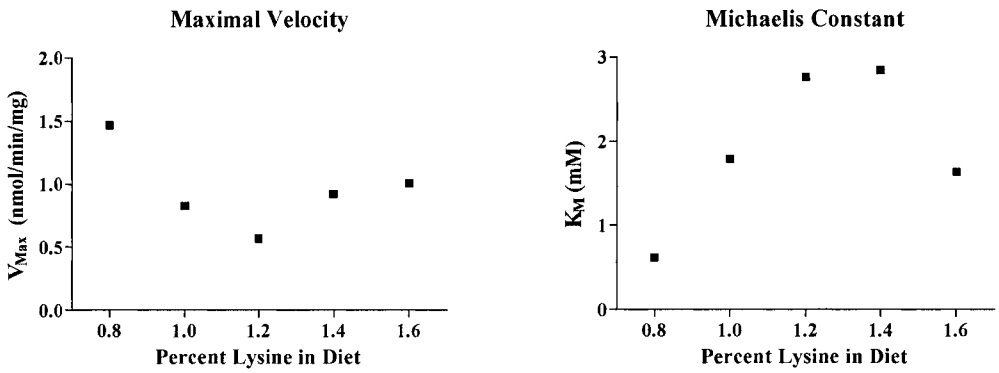


Table 5.4 Kinetic Constants for Hepatic LKR Activity in Chickens Fed Different Lysine Levels

		Percent Lysine in Diet				
		0.8%	1.0%	1.2%	1.4%	1.6%
V_{Max}[1,2]	LB[3]	1.47±10.06	0.83±5.14	0.57±4.77	0.92±4.73	1.01±6.51
	MM	1.56±0.39	1.19±0.24	0.59±0.10	2.41±0.91	1.40±0.27
K_M[4]	LB	0.62±1.53	1.80±1.11	2.77±2.03	2.85±1.97	1.64±1.55
	MM	0.79±1.84	8.50±5.60	2.70±2.14	44.2±35.0	7.15±4.88

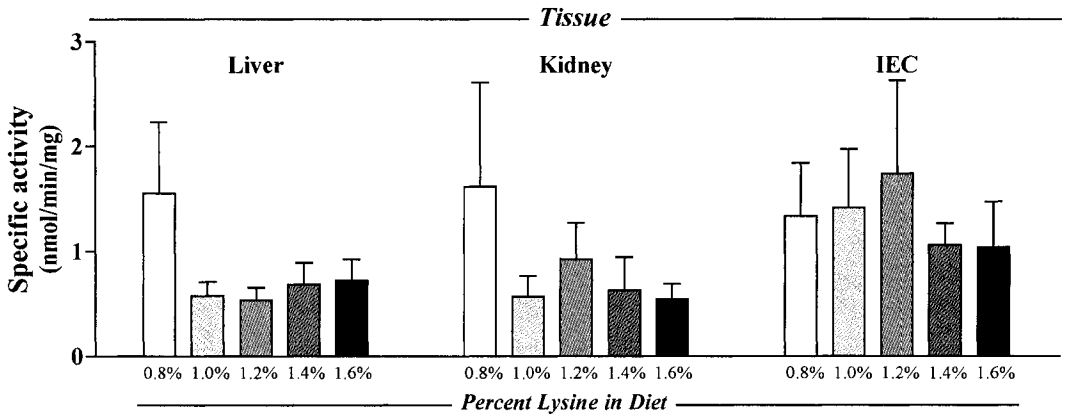
[1]Values are derived from the double-reciprocal plots (Lineweaver-Burke analyses) of data presented in Figure 5.3;
[2]V_{Max} data: nmol/min•mg mitochondrial protein;
[3]LB – Lineweaver- Burke analysis calculation, MM – Michaelis-Menten calculation;
[4]K_M data: mmol/L.

Figure 5.6 Effect of Dietary Lysine Intake on Kinetic Parameters of LKR Activity



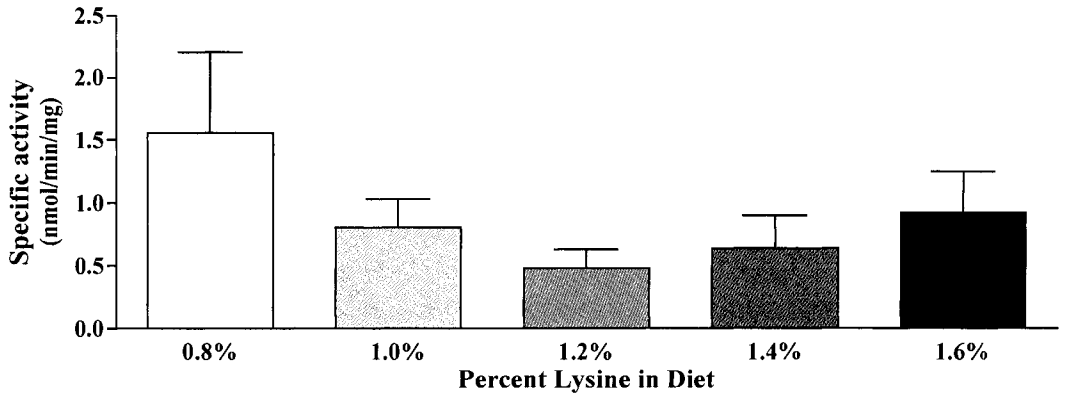
V_{Max} and K_M were calculated using the Lineweaver-Burke double reciprocal derivation. Data are plotted as Mean values from Table 5.4 and represent the activity data in Figure 5.3.

Figure 5.7 Effect of Dietary Lysine on LKR Activity in Broiler Tissues



LKR activity was measured using 10 mM L-lysine in solubilized mitochondria prepared from chicken tissues. Data are presented as Mean ± SEM (n=10; samples were analyzed in triplicate). Increasing depth of bar shading represents increasing dietary lysine content.

Figure 5.8 Effect of Dietary Lysine on Hepatic LKR Activity: Saturating Lysine (?) Conditions



LKR activity was measured using 25 mM L-lysine in solubilized mitochondria prepared from chicken liver. Data are presented as Mean ± SEM (n=10; samples were analyzed in triplicate). Increasing depth of bar shading represents increasing dietary lysine content.

5.4.5 Inhibition of Lysine α-Ketoglutarate Reductase Activity

LKR activity was measured in the presence of selected compounds to assess potential inhibition (Table 5.5). In the liver, aminoadipate significantly decreased LKR activity at all diet levels except 1.0%. In the kidney, aminoadipate addition was similarly effective, decreasing LKR activity at the 0.8%, 1.0% and 1.6% diet levels of lysine. However in the IEC mitochondria, aminoadipate was only effective at the higher diet levels of lysine (1.4% and 1.6%; Table 5.5).

Ketoadipate addition reduced LKR activity in the 0.8% and 1.4% solubilized liver mitochondria as well as 1.4% IEC mitochondria. Homocitrulline decreased liver LKR activity at the 1.4% and the 1.6% lysine levels. In the kidney and IEC, homocitrulline decreased LKR activity at the 1.6% and 1.4% lysine levels respectively. Potassium fluoride addition was effective at decreasing LKR activity only in the liver (0.8%, 1.4% and 1.6% dietary lysine).

Table 5.5 Effect of Different Compounds on LKR Activity in Chicken Tissues

	Liver				
	Percent Lysine in Diet				
Inhibitor	0.8%	1.0%	1.2%	1.4%	1.6%
Aminoadipate	3.6±4.3*	31.4±32.4	11.5±37.9*	28.9±8.9*	35.0±11.4*
Ketoadipate	55.9±23.1*	93.5±41.6	81.1±13.8	17.1±5.1*	70.3±23.7
Homocitrulline	71.7±29.2	70.7±43.1	96.8±15.9	39.4±28.8*	58.3±13.3*

	Kidney				
	Percent Lysine in Diet				
Inhibitor	0.8%	1.0%	1.2%	1.4%	1.6%
Aminoadipate	18.2±6.7*	27.1±13.8*	63.1±29.6	68.0±13.5	17.6±16.9*
Ketoadipate	59.7±16.1	67.2±30.1	105.9±31.4	70.0±18.5	39.2±19.7
Homocitrulline	44.0±12.1	89.8±30.0	100.6±36.8	62.6±10.0	29.7±22.7*

	Intestinal Epithelial cells				
	Percent Lysine in Diet				
Inhibitor	0.8%	1.0%	1.2%	1.4%	1.6%
Aminoadipate	76.9±27.0	121.3±85.9	125.4±81.6	24.3±17.9*	24.5±12.8*
Ketoadipate	79.0±37.5	128.9±48.9	67.5±28.4	20.8±65.0*	65.0±14.8
Homocitrulline	55.8±18.7	95.4±49.2	74.6±28.8	30.4±30.4*	49.6±26.7

LKR activity was determined in the presence or absence of various potential inhibitory compounds. α-Aminoadipate, α-ketoadipate or homocitrulline were added (to a final concentration of 5 mM) prior to addition of protein. Control tubes had water substituted for inhibitor and were run each day for each bird; data are presented as Percent of Control Activity: Mean ± SEM for n = 5 separate experiments (samples were measured in triplicate). Control activities (nmol/min/mg) are illustrated in Figure 5.7. Asterisks (*) indicate values are significantly different from control ($p<0.05$), as assessed by ANOVA and Dunnett's Multiple Comparison Test.

5.5 Discussion

The degradation of lysine has a major impact on the proportion of dietary lysine that is available for protein synthesis by growing chickens and swine. Until recently, lysine catabolism in the chicken was thought to occur almost entirely in the liver and kidney. Experiments by Manangi and colleagues (289) have shown that several extra-

hepatic tissues can account for significant proportions of lysine catabolism. The experiments conducted by these authors suggests that the regulation of lysine degradation, and hence utilization, is more complex than previously thought. This is most clearly shown by the demonstration of lysine catabolism in the intestine and breast muscle tissues – previously considered to not have the enzyme pathways required for lysine degradation.

5.5.1 Performance parameters

Birds fed the diets limiting in lysine would not be expected to perform as well as birds fed diets with higher concentrations of dietary lysine. Birds consuming the 0.8% lysine diets were significantly smaller at six days than birds fed the 1.4% lysine diet (Figure 5.1) and had a significantly lower average daily gain (Figure 5.2). The 0.8% lysine diet is considered limiting for broiler poults at this age according to NRC requirements (330).

5.5.2 Plasma amino acids

As expected, plasma lysine concentrations were greater in birds fed the excess lysine diets, 1.4% and 1.6% than the other diets. However, in the birds fed 0.8% lysine, plasma lysine levels were significantly greater than birds fed the 1.0% or 1.2% lysine levels. There are two possible explanations for this difference: either birds receiving 0.8% lysine reduced lysine degradation or more lysine was entering the plasma pool. Our data indicate that birds were initially mobilizing lysine from endogenous protein sources (i.e. muscle) to adapt to the limiting dietary levels of the amino acid. This suggestion is

supported by the fact that by day 10, the plasma levels of lysine in these birds was similar to birds fed the 1.0% and 1.2% levels of lysine and that LKR activity was not lower, in fact it was (numerically) higher. The increase in plasma lysine concentration may be the reason for the apparent increase in LKR activity, however plasma lysine was not correlated with LKR activity. It should be further noted that plasma lysine concentrations on day 10 were significantly reduced compared to day 6 concentrations among diets (except 1.0% and 1.2%).

The plasma lysine levels on day 10 were lower than those on day 6 in birds fed the 0.8%, 1.4%, and 1.6% lysine diets. These decreases were most likely in response to different factors: metabolic adaptation to the low dietary lysine in the 0.8% group as increased mobilization of lysine and other amino acids from protein and in the higher dietary levels presumably increased oxidation caused the decreases. While an increase in LKR was expected, it is possible that at the higher dietary excesses, lysine flux through the pipecolic acid pathway contributed to the plasma lysine decrease. Similar decreases in plasma lysine concentrations with time were described previously by D'Mello and Lewis (105) when they fed excess (1.5%) lysine to chicks for 5 days.

Additionally, we monitored the plasma concentrations of the lysine catabolites saccharopine and aminoadipate. We hypothesized that as dietary lysine increased from limiting to adequate to excess, that lysine catabolism would initially be similar and then increase in response to the excess dietary lysine. We further hypothesized that the concentrations of saccharopine and aminoadipate would follow the same pattern as the plasma lysine concentrations. This was not the case however. Birds fed the most limiting lysine diet (0.8%) had the highest concentrations of saccharopine and aminoadipate on

days 3 and 6 respectively, compared to all other treatment groups on these days. These data agree with the (numerically) increased LKR activity in the 0.8% diet group (Figures 5.7 and 5.8). The elevated catabolite concentrations do follow the same pattern as plasma lysine concentration over time in birds receiving 0.8% lysine; after the diet was introduced levels increased and then subsequently began to decrease. However the concentration of plasma saccharopine was not correlated with plasma lysine concentration (Figure 5.4A), suggesting that, like most amino acids, the concentration of this catabolite is regulated (104). Plasma aminoadipate concentrations were positively (albeit slightly) correlated with plasma lysine concentrations (Figure 5.4A) perhaps implying less regulation. Plasma aminoadipate concentrations were affected more by the appearance of plasma saccharopine than by plasma lysine (Figure 5.4). Plasma aminoadipate could also be increased in response via lysine catabolism through the pipecolic acid pathway. Additionally, it must be noted that excess tryptophan may be catabolized to α-ketoadipate, which in turn can be metabolized to aminoadipate by the cytosolic isoform of aminoadipate aminotransferase (AADATI) (345). The merging of the two catabolic pathways of lysine and tryptophan could lead to the increased aminoadipate concentrations in plasma.

The high catabolite levels seen in the 0.8% group are in agreement with the idea of an increased rate of LKR activity (Table 5.2, Figures 5.3), but this argument appears to be counter-intuitive considering that lysine catabolism was expected to be reduced when dietary lysine was deficient. However, the activity of LKR was not significantly different between diet treatment groups (Figure 5.7, 5.8). The mean activity of LKR is (numerically) greater for the 0.8% group than for the other dietary treatment groups. It is

possible that LKR activity was induced by higher concentrations of other amino acids. Both arginine and glutamate (glutamate + glutamine) were increased in the plasma of birds fed the 0.8% lysine. Arginine and lysine are structurally similar, and share transport mechanisms, thus it is plausible for arginine to stimulate LKR. But again the lack of significantly increased LKR activity confounds this idea.

Very little research has been reported in mammals or birds regarding whether the catabolites of lysine have regulatory roles in the cell, in addition to being intermediate products. A role for saccharopine, if one exists, in any species is currently unknown. However, an alternate use for this or other catabolites may be one viable hypothesis as to why such a high concentration was found in the plasma of the birds fed the low lysine diet. A second possible hypothesis could involve the interference from excess dietary amino acids with the ability of the second enzyme in the pathway, saccharopine dehydrogenase (SDH, EC 1.5.1.9) to function normally. The viability of this hypothesis may be dependant on whether or not substrate channeling is used to shuttle saccharopine from LKR to SDH because the two enzymes exist as a bifunctional protein. Substrate channeling can be advantageous in transporting unstable intermediates, such as saccharopine between the two active sites (310, 428). However, both Falco et al (144) and Miron et al (312) have found evidence in plants against the occurrence of substrate channeling in LKR/SDH. However, substantial differences in regulation and structure of LKR/SDH may exist between plants and animals (518). Therefore further research on both the structure of the enzyme as well as the fate of catabolites in mammals and birds is needed to test these hypotheses.

The possibility also exists that an alternative change in regulation may occur in response to the limiting amounts of lysine. The build up of saccharopine may be a result of increased flux through the pipecolic acid pathway, which is known to catabolize a small portion of lysine in the chick (190). Such an increase could lead to increased levels of aminoadipate. The increase in aminoadipate could in turn inhibit LKR activity, which may modulate saccharopine levels. The idea is supported by our data (Table 5.10) that showed aminoadipate was a potent inhibitor of LKR activity in the liver and kidney.

5.5.3 LKR Activity and Enzyme Kinetics

Because of significant changes in the plasma concentration of lysine and lysine catabolites, as well as performance parameters of the birds, significant differences in LKR activity were anticipated. No significant differences were detected in any tissue, at any diet level. The failure to show significant differences in LKR activity in either tissue or dietary treatment is an important observation in itself. Activity of a catabolic enzyme normally fluctuates depending on the dietary availability of its substrate. With excess lysine supplied, LKR was expected to exhibit a significantly greater activity than when supplied with a suboptimal amount, as reported in the poultry experiments of Wang et al (484, 485). As lysine concentration in the diet decreased below requirement, lysine catabolism and hence LKR activity, was expected to similarly decrease toward the basal rate. Reports by others have suggested that increased consumption of lysine and protein (from deficient to excess lysine) increased LKR activity in chicken and rat liver (34, 81, 160, 219). Results from three separate experiments agree with our findings however. Moehn et al (313) showed that lysine oxidation in pigs was not decreased until the lysine level reach <70% of requirement. Walton and colleagues (483) found no difference in

liver LKR activity after feeding dietary lysine ranging from 1.0% to 2.6% of the diet. Lysine requirement was determined to be 1.9% in these studies, in agreement with the 1.8% dietary lysine level suggested by NRC (328). Unpublished data by Manangi (288) showed that when chickens were fed deficient (0.69%) lysine diets, hepatic LKR activity was more than 2 fold decreased when compared with LKR activities from birds fed adequate (1.1%) or excess (2.1 % and 3.1%) lysine diets. However no differences in hepatic LKR activity were found among the adequate- and excess-lysine fed birds. Furthermore, no significant diet differences were seen for hepatic (in vitro) lysine oxidation. This report, while contrasting our deficient lysine diet LKR activity data, does show that lysine degradation is not necessarily proportional to dietary intake, which is in agreement with our data and conclusions. In Figure 5.6, the kinetic parameters of hepatic LKR were illustrated as a function of dietary lysine intake. At requirement (1.2%) maximal velocity is the lowest of any diet level and is paired with a high Michaelis constant. Thus at requirement, hepatic LKR activity has a lowered affinity for lysine and the maximal rate of degradation is lowest. At a dietary deficiency of lysine, the maximal velocity increased as lysine intake decreased, and the affinity for lysine increased, represented by a decreased Michaelis constant (K_m). As lysine intake increased past requirement, the maximal velocity of LKR activity increased. The increase however, was not as large as observed at deficient lysine intakes. Again the affinity for lysine decreased. These results imply different regulatory mechanisms control lysine degradation at deficient, adequate and excess levels of lysine intake,

Taken with our data, these reports imply that regulation of lysine degradation is not simply a dose-response mechanism clearly indicating that other factors are involved.

Other parameters, possibly transport of lysine into the mitochondria (34), may be a rate-limiting feature for lysine degradation when lysine is in limited or excess supply. The results, especially the lack of decrease in LKR activity at deficient dietary intake, also suggest that the degradation of lysine may be a necessary and indispensable process.

Genetic differences in poultry have been shown to influence the activity of LKR (484, 485). Birds selected for low arginine requirements (LA) had significantly higher hepatic LKR activity than birds with higher arginine (HA) requirements. When either lysine (0.96% basal diet supplemented with 0.25%, 0.50%, 0.75% and 1.0% L-lysine-HCl) or arginine (0.88% basal diet supplemented with 0.5%, 1.0%, 1.5% and 2% -L-arginine-HCl) was supplemented in the diet at various concentrations, the LKR activity in 10% liver homogenates from the low arginine requirement (LA) birds was greater. When fed the arginine supplements, the LA birds had a 3.3 fold increase (on average) in LKR activity compared to the HA birds, with no apparent differences as the arginine concentration in the diet increased. When fed the lysine-supplemented diets, the LA birds had a 1.8-2-fold higher LKR activity than the HA birds. As the dietary lysine concentration increased, the relative fold increase in LKR activity decreased with lysine concentration. This showed that the HA birds could induce an increase in LKR activity to a greater extent than the LA birds. The "better" increase in HA LKR activity coincided with a 5.6 fold increased in kidney arginase activity, compared with a stable rate of arginase activity in the LA birds. These results imply that different ratio of Lys:Arg was optimal to these strains, which would potentially alleviate transport competition. Although the plasma ratios of Lys:Arg in these birds did not show this, the effects would possibly be more pronounced at the intracellular level. In the current study, we have used

Ross x Ross 308 pullets, however whether this genetic line has a high or low requirement for lysine is unknown. Preliminary experiments in our laboratory utilized Cobb 500 poults fed a synthetic amino acid diet (Appendix 8.5.1). These COBB x COBB birds demonstrated greater hepatic LKR activity in the range (~6.5 nmol/min/mg) reported earlier (484, 485) compared to LKR activity (0.5-1 nmol/min/mg) observed in the Ross 308 birds in the current experiments. However no large change in LKR activity in the preliminary experiments due to diet was seen (Figure 8.11). Therefore it is possible that breed or strain differences play a significant role in the regulation of LKR activity and hence lysine degradation.

Experiments in pig tissues preceding the current study demonstrated that LKR activity was subject to substrate inhibition (Figures 3.2, 3.2, 4.2-4.8; (364, 365). Additionally, pilot studies in poultry also suggested that substrate inhibition was a part of the kinetic profile of LKR in poultry liver (Appendix 8.5, Figure 8.10). In the present experiments, liver LKR demonstrated the classical Michaelis-Menten response to increasing concentrations of L-lysine (Figure 5.3). Contrasting the liver response, LKR activity in mitochondria prepared from kidney and IEC clearly demonstrated substrate inhibition at high concentrations of lysine. While this response has not been described previously in chickens, Wang and Nesheim (485) demonstrated that microsomal L-amino acid oxidase prepared from chicken liver was substrate inhibited by lysine. LKR enzyme purified from human placenta was shown to be substrate inhibited by high concentrations of α-ketoglutarate (155). That we have demonstrated substrate inhibition of LKR is therefore not without precedent. It is possible that a high concentration of either of these substrates could negatively affect LKR activity. Using the assay system presented in this

study is not unique, however it may provide the appropriate environment (mitochondrial preparations versus broken cell homogenates) for these kinetic characteristics to be recorded. Furthermore, our studies (Chapters 3, 4, 5, 8) suggest that the substrate inhibition mechanism is quite possibly influenced by tissue, age and strain.

Despite significant variation in the dietary lysine concentration between treatments, LKR activity did not significantly vary in any of the tissues studied. L-amino acid oxidase, the initial enzyme in the pipecolic pathway, was also shown to not respond to increased L-lysine•HCl levels (1% and 2%) added to a base soybean meal diet (485). However these reports do not agree with results presented by Lewis (280, 417) that showed chick liver L-amino acid oxidase activity increased in response to high levels dietary lysine. These reports, and our own data, suggest that there is a complex level of regulation of lysine catabolism that most likely has a genetic component; that either strictly controls the intracellular levels of lysine itself, or one or more of its catabolites.

5.5.4 LKR Inhibition

To better understand the parameters regulating the activity of LKR, we investigated the response of the enzyme to known inhibitors (Table 5.5). α-Aminoadipate was a potent inhibitor of LKR in both the liver and kidney isoforms. Similarly, α-ketoadipate significantly reduced LKR activities in both tissues. Interestingly, it appeared that both α-aminoadipate and α-ketoadipate were more effective inhibitors at the dietary extremes of deficiency and excess. Thus it may be that LKR is regulated by different mechanisms when lysine intake is deficient, adequate, or in excess of requirements. A feedback mechanism would be more important during deficiency and excess, than when lysine is provided at requirement and being used for protein synthesis at an optimal rate. Because

the compounds were not as effective inhibitors in mitochondria isolated from IEC ay deficient lysine intakes (0.8% and 1.0%), this mechanism may be tissue specific. It also implies the continuous use of lysine by the IEC. Furthermore, intermediate concentrations of saccharopine, aminoadipate and ketoadipate may have to be balanced with intermediates generated from other pathways suggesting that the catabolism of lysine may be a necessary and indispensable process. For example, the degradation of tryptophan results in increases in kynurenine, kynurenate, ketoadipate and aminoadipate. The possible importance of this balance has been discussed previously (sections 3.5 and 4.5) but again emphasizes the importance of lysine degradation.

Homocitrulline, a lysine molecule with a carbamoyl moiety attached to the epsilon-amino group, was used as a possible inhibitor due to its structural similarity to lysine and reported metabolism (141, 212, 239, 240, 373). In liver, homocitrulline significantly reduced LKR activity at the higher (1.4% and 1.6%) diet levels of lysine. Similar results were noted in the kidney and IEC analyses. This contrasted to experiments in pigs in which homocitrulline had no effect on LKR activity (364). The effect of homocitrulline appears to be a species linked effect. It is possible that because birds and mammals dispose of ammonia differently, that the response to urea precursors would be different as well.

5.6 Implications

These studies indicate that the regulation of lysine degradation in the growing chicken is more complex than previously recognized. Our results, in combination with others, suggest that the regulatory mechanisms controlling lysine catabolism are influenced by genetic differences, dietary levels of protein and lysine, interorgan

metabolism, and transport mechanisms. The resistance to decrease LKR activity at deficient lysine intakes in both chicken (reported here) and in trout (483), is paralleled by a lack of reduction in lysine oxidation at deficient lysine intakes reported in pigs (313) and chickens (288). These reports strongly suggest that lysine catabolism may be a necessary and indispensable process. Furthermore, interactions with other amino acids and intermediates appear to result in antagonistic relationships that increase the level of complexity. The concept, often assumed in production settings, that lysine is used simply as a substrate for protein synthesis, is most likely inaccurate and overly simplistic. Such a complex level of regulation for degrading this indispensable amino acid would indicate a more diverse role in metabolism either for lysine or one of its catabolites.

6 General Discussion and Conclusions

6.1 Introduction

The isolation and identification of lysine from casein hydrolysates in 1889 by Edmund Drechsel (128) marked the beginning of nutritional research into this amino acid. In the early decades of the next century, Abderhalden (2) began experiments to investigate the role of lysine in protein synthesis. After growth experiments, which unsuccessfully replaced [wheat gluten (gliadin)] protein by the complete digestion products of gliadin protein and abundant lysine, Abderhalden remarked

"*...that the proportion in which the various amino acids are present is probably too little adapted for the reconstruction of tissue*" (1).

A short time later, Osborne and Mendel (347) reinvestigated Abderhalden's experiments in rats and were able to promote growth in rats when a maintenance ration containing gliadin as the sole protein was supplemented with (0.54%) lysine. These studies, in conjunction with other investigations showing almost complete cessation of growth of rats fed lysine-free protein diets (347) led to their idea (and now dogma) that lysine was indispensable for the functions of growth.

The first experimental evidence that lysine is an essential factor for the growing pig was offered by Mertz and colleagues (305, 408) when they demonstrated that deficiency symptoms were alleviated by the addition of 2.0% DL-lysine-HCl. Experiments by Rose (383, 384) during this period were providing the initial requirements for lysine in man. Similarly, lysine supplementation of practical swine diets (37, 63, 229) and poultry diets (85, 186, 401, 432) were also being evaluated during this period.

Early investigations examining the metabolism of lysine showed that lysine was degraded to glutaric acid (42, 332, 381) and α-aminoadipic acid (43, 332) although the metabolic route to arrive at these catabolites remained obscure (387). Work in the early 1950s demonstrated that lysine could also be converted to glutamate (6, 311). These studies pointed toward the, as yet, undescribed saccharopine pathway. Rothstein and Miller's experiments (385-387) led to the conclusion that pipecolic acid was a major metabolite of lysine degradation in the rat. However, by the late 1960s, several studies had concluded that saccharopine was the key intermediate in the degradation of lysine (187-189, 204, 220). The pipecolic acid pathway was then, and is still regarded as the secondary overflow pathway, activated when the lysine load overwhelms the capacity of LKR (99).

The identification of lysine α-ketoglutarate reductase as the key regulatory enzyme in lysine degradation initiated research that has defined this catabolic pathway to be far more complex than many of the other amino acid degradative pathways. In the current work, we have tried to elucidate aspects of the regulatory story and solve a few pieces of the puzzle.

6.2 Regulation of Lysine α-Ketoglutarate Reductase

After definition of the rate-limiting enzyme in a degradative pathway, delineation of the factors which control the enzymic activity are critical to understand fully the role of that enzyme in metabolism. This fact is far more important in our scenario because the key enzyme catalyzes an essential amino acid; an amino acid whose intake regulates the protein synthetic rate of many populations around the world (218, 405, 419, 517).

The current studies focused on several aspects of regulation of LKR and to a lesser degree SDH. These parameters included tissue distribution and subcellular location of the enzyme protein, kinetic parameters of the enzyme in response to different levels of substrate, age and development of the growing animal, dietary levels of lysine and sensitivity of enzyme activity to catabolites or analogues of enzyme substrates.

6.2.1 Tissue Distribution of Lysine Degradation

The liver is most often considered the main tissue for the degradation of amino acids (27). While the liver does appear to be the primary tissue for lysine degradation (374), this dogma has led most researchers to focus only on the hepatic venue and neglect other tissues as possibly significant and important sites of degradation. Typically the liver and kidney have been cited as having the most substantial LKR and SDH activities (Table 1.2) and hence the only tissues with roles in lysine degradation. However our studies, in both pig and chicken, showed that although the liver does have substantial LKR activity, other tissues including the kidney have significant LKR/SDH activity. Of these extra-hepatic tissues, the intestinal epithelial cells and the muscle are the most important (Tables 3.1, 4.1, 4.2, Figures 5.4, 5.5). In agreement with our findings, recent work from the laboratory of Dr. Ken Blemings (Univ. West Virginia) has established that while the liver accounts for 20% of total body LKR activity, the muscle and intestine account for 33% and 40% respectively (Table 1.4). Our own calculations indicate that the intestine accounts for between 2%-17% of the total LKR activity and the muscle LKR activity accounted for ~15%- >80% of the total activity (Table 8.8). When considering lysine oxidation, the liver accounted for ~17% of the total, the intestine 8% and the breast muscle a staggering 63% of total lysine oxidized.

These same authors (289) demonstrated that LKR/SDH activity was not a good predictor of L-1-^{14}C-lysine oxidation to $^{14}CO_2$. Potential explanations for this apparent discrepancy may relate to the inability to optimize the in vitro activity of all five enzyme activities required to generate the first carboxyl group released as CO_2. While trying to optimize the conditions for LKR in the oxidation medium, a less than optimal microenvironment for the catalytic activity of other enzymes in the pathway may occur. For example, different enzymes in the saccharopine pathway have different optimal pHs: LKR pH_{opt}= 6.7-8.4 (220, 221, 337); SDH pH_{opt}= 8.5-8.9 (154, 222); α-aminoadipate aminotransferase pH_{opt}= 7.5 (327). In addition, the effect of rate of lysine transport into the mitochondria, potentially a key regulator of lysine oxidation (34)is not accounted for. Lysine transport may be more important as a regulator of lysine oxidation in vivo rather than in vitro. Additionally, the contribution of L-amino acid oxidase, the first enzyme in the pipecolic acid pathway, to lysine oxidation in tissues of different species is unclear. Although considered a secondary catabolic route for lysine degradation, the pipecolic pathway has been shown to be active in several tissues (68). Therefore, while the rate of LKR clearly indicates a significantly higher lysine flux through the saccharopine catabolic pathway, the regulation of several other catabolic enzymes may also contribute to the tissue and whole-body oxidation of lysine in vivo. In agreement with this idea, Benevenga and colleagues (28) suggested that although the saccharopine pathway is the main catabolic pathway, flux through this pathway could not account for all of whole-body lysine oxidation. Additionally, studies using fibroblasts from hyperlysinemic patients suggested that lysine transport into cells and cellular compartments was a significant in vivo regulator of lysine catabolism during this period of dysfunctional

lysine metabolism. These cells had normal enzyme activities of LKR and SDH, but demonstrated a 50% decrease in the oxidation of lysine (350) showing that while LKR was the rate-limiting enzyme for lysine degradation, other factors have significant regulatory effects on the total rate of lysine oxidation.

LKR protein was shown to be present in the liver, kidney, IEC and brain using western blot analyses. Identification of LKR activity and protein in the brain of young growing pigs clearly suggests a functional role for lysine degradation in this organ and is in agreement with the findings of others (68, 354, 374, 440). The question of whether there is a functional role for neuronal lysine degradation needs to be resolved. However, the story is becoming clearer through the work of researchers like Miro Smriga (Institute of Life Sciences, Ajinomoto Co., Inc. Kawasaki, Japan) who has linked dietary lysine levels to the levels of stress hormones such as cortisol (419-424, 429) and Pablo Arruda and Fabio Papes (State University of Campinas, Brasil) (354) who suggested the possibility that glutamate production from lysine degradation regulates nerve transmission signals via glutamate receptors. A more complicated explanation for neuronal lysine degradation could involve a need to regulate a homeostatic balance between kynurenate and aminoadipate production from tryptophan and lysine degradation, respectively (see below for details).

In future experiments, the location of the various lysine catabolic enzymes in different cell types would be beneficial. For example, the localization of enzyme protein for LKR, SDH, AADATI, AADATII and KADh using immunohistochemical techniques could further resolve the cellular sites for synthesis of aminoadipate and ketoadipate in the various cell populations in the brain. Similar experiments localizing the tryptophan

catabolic enzymes indoleamine 2,3 dioxygenase (388), and the kynurenine aminotransferases I and II could provide stronger evidence to support our ideas about the potential necessary catabolism of lysine for aminoadipate and ketoadipate synthesis to balance kynurenate synthesis. These experiments could be replicated in a similar fashion to investigate whether cells of the enteric nervous system have the potential for this mechanism. This could greatly increase our understanding of the development of the enteric and central nervous systems and the role of secondary amino acid functions in this process. Additionally, these types of experiments could be utilized to investigate LKR/SDH protein expression in compromised metabolic states such as when intravenous nutrition is required. For example, following the initiation of intravenous total parenteral nutrition (TPN) feeding, the gut atrophies; does the atrophy of the gut alter the expression pattern of LKR/SDH in the epithelial cells? As TPN is often a consequence of preterm birth, one could investigate the expression of enzyme in the liver, as well as the zonal pattern. While pig liver LKR activity did not change with age, we could determine whether the enzyme becomes localized to a particular zone in the liver at a specific age, which could help identify alternate roles for lysine catabolism. Similar experiments could investigate the expression of the other catabolic enzymes and show the development of a metabolic pathway in normal and metabolically stressed tissue (post TPN feeding).

Additionally, determining whether regulation of the enzyme-protein expression involves a short-term induction of monofunctional LKR or SDH expression (and or activity) in response to metabolic stressors, in conjunction with long-term expression of the bifunctional protein, will help explain some of the more complex regulatory characteristics (lysine degradation during lysine deficiency) of lysine degradation.

6.2.2 Kinetics of the Main Degradative Enzymes

The investigation of enzyme kinetics from in vitro experiments serves several purposes. It provides the researcher with estimates of K_M and V_{Max}, thus describing the relative ability of an enzyme to metabolize a particular substrate. These parameters are of considerable interest because an enzyme may utilize more than one compound as a substrate or different isoforms of the enzyme may exist in different cellular compartments; the relative differences in K_m can shed light on the preferred substrate and catalytic direction, and hence regulation *in vivo*.

The Michaelis constants of LKR have been described for several species. In rat liver, Blemings (34) estimated the K_M to be 5 mM. This was comparable to the estimate of 6.96 mM for chicken liver homogenate (289). For the partially purified human liver isoform, Hutzler and Dancis estimated the K_M to be 1.5 mM (221). Fjellstedt and Robinson (154) estimated the K_M of SDH to be 1.15 mM for human placenta. We found that the activity of LKR in adult pig tissues did not always follow classical Michaelis-Menten kinetics and was actually subject to substrate inhibition at lysine concentrations above 15 mM (Figures 3.3, 3.4, 4.3-4.9,5.5,8.10). In contrast, SHD followed classical Michaelis-Menten kinetics (Figure 3.3). Using the Lineweaver-Burke derivation we calculated a K_M of 12.7 mM for LKR and 1.41 mM for SDH in the adult pig liver. In IEC of the adult, the K_M was only 1.03 mM. In the developing pig liver, the K_M values for liver LKR were more variable but were estimated to be between 8 mM and 55 mM. In other tissues, the enzyme appeared to follow either classical kinetics or substrate inhibition, and this inhibitory mechanism was dependent upon tissue and age (Figures 4.2

–4.8, Table 4.3). Regardless of tissue or age, the K_M was typically in the millimolar range, consistent with the role of LKR as a catabolic enzyme.

In our poultry studies (Chapter 5 and section 8.5), liver LKR appeared to follow Michaelis-Menten kinetics in the Ross 308 birds and K_M was between 0.6 and 2.9 mM (Table 5.9, Figure 5.3). Preliminary studies used Cobb 500 poults; liver LKR from these birds had K_M values estimated at 2-2.65 mM. These data suggest a level of regulation dependant upon both the substrate concentration and catabolic tissue. That LKR may be prone to substrate inhibition has been described previously by Fjellstedt and Robinson (155) who showed that purified LKR from human placenta was substrate inhibited at high concentrations (> 1mM) of α-ketoglutarate.

Substrate inhibition is believed to be due to multiple substrate molecules binding within the active site simultaneously, resulting in decreased turnover of substrate at one of these sites. Mechanistically, it is unclear whether the inhibition is due to steric hindrance by a substrate at one site blocking the catalytic action at the primary site or whether the multiple binding results in a conformational change in the protein that blocks or inhibits substrate binding at the key catalytic site. Regardless, the end result is the diminished flux of substrate through the pathway.

In the case of regulation of amino acid catabolism, the tetrahydropterin-dependent amino acid hydroxylases are an interesting group to consider. In the presence of tetrahydropterin (BH_4) and lysolecithin, a potent activator of the enzyme, phenylalanine hydroxylase was substrate inhibited by increasing concentrations of phenylalanine (152, 241, 242). Closer to the argument at hand, tryptophan hydroxylase was similarly susceptible to substrate inhibition when the naturally occurring BH_4 replaced the

synthetic cofactor substitute $DMPH_4$ (163). The hydroxylation of tryptophan is the initial step in the synthesis of serotonin whereas the action of (tryptophan) indoleamine-2,3-dioxygenase, the rate limiting enzyme for tryptophan degradation (193), results in the formation of formyl-kynurenine, preceding the formation of kynurenine. High levels of tryptophan will inhibit the hydroxylase action via substrate inhibition (152), and increase the synthesis of kynurenine, which also results in competition for cellular uptake of tryptophan (167). Kynurenine can be transaminated to kynurenic acid, a neuroprotective excitatory amino acid receptor antagonist, by α-aminoadipate aminotransferase. Catabolism of lysine to aminoadipate, which can compete with kynurenine for transamination, could then decrease kynurenate production (505). Depletion of kynurenate would therefore enhance the responses of excitatory amino acid receptors (462). Conversely, the presence of increased aminoadipate concentrations have been shown to be both neurotoxic and gliotoxic (45, 248, 299). Transport systems in astrocytes appear to act in concert to limit aminoadipate accumulation and prevent detrimental effects in these cells, while permitting concentrations of the lysine catabolite to effectively limit kynurenate production (462). This substrate inhibition mechanism utilized by LKR, as shown by our data would effectively limit lysine degradation and production of aminoadipate, comparable to the inhibition of tryptophan hydroxylase by increased levels of tryptophan (152). Whether this scenario is limited to the brain or relevant to the development of particular tissues or the animal/bird as a whole remains unclear.

The future experiments described in the previous section would provide the enzyme expression patterns to validate this last set of ideas suggesting a homeostatic

balance between the synthesis of lysine and tryptophan catabolites. The substrate-inhibition mechanism of LKR/SDH needs to be further investigated to validate a function for this unusual regulatory mechanism. Purification and sequencing of the enzyme protein from different tissues might explain why some tissues demonstrate classical Michaelis-Menten kinetics whereas others demonstrate substrate inhibition and answer questions about different isozymes of LKR or SDH. For example, does the monofunctional LKR protein exhibit substrate inhibition while the bifunctional isoform use a substrate-inhibited mechanism?

6.2.3 Diet and Developmental Effects

As animals grow, the lysine requirements for optimal growth (expressed as % of diet) gradually decline. In growing pigs the initial requirements start at 1.34 % (based upon the true ileal digestible basis) for 3-5 kg piglets and decline to 0.52% for 80-120 kg pigs (331). Comparatively in broilers, lysine is supplied at 1.1% for the first 3 weeks. This level declines to 1.0% (3-6 weeks) and finally to 0.85% for birds 6-8 weeks of age (330). In those birds bred for white egg-laying capacity, lysine is held at 0.85% for the first 6 weeks and gradually decreases to 0.52% during the period between 18 weeks to first egg lay (330). The requirements for brown-egg layers follow the same trend but are slightly (0.3-0.5%) lower at each age.

In a rat study conducted by Rao and colleagues (374), LKR activity in the liver increased significantly from birth through to 30 days of age. In another rat study, LKR activity in the liver increased dramatically from 3 days before birth to 3 days after birth, decreased until day 5 and then the LKR activity was stable through adulthood (411). These mark the only studies, to the best of the author's knowledge, examining

developmental influences on LKR activity in mammalian systems. In the current studies we investigated the activity of LKR and lysine oxidation at ages that mark key dietary transitions in the growing pig, such as birth and weaning. We also investigated the effects of dietary levels of lysine in young growing broiler-breeder poults.

In growing pigs, tissue activities of LKR were affected by age and concentration of lysine. Liver LKR had a mean activity of 1.86 nmol/min/mg during the experimental period, but also demonstrated a significant (~180%) increase in LKR activity on day 14 (Table 4.1). Adults pigs had a liver LKR activity of 4.26±1.80 nmol/min/mg (Table 3.1) which was significantly different from the mean activity of growing pigs. However, it should be noted that adult animals were obtained from a local abattoir, and thus we could not control for stress levels associated with transport or the diet of the animals. Of particular interest, muscle LKR activity was highest on day 0 and then significantly decreased to approximately 30% of the day 0 activity for the remainder of the experimental period. The oxidation of lysine to radioactive carbon dioxide in muscle mitochondria also significantly changed during the first week. The rate of $^{14}CO_2$ production by muscle mitochondria was significantly greater on day three and seven compared to both liver and IEC. These results highlight that different tissues in the growing animal vary in their contribution to the degradation of lysine according to age. As the animal grows, different tissues become more important for degrading lysine. For example, during the first week, the changes in LKR activities and lysine oxidation may reflect the shifts in tissue and whole-body metabolism that are occurring for the animal to adapt to ex-utero nutrition. As discussed, because the body reserves of lipid and glycogen are insufficient to meet energy demands after birth (30, 139, 273), a resistance to the

depression of protein catabolism occurs to insure provision of adequate concentrations of amino acids for energy metabolism and thermogenesis (367, 368). The potential for an important metabolic role of any lysine catabolites, namely saccharopine, aminoadipate or ketoadipate, remains unclear but cannot be wholly discounted as a reason for lysine degradation.

Regarding the influence of dietary lysine intake on the activity of LKR, it is intuitive that when lysine, an essential amino acid, reaches excess levels in the diet its degradation increases. In contrast, if levels fall below requirement, lysine should be conserved for maintenance protein synthesis, and hence flux through LKR should be diminished. We fed Ross 308 poults lysine ranging from 0.8% (limiting) to 1.6% (excess) levels in the diet. Birds fed the lowest level were smaller and did not grow as well as birds on other dietary lysine intakes. However, several interesting things occurred. Plasma levels of lysine and the catabolites saccharopine and aminoadipate were generally higher in the birds fed the lowest diet level of lysine (Table 5.7). More importantly, low lysine levels were enough to blunt growth, but the activity of LKR in the liver, the kidney and in IEC were not different (Figures 5.4, 5.5). Furthermore the activity of LKR at the 0.8% level more closely resembled the LKR activity of birds fed a high lysine diet. These results do not agree with other poultry data demonstrating increases in LKR activity with increasing dietary lysine (484, 485). However, birds used in those studies had been selected for high/low arginine requirements and demonstrated that genetic differences affected LKR activities. Results from three separate experiments agree with our findings however. Moehn et al (313) showed that lysine oxidation in pigs was not decreased until the lysine level reached <70% of requirement. Walton and colleagues (483) found no

difference in trout liver LKR activity after feeding dietary lysine ranging from 1.0% to 2.6% of the diet. Unpublished data by Manangi (288) showed that when chickens were fed deficient (0.69%) lysine diets, hepatic LKR activity was more than 2 fold decreased when compared with LKR activities from birds fed adequate (1.1%) or excess (2.1 % and 3.1%) lysine diets. They found no differences in hepatic LKR activity among the adequate- and excess-lysine fed birds. Furthermore, no significant diet differences were seen for hepatic (in vitro) lysine oxidation. Although the report by Manangi (288) described a decrease in LKR activity when lysine intake was deficient, in contrast to the (numerical) increase observed in our data, it does show that lysine degradation is not necessarily proportional to dietary intake, which is in agreement with our data and conclusions. It is possible that genetic differences were also a factor; Manangi (288) used a COBB x COBB strain while Ross 308 poults were used in the current experiments. Our own studies provide additional evidence showing that differences in strains affect LKR activity (compare Figures 5.7 and 8.11). In addition, it must be noted that much further genetic selection for optimal performance properties (e.g. increased feed efficiency) has been performed since the early 1970's, of these early studies (484, 485). The possible effects of this selection process on LKR activity or on lysine oxidation have not been documented and represent a large field of study to explore.

Regarding diet and development, our results clearly indicate that this is an area to be further investigated. One of the main questions has to be – Why does lysine catabolism not necessarily follow a dose-response mechanism? Why does LKR activity not change when lysine intake is deficient or in excess? The answers to these questions

may be answered once the monofunctional versus bifunctional characteristics of the enzyme are clarified.

Does lysine degradation play a necessary role in development? To the best knowledge of the author, no one has described the plasma, tissue or intracellular concentrations of lysine catabolites as a function of age, especially during the periods of significant nutritional transitions. Comparing the lysine catabolite profiles with tryptophan catabolite profiles (especially kynurenine and kynurenate), arginine (regarding the antagonism between lysine and arginine), hormone concentrations (serotonin, melatonin, cortisol as well as glucagon and insulin) may provide more insight into the potential developmental role of these compounds.

In terms of genetic selection, the University of Alberta holds several rare breeds (older genetic strains) of poultry. Experiments comparing the lysine requirements of these rare birds to modern strains could be paired with determinations of tissue LKR activity and enzyme expression. These experiments could provide an answer to questions of whether genetic selection for increased rate/efficiency of protein synthesis has altered the regulation of lysine degradation in different tissues. Diet experiments similar to those described in Chapter five could be integrated into the design to further elucidate the effects of genetic selection.

An interesting route of investigation may coincide with advents of the Barker hypothesis (21-23, 123, 194). If an animal is raised in utero and post-parturition with a limiting lysine diet fed to the mother and then to the neonate, what are the consequences for future generations if these (future) individuals are exposed to deficient, optimal and

excess dietary lysine? What are the responses of LKR activity in different tissues? Does whole-body lysine oxidation or utilization efficiency change?

6.2.4 Exogenous effectors of LKR activity

The mechanism by which any enzyme is controlled or functions may be examined through the addition of exogenous compounds that are in some way related to a key substrate of that particular catalytic activity. When compounds are shown to affect the activity, we may begin to describe the preferred reaction direction or determine Michaelis constants for example. This is especially important if isoforms of enzymes work in tandem across different organelles of the cell to facilitate a particular metabolic fate for some compound. A good example of this would be AADATs I and II (section 1.3.2) that work to balance the flux of common metabolites from two merging catabolic pathways.

In the case of LKR, we tested the activity of the enzyme in the presence of the catabolites saccharopine, aminoadipate and ketoadipate as well as numerous structurally related compounds. Of the catabolites, aminoadipate generally appears to be the most potent inhibitor. The effects of aminoadipate and indeed the other catabolites may reflect some form of feedback regulation on LKR activity. This is more relevant if one considers that aminoadipate is considered extremely toxic. These and other compounds were tested for potency at different ages (or nutritional transitions) and it appeared that there might be some effect of development on the potency of any particular compound (Tables 4.4 –4.10, Section 4.5.3). Contrasting the adult data, the catabolites essentially had no effects during development. Could these compounds be important in the development of particular tissues? Van Kempen and colleagues (471) unsuccessfully provided adipic acid

in pig diets to reduce lysine requirements. Unfortunately, these authors did not investigate any aspect of lysine degradation at the cellular or enzymic level. In addition, our developmental data suggests that the animals may be influenced by dietary adipate only at specific periods. Therefore additional experiments are required to explain the basis for the failure of this compound to reduce lysine requirements. The effects of metals (particularly zinc and copper) on LKR activity were dramatic (Figure 3.9) and may prove to be an important area of future investigation as these metals are commonly added to production diets as immuno-facilitating agents.

When LKR activity was examined after birds were exposed to different dietary levels of lysine, it appeared that when birds were fed the lowest or highest levels of lysine, LKR activity was more susceptible to inhibition by compounds like aminoadipate and ketoadipate than when birds were fed concentrations of lysine closer to requirement (Table 5.10). Thus it might be that the sensitivity of the enzyme to inhibition is dependent upon dietary level of lysine, the intracellular concentration of lysine or some other catabolite.

Regarding the tryptophan catabolites, one series of experiments to perform would include incubating cells/mitochondria from different tissues with increasing concentrations of lysine first as tryptophan concentration is held constant, and then increased proportionally to lysine and finally in excess of lysine. Following the incubations, the production of saccharopine and aminoadipate can be correlated with the production of kynurenine and kynurenate as a function of either lysine or tryptophan concentration. These experiments should provide more insight into the relationship between lysine and tryptophan catabolism.

For the investigation of the calcium-based mechanism illustrated in Figure 1.6, the activity of LKR and SDH can be investigated in the presence of calcium chelators (e.g. EGTA) and in the presence of increasing concentrations of Ca^{2+}. These experiments, especially in muscle tissue, will provide considerable knowledge toward a more complete understanding of LKR.

6.3 Concluding Thoughts and Future Directions

These studies have investigated regulatory aspects of lysine catabolism in growing swine and poultry. We have shown that extra-hepatic tissues in both pig and chicken possess LKR activity. Of these, the intestine and muscle represent two of the most important tissues for consideration. Animal nutrition has focused on enabling the synthesis of muscle protein, while often ignoring the nutrition of the gut. We showed that this tissue can degrade the most limiting nutrient and also one of the most expensive feed components in the livestock industry. The influence of the intestine should no longer be ignored when it comes to lysine metabolism. The question of why the gut possesses this capacity is unclear.

We have further shown that during development different tissues appear to play different roles in lysine catabolism. The true nature of these roles remains unclear and should be the subject of several future investigations. Adding to these questions is the role of lysine catabolites in the overall metabolism of the animal or bird. We know that aminoadipate can have detrimental effects on neural biology, however we do not yet understand fully the function of aminoadipate in different tissues. What is the true nature

of the interorgan relationships involved in lysine catabolism? Do particular organs work to partially degrade lysine in the manner of muscle catabolism of the branched-chain amino acids?

At the dietary level we have shown that while broiler poults do not grow as well on a limiting lysine diet, the activity of LKR does not seem to be affected by dietary lysine. Is this a phenomenon related to genetic selection for growth and feed efficiency? Have these birds been inadvertently selected for lower LKR activities? In pigs we know that lysine oxidation does not decrease until dietary lysine intake falls below 70% of requirement, so why is lysine catabolism such an important event? Why is the basal level of catabolism so high for this essential amino acid? These questions should continue to be the basis of future research.

At the kinetic level, we have shown that LKR is susceptible to substrate inhibition, at least in vitro. This mechanism was seen in different tissues and at different ages in the pig. It was similarly seen in different tissues of the broiler poult. Past research has shown that the enzyme can be susceptible to this type of inhibition, but it has not been described for pigs or chickens. The metabolic role for this type of kinetic mechanism may be involved in the homeostatic balance between aminoadipate, kynurenine and kynurenate, however much work remains to be performed to answer this question.

In summary, the level of complexity associated with lysine degradation has been acknowledged in plant biochemistry. However, scientists investigating the mammalian and avian forms of this enzyme, and the lysine degradative pathway as a whole, are only now starting to appreciate the diverse levels of regulation controlling catabolism of this essential amino acid.

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8 Appendix

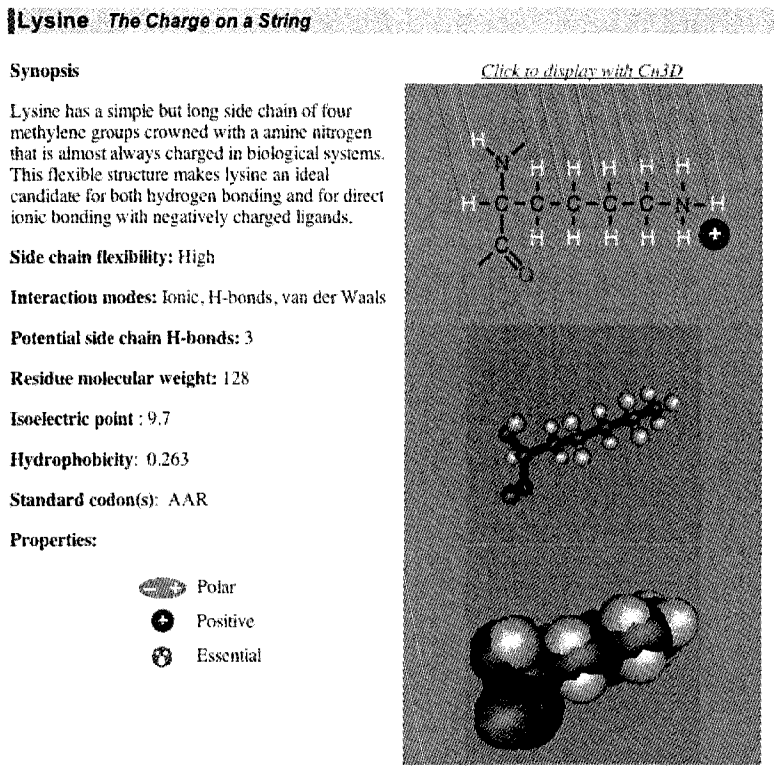
8.1 General Appendix to Chapter 1

Presented in this first appendix are items that add to the *Introduction* section on lysine metabolism. Included are fundamental chemistries of the amino acid and relevant considerations of the producer, the agricultural industry, researchers and students that may not fit ideally into the chapter descriptions.

8.1.1 Fundamentals of Lysine Chemistry

Lysine, an essential amino acid is characterized as a basic amino acid. The epsilon amino in the R-group of lysine affords many of the functionalities of this amino acid including polarity, (positive) charge, and reactivity of the amino acid.

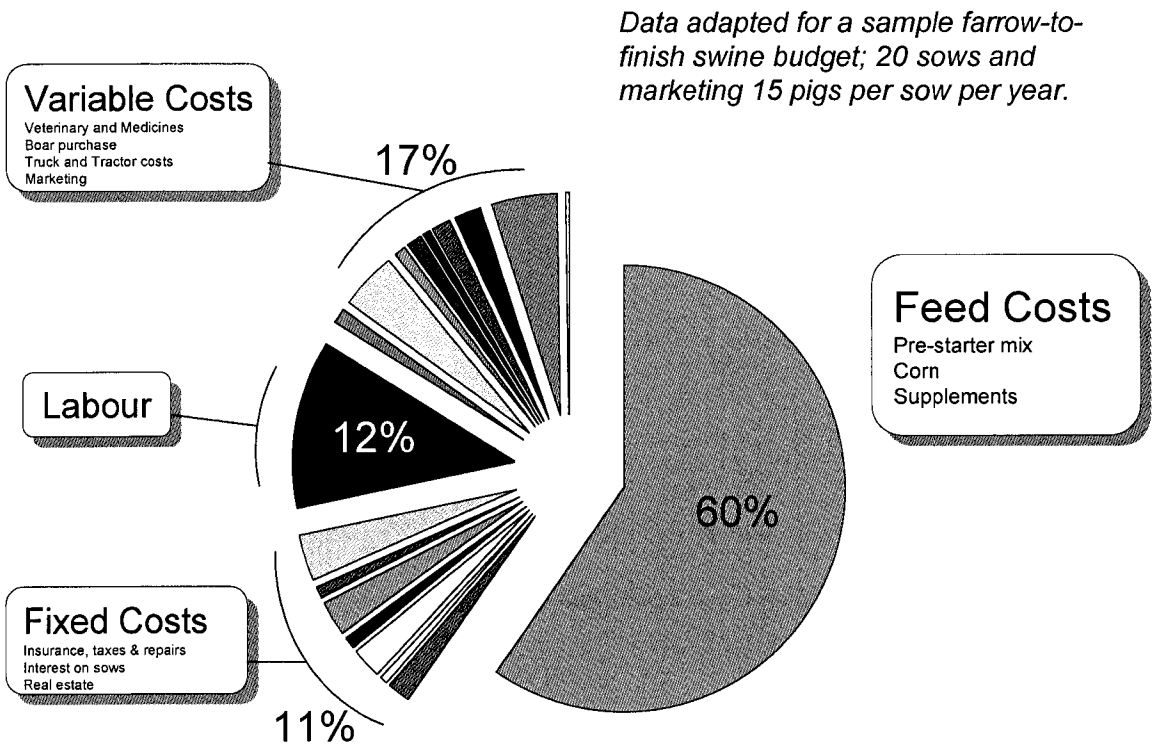
Figure 8.1 Fundamental Chemistry of the Amino Acid Lysine



8.1.2 Breakdown of Swine Production Costs

The typical breakdown of production costs is illustrated in the pie chart below. Data were adapted from agalternatives.aers.psu.edu/livestock/swine/swine.pdf.

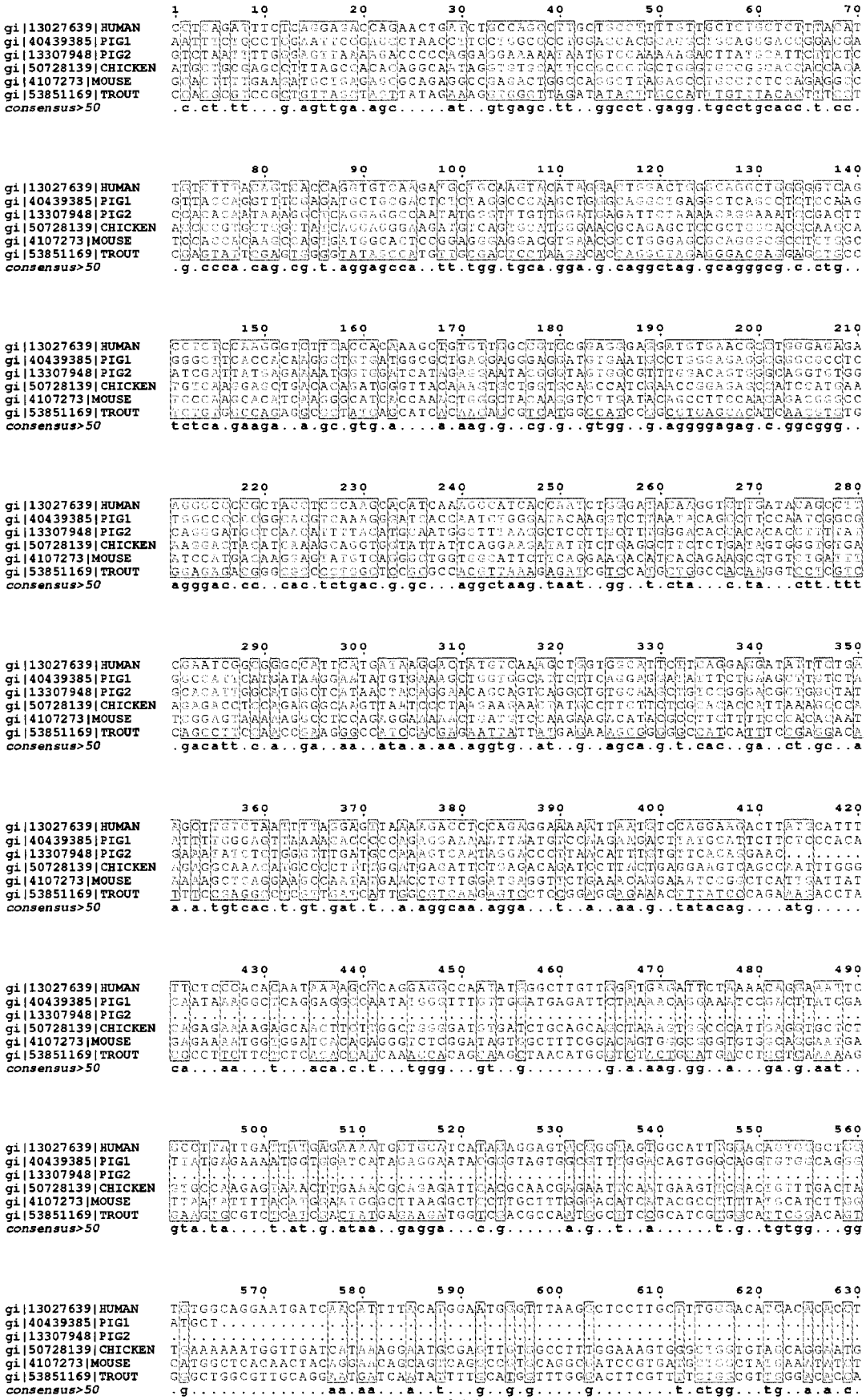
Figure 8.2 Production Costs of Farrow to Finish Operations

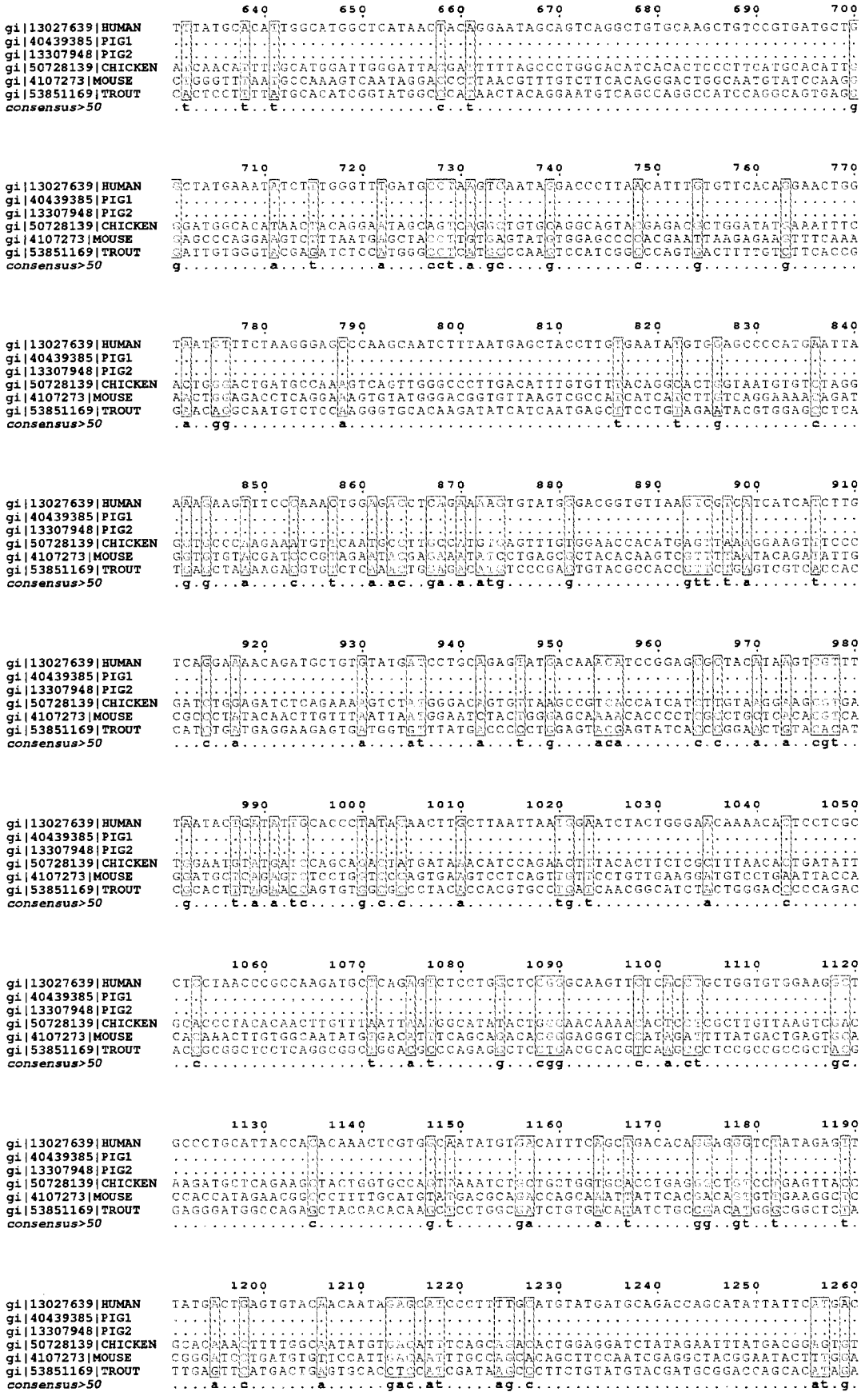


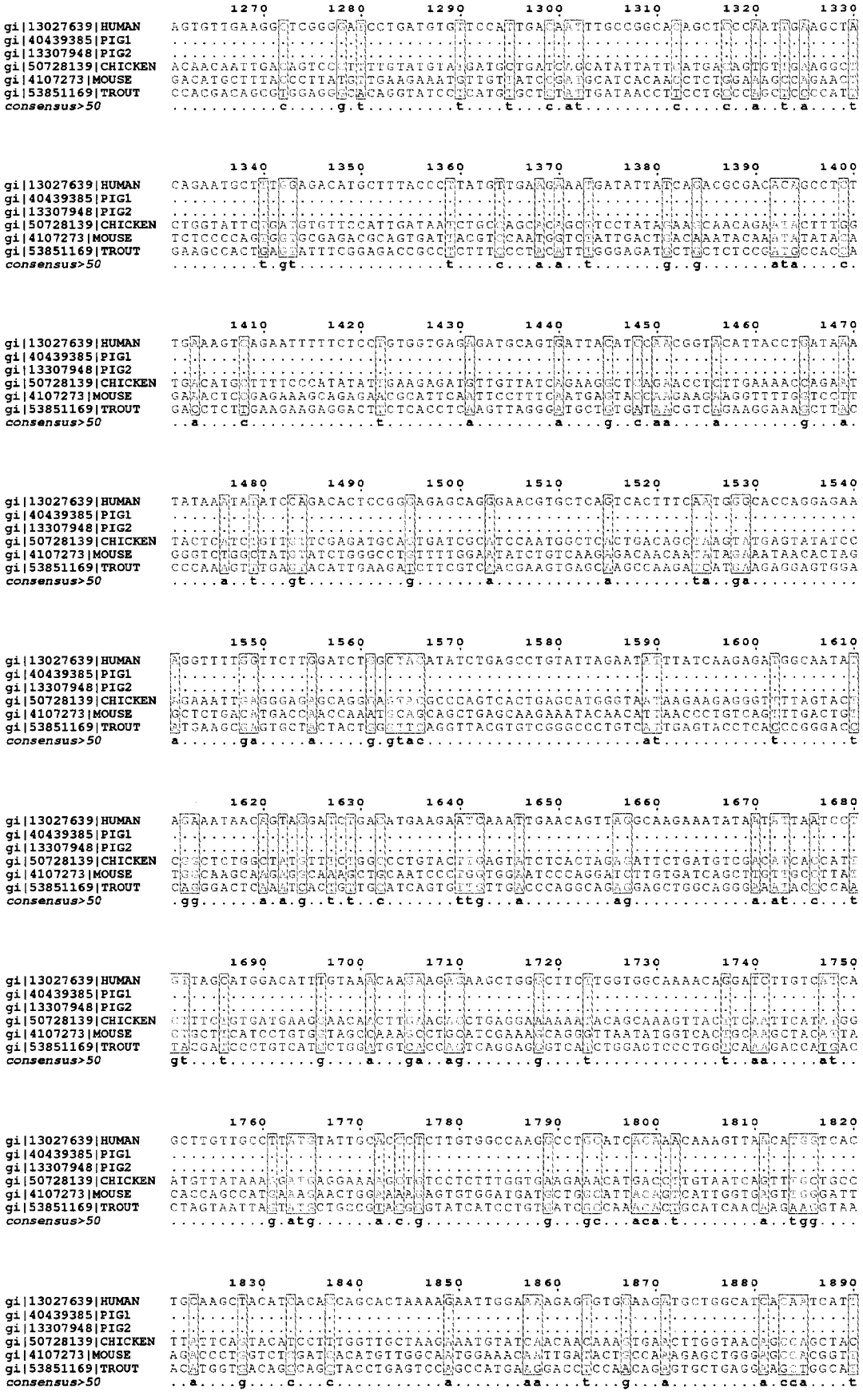
8.1.3 Multiple sequence alignments of AASS proteins

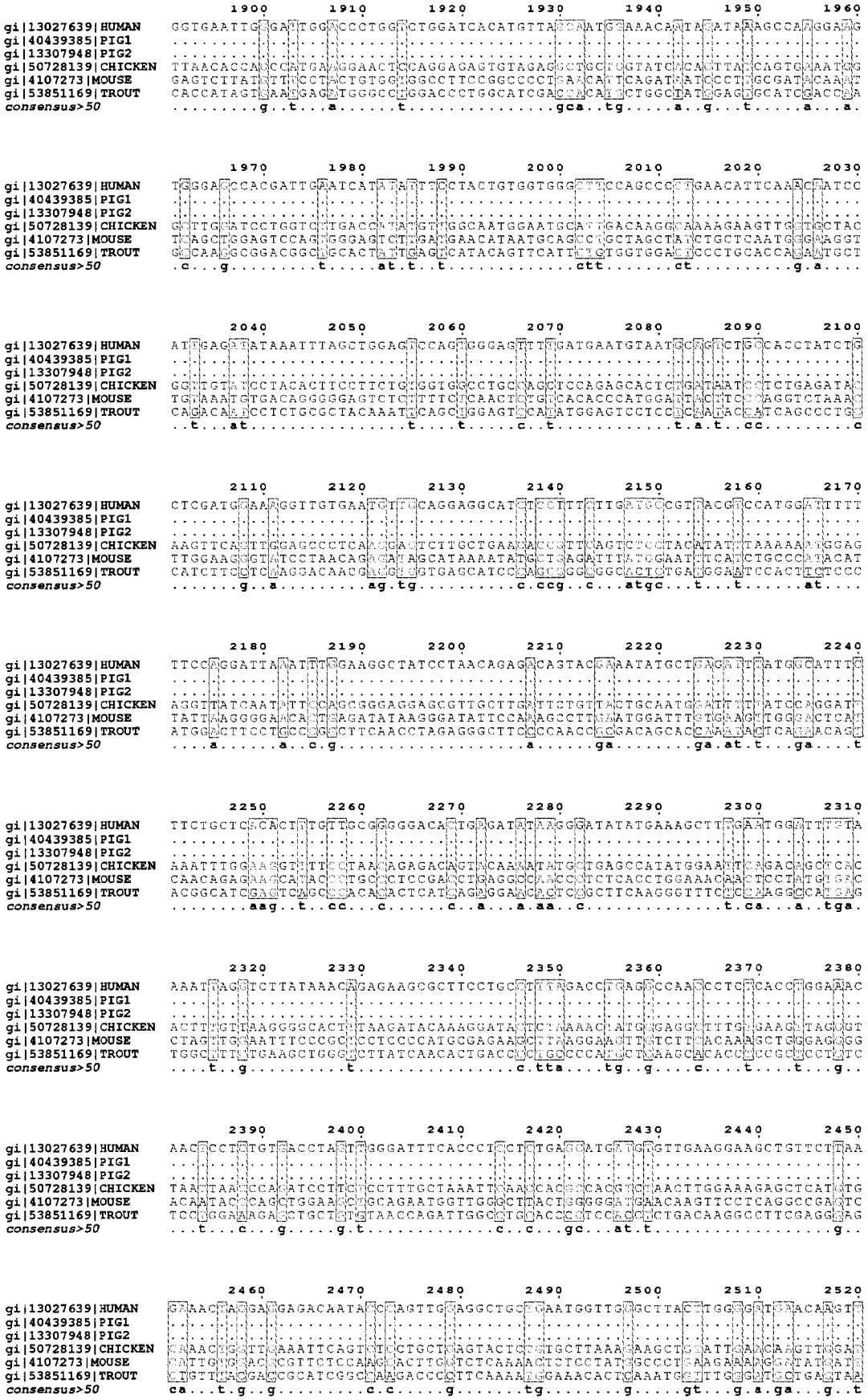
To avoid disruption and flow to Chapter 1, I have reserved the entire multiple sequence alignment for this appendix.

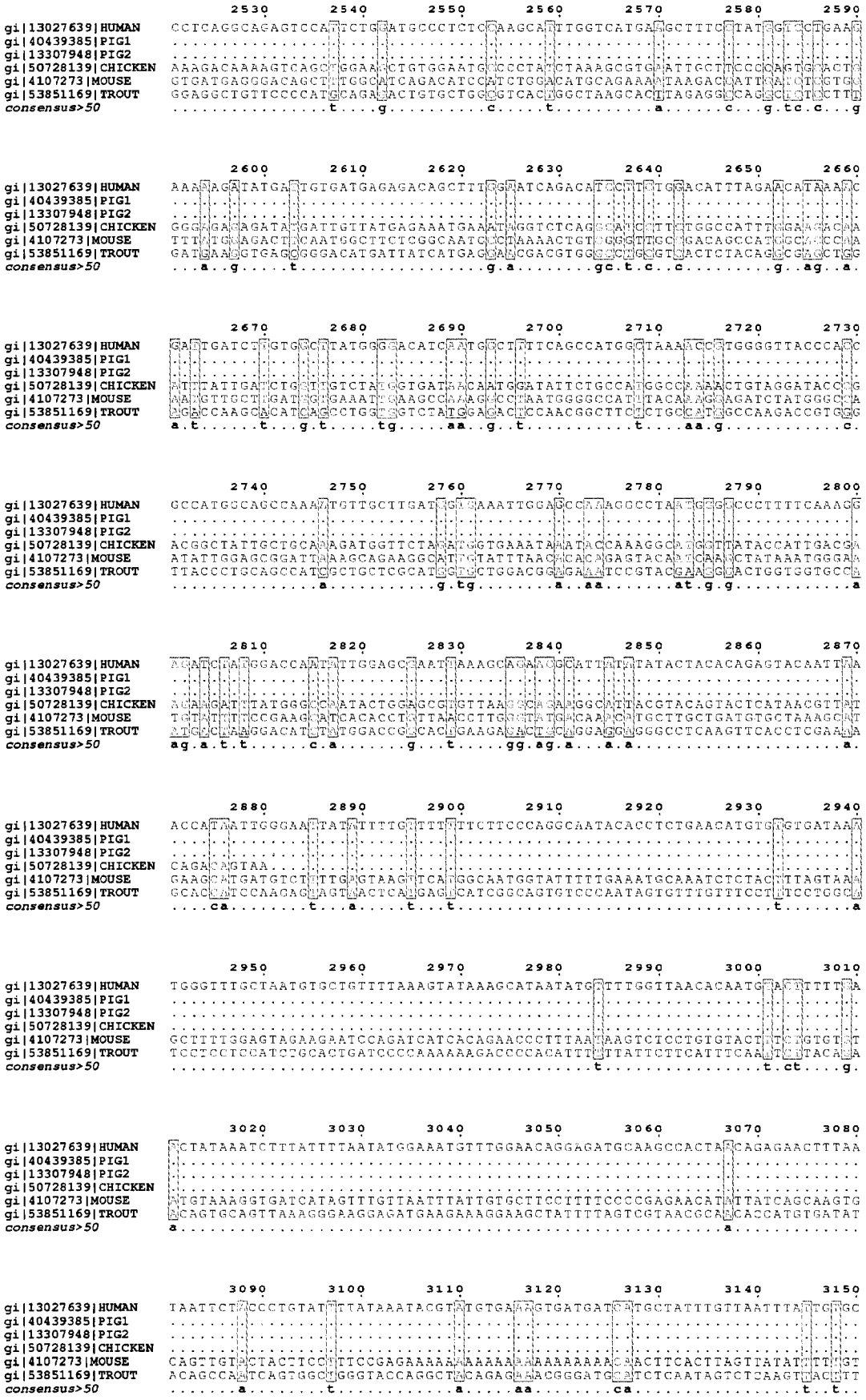
Figure 8.3 Sequence Alignment of AASS Proteins from Different Species

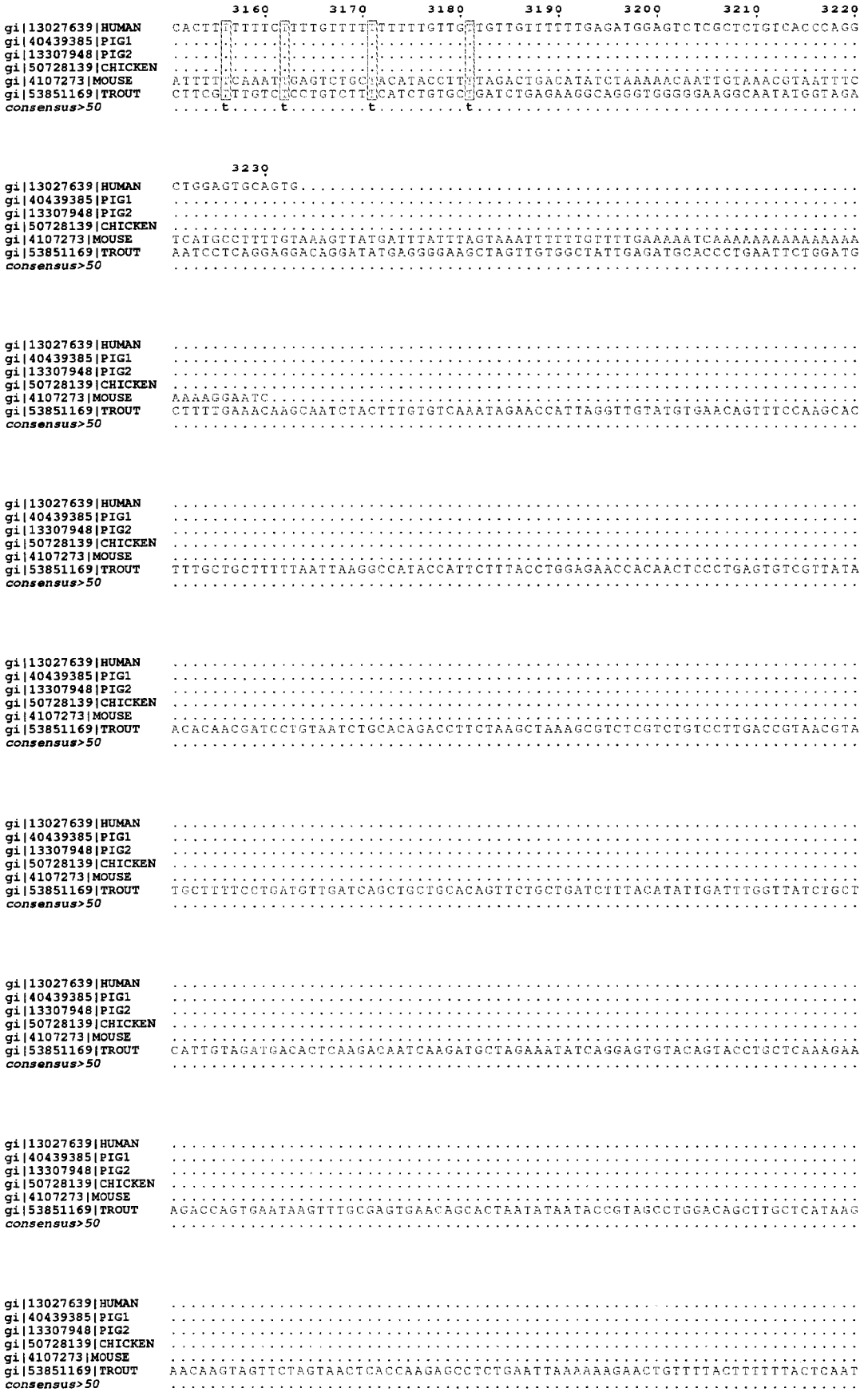


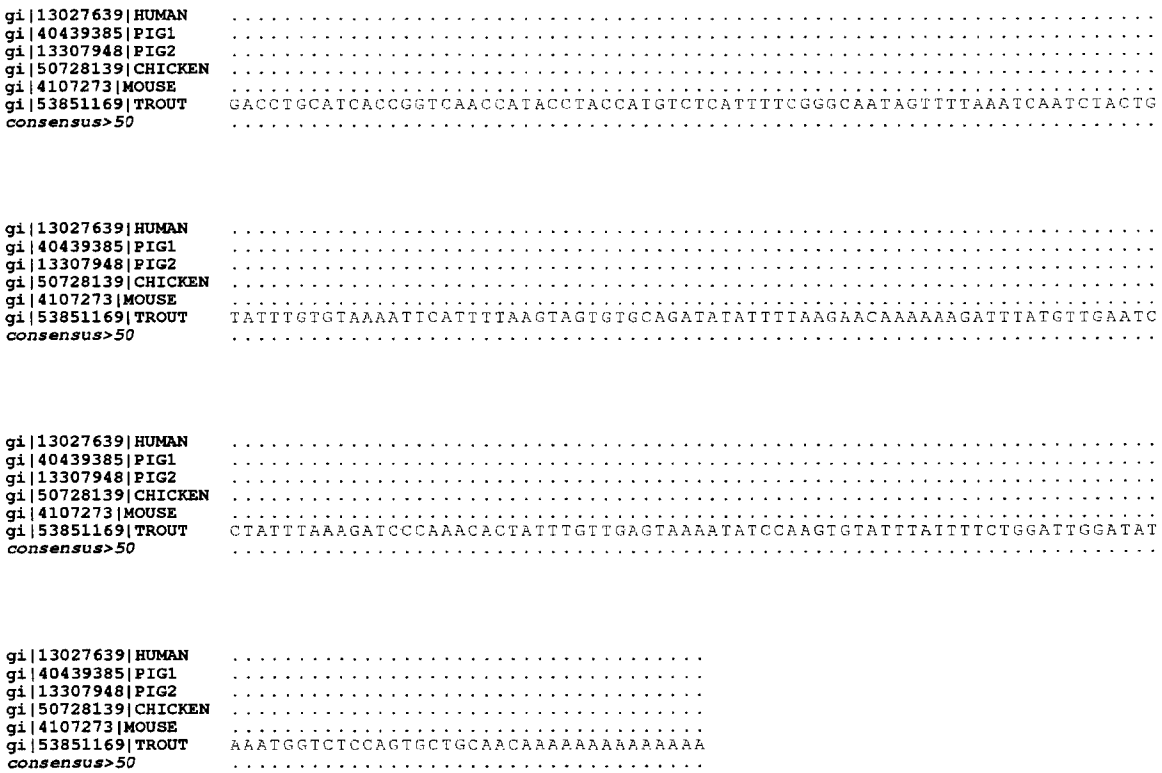










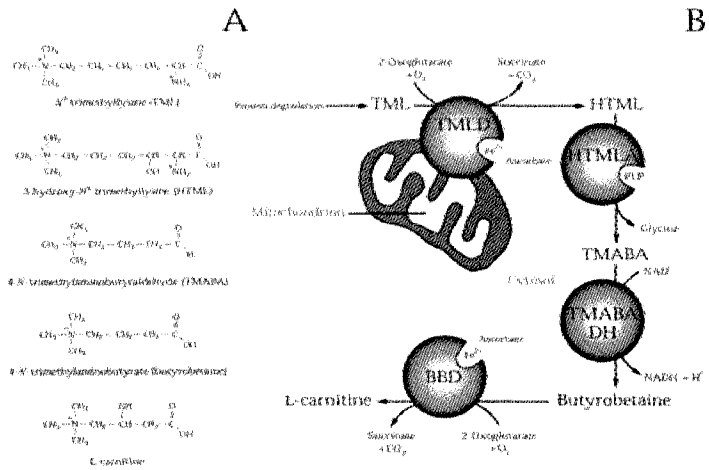


Multiple sequence alignments of protein sequences generated from different animal, bird and fish gene profiles for AASS. Letters in red text are highly conserved (≥90%), blue boxes indicate low consensus between sequences (≥50%). The consensus sequence is given on the bottom line. Sequences (human (353, 392, 398), pig (143, 463), chicken (predicted sequence based upon an annotated genomic sequence for chicken (369, 370, 452), mouse (353), and trout (205)) were identified by BLAST analysis and were aligned using MultAlin online software (95).

8.2 Additional Details on Carnitine Metabolism

Carnitine synthesis is a major function of endogenous protein-bound lysine. Highlighted below (Figure 8.4) are the main metabolites and also the synthetic pathway for synthesis of carnitine in mammals.

Figure 8.4 Carnitine Synthesis in Man

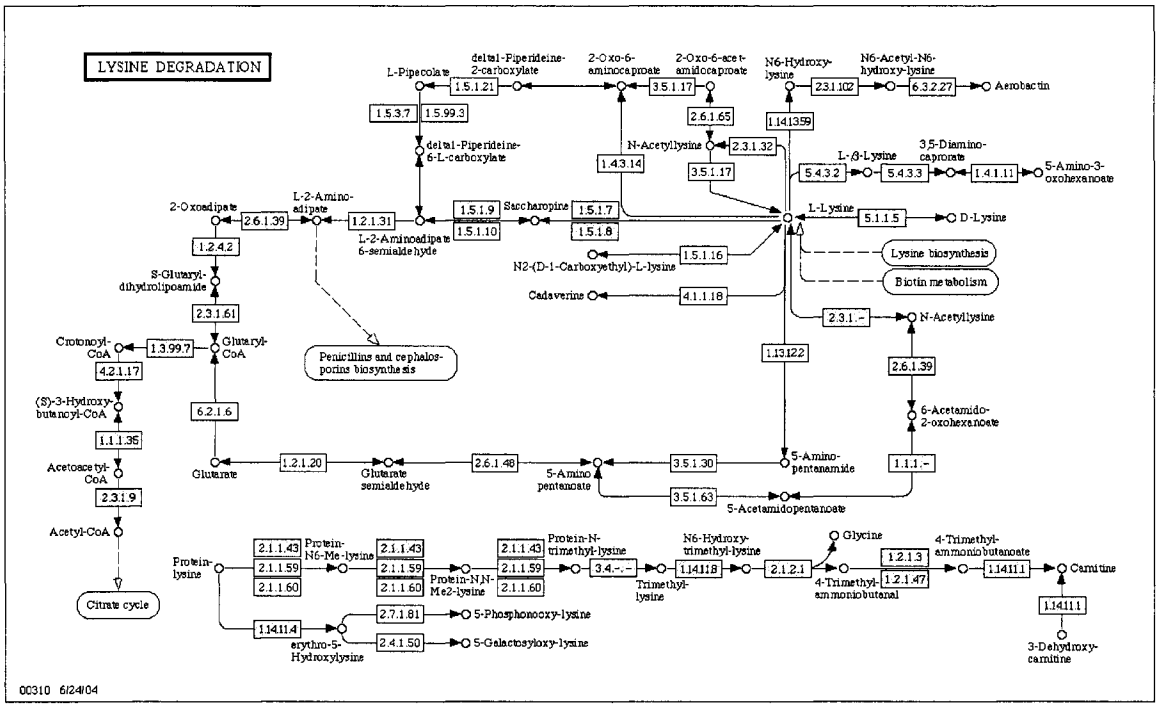


(A) The chemical structures of the five carnitine biosynthesis metabolites. **(B)** Carnitine biosynthesis from TML. After release of TML by lysosomal protein degradation, this compound is hydroxylated by TMLD, after which the resulting HTML is cleaved by a specific aldolase, which uses pyridoxal 5-phosphate (PLP) as a cofactor, into TMABA and glycine. Subsequently, TMABA is oxidized by TMABA-DH to form 4-N-trimethylaminobutyrate (butyrobetaine). In the last step, butyrobetaine is hydroxylated by BBD, yielding L-carnitine. (Figure taken from (477)).

8.3 Expanded View of the Lysine Catabolic Pathway

Presented in the following diagram: Lysine degradation in Homo sapiens as generated by the KEGG PATHWAY (243); the database is a collection of graphical diagrams (KEGG pathway maps) representing molecular interaction networks in various cellular processes. Each reference pathway is manually drawn and updated with the notation shown below. Organism-specific pathways (green-colored pathways) are computationally generated based on the KO assignment in individual genomes. Green in this case refers to Homo sapiens. Thus, in Figure 8.4, green blocks indicate steps known to occur in the human pathway.

Figure 8.5 Detailed View of Complete Lysine Catabolism



For access to clickable areas please see:
http://kegg.com/dbget-bin/get_pathway?org_name=hsa&mapno=00310

8.4 Methodology and Experimental Additions

8.4.1 Isolation of Intestinal mucosal epithelial cells

Reference:

1. Watford, M., Lund, P. and Krebs, H.A. (1979) Isolation and metabolic characteristics of rat and chicken enterocytes. *Biochemical Journal* **178**:589-596.
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The isolation procedure is basically a combination of Watford's method. Wu has used this method often with great success.

Procedure

1. A section of small intestine (60 cm) is obtained and transported to the lab in ice cold phosphate buffered saline with protease inhibitor cocktail added (PBS 137mM NaCl / 2.7mM KCl / 4.3 mM Na_2HPO_4 / 1.4 mM KH_2PO_4).

2. The section is rinsed (1X) with fresh cold PBS to remove any remnants of excreta.

3. The section is then rinsed (3X 100mL) with fresh oxygenated (19:1 O_2:CO_2)Krebs-Henseleit Ca^{2+}-Free buffer (121mM NaCl, 4.7mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 25.2mM $NaHCO_3$) containing 20 mM Hepes (pH 7.4), 5 mM EDTA, 20 mM DL-glucose, 2.1 mM lactate and 0.3 mM pyruvate (KHB buffer).

4. The section of small intestine is then filled with 100 mLs of pre-warmed (37°C) oxygenated KHB (Ca^{2+}-Free), closed with clamps and immersed in 400mls of the same buffer.

5. This system is then shaken for 20 minutes at 37°C after which time the contents are collected into a cooled beaker. The contents are then centrifuged at 400 *g* for 3 minutes at 4°C. These cells are washed 3 times in oxygenated KHB buffer (pH 7.4) containing 20 mM Hepes, 2.5 mM $CaCl_2$ but no EDTA.

6. The section of small intestine is refilled with 100 mLs of KHB buffer and shaken for another 20 minutes. This process releases more enterocytes. Collect the cells and wash as described in (5). Repeat for another 20 minutes.

7. Assess viability of the cells by Trypan blue exclusion.

8.4.2 Isolation of Mitochondria

References:

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2. Myers, D.K. and Slater, E.C. The enzymatic hydrolysis of adenosine triphosphate by liver mitochondria. *Biochemical Journal* **67**:558-572, 1957.
3. Hutzler, Joel and Dancis, Joseph Lysine-ketoglutarate reductase in human tissues. *Biochim.Biophys.Acta.* **377**:42-51,1975.

Protocol

1. Transport liver to lab on ice ~ 40 minutes from slaughter to lab.
2. Weigh a portion of liver and homogenize in 5 mLs ice cold 0.3M mannitol/1mM EDTA per gram liver in the VirTis homogenizer. Homogenize approximately 1 minute.
3. Centrifuge homogenate for 5 minutes at 800×g in Beckman refrigerated centrifuge, fixed angle rotor, 4°C.
4. Decant the supernatant carefully and centrifuge (the supernatant) for 10 minutes at 6000-7000×g, 4°C.
5. The pellet will contain the mitochondria and is resuspended in the homogenization buffer (mannitol/EDTA). Centrifuge the resuspended mitochondria at 18000×g, 10 minutes and at 4°C.
6. The fluffy layer is carefully removed from the firmly packed layer of mitochondria. The pellet is resuspended in the minimum amount of 0.3M mannitol.
7. The total protein content of mitochondrial suspension is determined using the bicinchonic acid kit for protein determination. (Sigma BCA-1).

** Mitochondria are isolated from kidney and intestine in a similar manner.

Use the activity of GDH as a positive marker of mitochondrial purity.

8.4.3 Microplate Assay for Lysine α-Ketoglutarate Reductase

Reference: Scislowski, P.W.D., Foster, A.D. Fuller, M.F. (1994) Regulation of oxidative degradation of L-lysine in rat liver mitochondria. *Biochemical Journal* **300**:887-891.

Microplate Assay Assay Medium	Stock Solution	Final Conc. in Assay	Comments
HEPES buffer, pH 7.8	1M	100 mM	light yellow in colour
α-Ketoglutarate	75 mM	7.5 mM	a.k.a. 2-oxoglutaric acid
NADPH (keep in ice)	1.875 mM	0.125 mM	Make just prior to use
L-Lysine in pig	500mM	10 mM	nb [Lys] >inhibit the enzyme

Microplate Assay

1. Porcine mitochondria are prepared as described elsewhere.
2. Solubilize mitochondria in 300 mM mannitol containing 0.2% (v/v) Nonidet P-40 to a final concentration of 3.0 mg/mL protein. Keep on ice until use.
3. Combine HEPES buffer (2.0 mLs), α-Ketoglutarate (2.0 mLs), L-lysine (400 µL) and add H_2O (5.6 mLs) to final volume of 10.0 mLs. This is the **WORKING ASSAY BUFFER**, keep on ice.
4. Mix 150 µL **WORKING ASSAY BUFFER** with 130 µL solubilized mitochondria protein per well.
5. Add 20 µL of NADPH solution to each well using a multi channel pipette.
6. Measure disappearance of NADPH at an absorbance of 340nm for 10 - 25 minutes. Spectrophotometer is preset to 30°C.

MICROPLATE ASSAY IN BRIEF

150 µL WORKING ASSAY BUFFER
130 µL protein suspension
20 µL NADPH solution
300 µL total volume

Slope is given as ΔmilliAbs / min in plate reader

Calculation

$$\text{Specific Activity} = \frac{\text{Total Assay volume}}{\varepsilon_{NAD(P)H} \times \text{path length} \times \text{volume}_{\text{protein suspension}} \times [\text{protein}]} \times \frac{\Delta\text{Absorbance}}{\text{time}}$$

$$\text{e.g.} = \frac{0.300\ \text{mL}}{6.22\text{mM}^{-1}\cdot\text{cm}^{-1} \times 0.638\text{cm} \times 130\ \mu\text{L} \times 3.0\ \text{mg/mL}} \times \frac{\Delta\text{Abs}}{\text{min}} \div 1000 \times 1000$$

e.g. = 0.19384 × slope value nmol/min/mg protein

8.4.4 Microplate Assay for Saccharopine Dehydrogenase

Reference: Scislowski, P.W.D., Foster, A.D. Fuller, M.F. (1994) Regulation of oxidative degradation of L-lysine in rat liver mitochondria. *Biochemical Journal* **300**:887-891.

Assay Medium	Stock	Final Conc. in Assay	Comments
Tris-HCl, pH 9.4	500 mM	50 mM	
NAD+	20 mM	2.0 mM	
Saccharopine*	30 mM	2.0 mM	*Make in 50 mM Tris-HCl pH 9.4*

*Saccharopine solution - *must be titrated to pH 9.4* before dilution to final concentration

1. Porcine mitochondria are prepared as described elsewhere.

2. Solubilize mitochondria in 300 mM mannitol containing 0.2% (v/v) Nonidet P-40 to a final concentration of 3.0 mg/mL protein. Keep on ice until use.

3. Combine Tris-HCl (2.0 mLs) and NAD^+ (2.0 mLs) and add H_2O (6.0 mLs) to final volume of 10.0 mLs. This is the **WORKING ASSAY BUFFER**, keep on ice.

4. Mix 150 µL **WORKING ASSAY BUFFER** with 130 µL solubilized mitochondria protein per well. Protein concentration ~3.0 mg/mL - have to do serial dilution.

5. Add 20 µL of Saccharopine solution to the cuvettes to start the reaction.

6. Measure appearance of NADH at 340nm over time. Spectrophotometer is preset to and maintained at 30°C.

Microplate Assay

150 µL Working Buffer
130 µL protein suspension
20 µL saccharopine stock solution
300 µL total volume

Let reaction run for 15 minutes.
Calculate as for LKR.

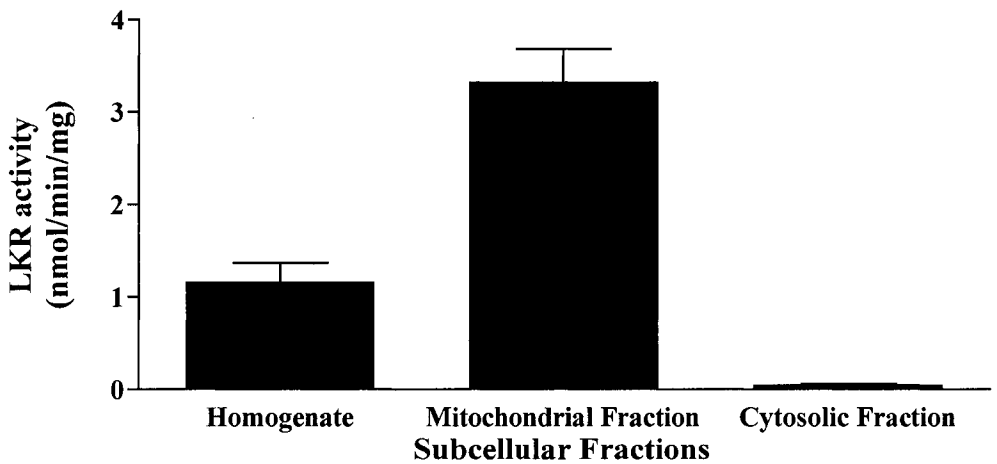
8.4.5 Experimental data additions

Shown below are the data that pertain to specific experimental sections.

8.4.5.1 Subcellular localization of LKR and SDH

Crude experiments were performed using pig liver to determine the subcellular localization of LKR and SDH activities. Based on previous work by Blemings and co-workers (35), we expected the enzyme to be localized to the mitochondrial fraction. In brief, we took liver homogenates, mitochondrial fractions and cytosolic fractions and assayed for LKR activity. The data in Figure 8.6 below led us to conclude that LKR was a mitochondrial enzyme in pig liver.

Figure 8.6 LKR Activity Is Localized to the Mitochondrial Fraction

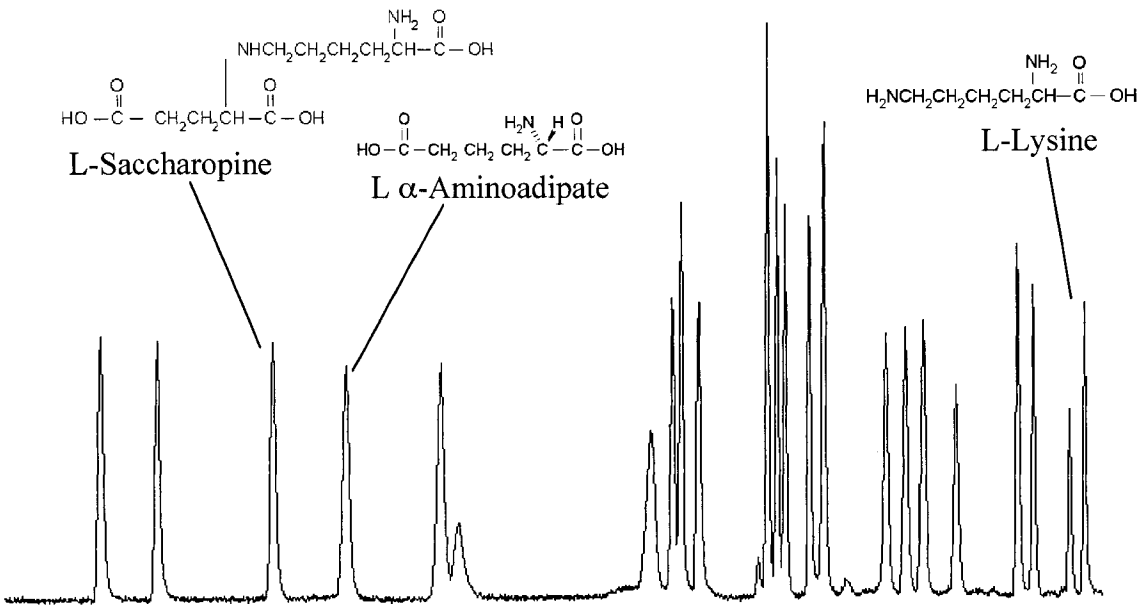


Portions of adult pig livers were homogenized to approximately 25% and from this mitochondrial and cytosolic fractions were prepared as described elsewhere (35). Data are presented as Mean±SEM for n=3 experiments.

8.4.5.2 LKR assay validation

To confirm that we were measuring the conversion of lysine to saccharopine in our assay we incubated solubilized mitochondria lysine as per the normal mitochondrial assay protocol and measured the appearance of saccharopine using HPLC. Figure 8.7 illustrates a typical HPLC chromatogram that was used to evaluate saccharopine production from the standard LKR assay and also plasma levels of these catabolites (Chapter 5).

Figure 8.7 HPLC Chromatogram Showing Amino Acid Profile with Saccharopine and Aminoadipate



Samples were prepared for amino acid analysis as described elsewhere (298, 406). Aminoadipate and saccharopine were run separately to ascertain the retention times and then added to standard amino acid solutions. If sample peaks were questionable, samples were spiked with the amino acid in question and re-run.

Table 8.1 Saccharopine Production by Mitochondria

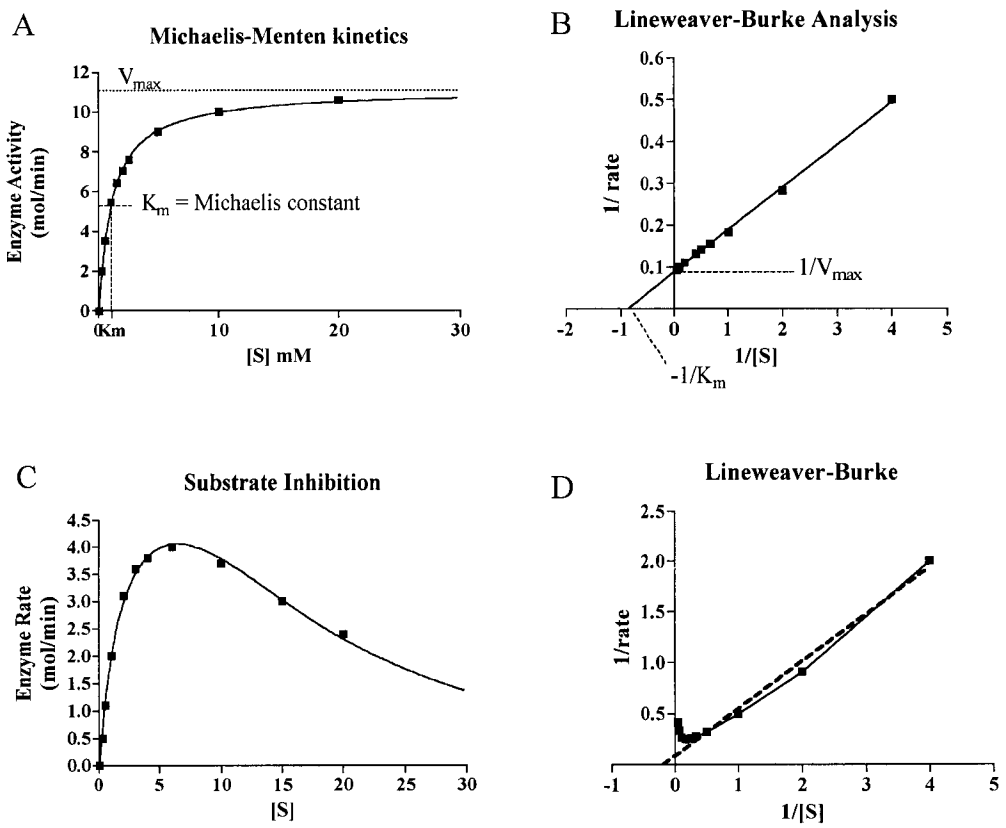
	Liver mitochondria	IEC mitochondria
Rate *nmol/min/mg*	1.23±0.68	0.56±0.39

Data are presented as Mean±SEM for n=2 animals. Samples were analyzed in duplicate and saccharopine quantified by HPLC (406). Appropriate blanks (zero time, protein, substrate) have been subtracted.

8.4.5.3 Review of Enzyme Kinetics

Enzymes are protein catalysts that like all catalysts, speed up the rate of a chemical reaction without being consumed in the processed. Enzyme activity refers to the rate of converting substrate to product (per unit of protein) and the study of this process is called *enzyme kinetics*. To examine the relationship between enzyme kinetics and substrate concentration, the rate of substrate conversion to product (initial velocity) is plotted against increasing substrate concentration [S], Figure 8.8 (A). Maximal velocity (Vmax) is estimated as the tangent to the line of highest activity and Km is the substrate concentration that yields 1/2 maximal activity. To provide a better estimate of V_{max} and K_m, the Lineweaver-Burke derivation (1/rate versus 1/[S]) is calculated. Linear regression of the data points provides an estimate of Vmax as the 1/ Y-axis intercept and –1/ x-axis intercept provides the estimate of Km. The equation for Michaelis Menten kinetics is given as: *velocity* = $(V_{max} \bullet X)/(K_m + X)$ where x = substrate concentration. Substrate inhibition, described by the equation, *velocity* = $(V'_{max} \bullet X/(K'_m+X+X^2/K_{si}))$ where K_{si} represents the point of substrate inhibition is shown in Figure 8.8(C). The Lineweaver-Burke graphical solution is given in Figure 8.8(D).

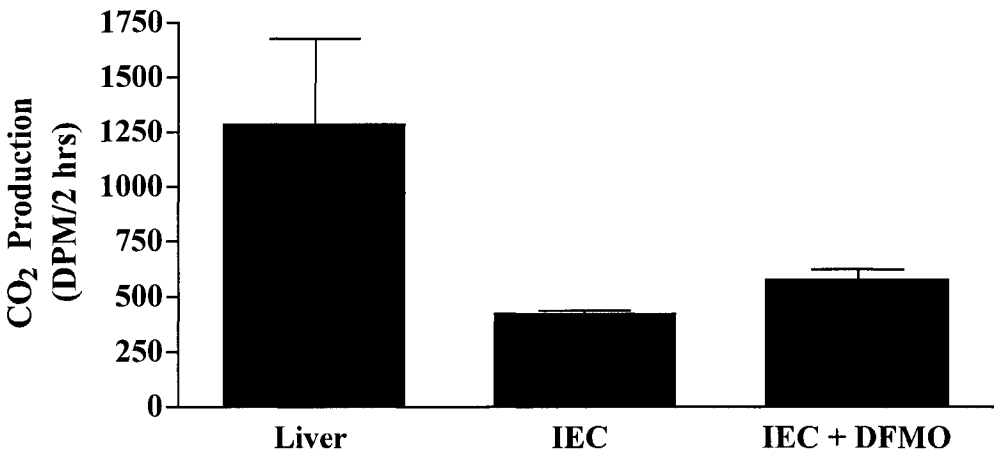
Figure 8.8 Examples of Enzyme kinetics



8.4.5.4 Oxidation of Lysine: $^{14}CO_2$ Production Experiments

To check that lysine oxidation in IEC mitochondria was not due to metabolism via ornithine decarboxylase (ODC) activity we incubated IEC mitochondria in the presence and absence of difluormethylornithine (DFMO), an inhibitor of ODC activity (482).

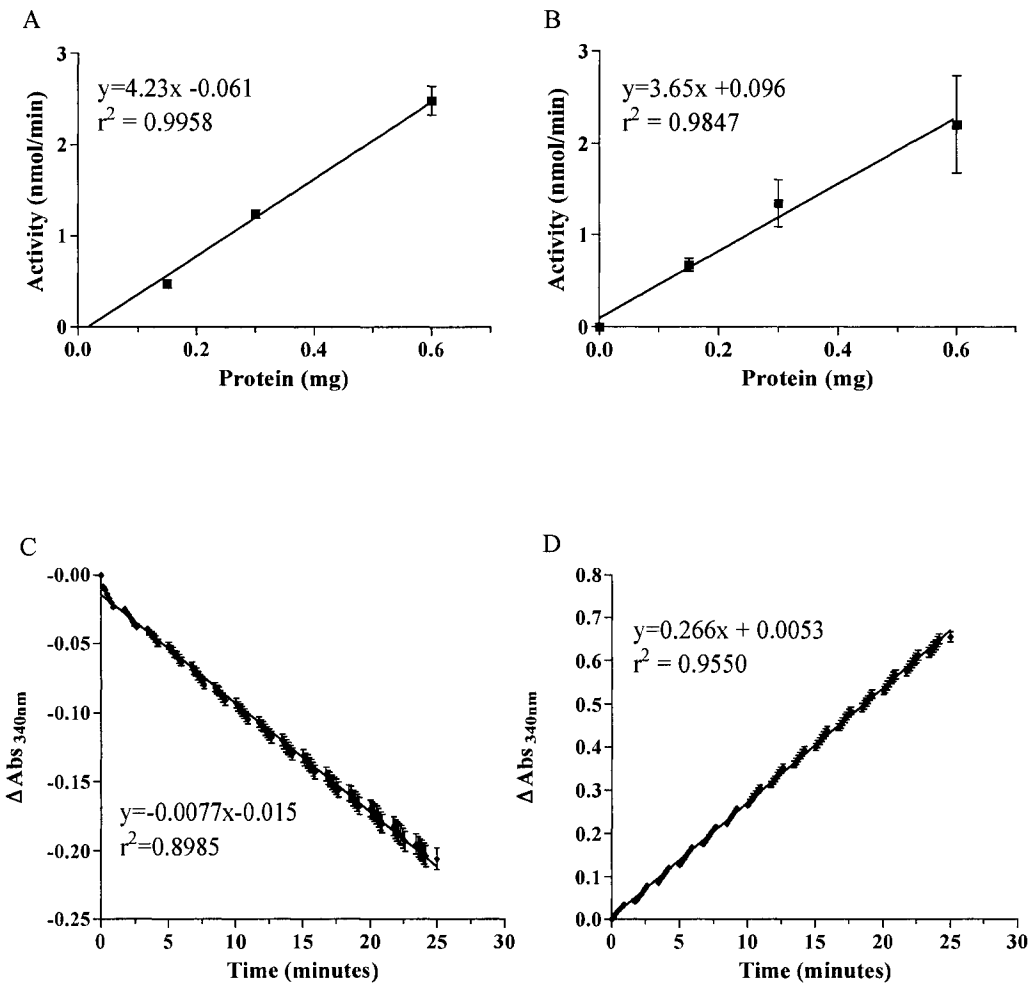
Figure 8.9 Effect of DFMO Addition on CO_2 Production by IEC Mitochondria



8.4.5.5 Linearity Curves: Developmental Study

For each animal studied in the developmental study, an analysis of LKR and SDH activity against time and protein was determined for each tissue. The graphs (Figure 8.9) below are representative of those analyses.

Figure 8.10 Liver LKR & SDH Activity is Linear with Protein Concentration & Time



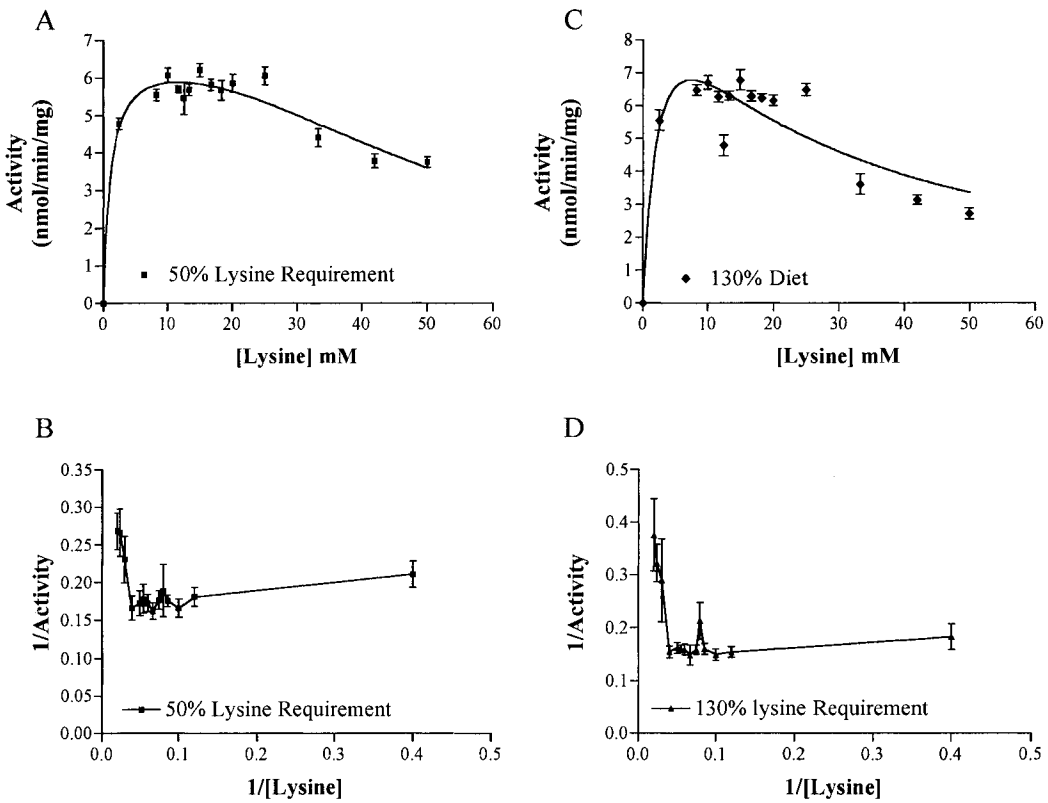
Representative graphs to illustrate enzyme activity linearity with protein content and time elapse. Panel A: LKR; Panel B: SDH; enzyme activity was linear with protein content in the incubation mixture (0.1 - 0.6 mg). Panel C: LKR; Panel D: SDH; rate of enzyme activity illustrated as change in absorbance versus time. Absorbance readings were measured at nine-second intervals in 96 well microplates (~200 measures/well/15 minutes). Data are presented as Mean ± SEM for 5 separate experiments.

8.5 Poultry Studies

8.5.1 Preliminary Studies

Because lysine metabolism is an integral component of poultry nutrition, we characterized the activity of LKR in various tissues of the growing chicken. Cobb 500 pullets were fed synthetic amino acids diets as part of on-going research to define the lysine requirements of poultry (92). At the end of trial, birds (n=2/treatment) were fed either a diet at ~50% of the lysine requirement (0.48% lysine) or at ~130% (1.12% lysine), based upon current NRC data. After 7 days on diet, birds were killed and their livers removed. Mitochondria were prepared and LKR activity was determined as described earlier.

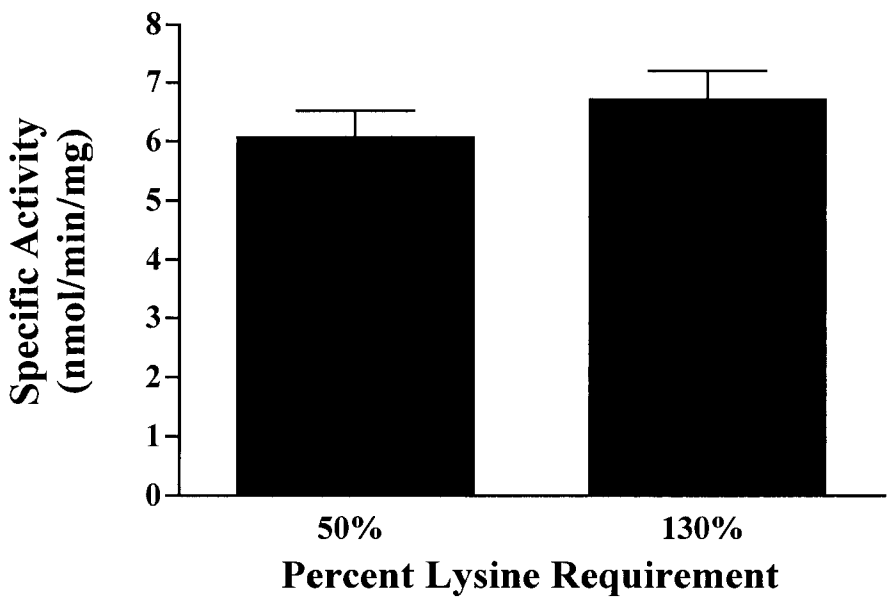
Figure 8.11 Kinetic Analyses of LKR in Cobb 500 Pullets Fed 50% or 130% of Lysine Requirement



Preceding page: LKR enzyme kinetics was investigated in solubilized chicken liver mitochondria using L-lysine concentrations ranging from 0 to 50 mM. Panels A,B illustrate the Michaelis-Menten analysis and the Lineweaver-Burke manipulation respectively for the 50% Diet; panels C,D are similarly arranged for the 130% diet. Data represent Mean ± SEM (n=2; samples were assayed in triplicate) and are fitted to substrate inhibition non-linear regression equations. Kinetic parameters for the analyses included: 50% Lysine diet V_{Max} = 8.56 nmol/min/mg and K_M = 2.02 mM; 130% lysine diet V_{Max} 11.65 nmol/min/mg and K_M = 2.65 mM.

The comparison between the 2 diets revealed no dramatic change in the specific activity of LKR (Figure 8.10), which prompted us to follow up on this line of research (Chapter 5).

Figure 8.12 Effects of Limiting Lysine on LKR Activity in Chicken Liver



LKR activity was determined in solubilized mitochondria prepared from the livers of Cobb 500 poults fed different lysine diets for 7 days. The specific activity was determined using 10 mM lysine as the substrate for LKR. Data presented as Mean±SEM for 2 birds per treatment. Analyses were performed in triplicate.

8.5.2 Analysis of Lysine Diets

An amino acid analysis of the diets fed to broilers is presented in Table 8.2.

Table 8.2 Analyzed Dietary Amino Acids For Broiler Experiment

	Percent Amino Acid (w/w) in Diets Fed to Broilers				
Amino Acid	**0.8%**	**1.0%**	**1.2%**	**1.4%**	**1.6%**
Aspartic Acid	1.36±0.21	1.83±0.17	1.61±0.10	1.68±0.12	1.53±0.06
Glutamic Acid	5.55±0.82	7.12±0.43	6.68±0.6	6.68±0.44	5.88±0.16
Serine	1.28±0.20	1.79±0.20	1.50±0.11	1.56±0.08	1.49±0.03
Histidine	0.42±0.08	0.52±0.05	0.46±0.03	0.50±0.01	0.46±0.02
Glycine	1.39±0.24	1.93±0.26	1.68±0.15	1.76±0.11	1.53±0.10
Threonine	0.90±0.25	1.37±0.11	1.19±0.10	1.26±0.07	1.23±0.06
Arginine	0.87±0.15	1.20±0.12	1.08±0.08	1.16±0.06	1.02±0.04
Alanine	1.74±0.28	2.44±0.26	2.15±0.14	2.27±0.14	2.19±0.09
Tyrosine	0.49±0.10	0.68±0.12	0.60±0.12	0.56±0.11	0.49±0.08
Methionine	0.15±0.00	0.15±0.02	0.17±0.01	0.18±0.01	0.20±0.02
Valine	1.12±0.31	1.77±0.18	1.62±0.12	1.67±0.11	1.59±0.04
Phenylalanine	0.89±0.15	1.25±0.13	1.11±0.08	0.97±0.18	1.10±0.03
Isoleucine	1.02±0.17	1.42±0.13	1.30±0.08	1.37±0.09	1.29±0.04
Leucine	1.96±0.56	3.08±0.31	2.73±0.20	2.86±0.20	2.71±0.09
Lysine	0.82±0.15	1.07±0.08	1.24±0.07	1.35±0.10	1.53±0.03

Data presented as Mean±SEM for 3 replicates of dietary amino acid analyses.

8.5.3 Plasma Amino Acid Profiles of Broilers Fed Different Lysine Diets

During the course of the experiments described in Chapter 5, amino acids were quantified by HPLC to detect changes due to the effects of the different lysine contents of the diets, particularly saccharopine and aminoadipate (Tables 8.2 – 8.6).

Table 8.3 Plasma Amino Acid Profile of Broiler chicks fed 0.8% Lysine for 10 Days

	Plasma Amino Acid Values at			
Amino Acids	**Day 0[1]**	**Day 3**	**Day 6**	**Day 10**
Aspartic Acid	59.5 ± 5.3	58.7 ± 9.8	44.2 ± 7.7	51.1 ± 7.0
Glutamic Acid	158.5 ± 28.0	218.1 ± 29.7	227.2 ± 41.9	186.6 ± 25.3
Serine	336.7 ± 39.3	346.3 ± 33.3	428.1 ± 64.4	369.5 ± 42.4
Histidine	89.3 ± 12.0	117.8 ± 13.4	136.4 ± 32.7	114.1 ± 6.7
Glycine	261.7 ± 22.3	337.6 ± 19.6	345.0 ± 36.1	405.6 ± 28.7
Threonine	439.6 ± 79.0^{a}	805.7 ± 97.1^{b}	782.2 ± 78.8^{b}	811.4 ± 76.1^{b}
Arginine	281.2 ± 29.4	327.0 ± 40.4	273.3 ± 22.2	310.8 ± 44.5
Alanine	431.5 ± 96.2	526.2 ± 32.6	510.4 ± 50.9	466.7 ± 56.2
Tyrosine	158.6 ± 59.1	248.0 ± 52.7	297.0 ± 47.4	230.8 ± 40.9
Methionine	67.1 ± 11.9	104.6 ± 16.7	107.2 ± 15.0	95.4 ± 16.1
Valine	178.3 ± 22.4	206.0 ± 28.3	206.8 ± 24.6	202.5 ± 31.0
Phenylalanine	110.1 ± 11.2	149.3 ± 19.3	146.0 ± 16.0	144.1 ± 22.1
Isoleucine	94.1 ± 12.0	113.9 ± 20.1	119.3 ± 22.6	101.0 ± 15.9
Leucine	176.7 ± 32.5	226.9 ± 32.7	260.6 ± 46.9	226.0 ± 32.8
Lysine	70.8 ± 14.1^{a}	193.4 ± 63.9^{b}	217.8 ± 56.9^{b}	92.4 ± 23.3^{a}
Saccharopine	3.7 ± 0.9^{a}	5.4 ± 0.7^{b}	4.8 ± 0.3^{c}	3.9 ± 0.6^{a}
Aminoadipic acid	2.2 ± 1.0^{a}	8.5 ± 1.8^{b}	10.0 ± 2.1^{c}	5.5 ± 0.6^{d}

[1]Data are presented as Mean±SEM for n = 5 –10 birds. Samples were analyzed in duplicate and significant differences ($p<0.05$) are denoted with different letters.

Table 8.4 Plasma Amino Acid Profile of Broiler chicks fed 1.0% Lysine for 10 Days

	Plasma Amino Acid Values at			
Amino Acids	**Day 0[1]**	**Day 3**	**Day 6**	**Day 10**
Aspartic Acid	70.7 ± 12.1	56.9 ± 7.9	63.2 ± 7.6	59.6 ± 6.8
Glutamic Acid	114.5 ± 10.0	144.5 ± 11.1	143.2 ± 10.0	136.4 ± 10.4
Serine	232.7 ± 57.9	248.3 ± 21.7	245.2 ± 15.7	276.5 ± 24.9
Histidine	87.4 ± 18.9	111.7 ± 20.0	97.1 ± 9.8	104.8 ± 14.1
Glycine	306.8 ± 76.5	335.8 ± 90.3	251.3 ± 32.1	294.1 ± 52.5
Threonine	293.4 ± 52.2^{a}	613.9 ± 103.1^{b}	594.9 ± 82.1^{b}	640.8 ± 100.0^{b}
Arginine	191.0 ± 24.5	175.9 ± 12.7	184.3 ± 12.6	162.4 ± 10.9
Alanine	328.5 ± 39.3	326.2 ± 41.4	281.6 ± 26.2	294.5 ± 31.9
Tyrosine	72.3 ± 14.8	166.3 ± 25.5	183.2 ± 25.6	185.8 ± 23.6
Methionine	49.7 ± 5.3	87.8 ± 6.7	74.4 ± 6.6	83.6 ± 7.0
Valine	148.3 ± 30.0	144.0 ± 7.8	131.0 ± 7.5	126.6 ± 4.7
Phenylalanine	93.4 ± 15.3	99.8 ± 3.9	98.7 ± 5.1	93.2 ± 3.5
Isoleucine	74.7 ± 15.1	78.1 ± 4.7	71.9 ± 4.0	70.9 ± 4.4
Leucine	112.3 ± 21.9	212.6 ± 18.7	221.0 ± 19.8	194.6 ± 24.3
Lysine	44.3 ± 4.9^{a}	118.5 ± 24.4^{b}	119.3 ± 15.9^{b}	117.4 ± 30.5^{b}
Saccharopine	2.4 ± 0.8^{a}	3.5 ± 0.3^{b}	4.1 ± 0.3^{c}	2.9 ± 0.2^{a}
Aminoadipic acid	3.8 ± 0.3^{a}	5.5 ± 0.5^{ab}	6.3 ± 0.5^{b}	3.7 ± 0.5^{c}

[1]Data are presented as Mean±SEM for n = 5 –10 birds. Samples were analyzed in duplicate and significant differences ($p<0.05$) are denoted with different letters.

Table 8.5 Plasma Amino Acid Profile of Broiler chicks fed 1.2% Lysine for 10 Days

	Plasma Amino Acid Values at			
Amino Acids	**Day 0[1]**	**Day 3**	**Day 6**	**Day 10**
Aspartic Acid	59.6 ± 8.3	31.9 ± 7.9	25.3 ± 5.8	36.7 ± 2.9
Glutamic Acid	114.0 ± 8.8	130.5 ± 8.4	103.7 ± 6.4	119.4 ± 11.0
Serine	267.3 ± 26.1	257.2 ± 19.6	226.8 ± 21.3	282.5 ± 17.4
Histidine	89.8 ± 35.4	91.9 ± 12.2	106.6 ± 23.6	96.4 ± 11.7
Glycine	330.0 ± 59.8	263.6 ± 21.3	257.8 ± 36.7	258.3 ± 23.0
Threonine	300.4 ± 55.1^{a}	855.2 ± 61.8^{b}	642.3 ± 90.0^{c}	696.2 ± 59.3^{c}
Arginine	191.6 ± 25.2	157.9 ± 18.4	141.5 ± 17.6	204.2 ± 23.6
Alanine	297.3 ± 28.3^{a}	463.9 ± 29.3^{b}	378.2 ± 37.1^{ab}	348.8 ± 34.4^{a}
Tyrosine	68.2 ± 14.7	173.7 ± 11.2	162.8 ± 20.4	158.2 ± 13.3
Methionine	42.2 ± 7.0	86.4 ± 6.1	66.9 ± 6.1	74.8 ± 7.8
Valine	130.6 ± 13.6	137.5 ± 9.6	121.3 ± 14.6	140.9 ± 16.1
Phenylalanine	76.9 ± 8.5	98.0 ± 4.7	88.2 ± 9.2	95.1 ± 8.1
Isoleucine	65.5 ± 8.5	73.5 ± 5.4	64.4 ± 6.1	78.7 ± 9.7
Leucine	109.0 ± 19.5^{a}	222.8 ± 18.5^{ab}	205.6 ± 27.0^{ab}	245.6 ± 37.1^{b}
Lysine	62.9 ± 11.7	88.0 ± 14.88	106.9 ± 20.4	64.9 ± 10.4
Saccharopine	3.4 ± 0.3	3.6 ± 0.1	3.5 ± 0.2	3.5 ± 0.4
Aminoadipic acid	2.5 ± 0.5^{a}	5.6 ± 1.0^{b}	4.7 ± 1.0^{b}	4.7 ± 0.6^{b}

[1]Data are presented as Mean±SEM for n = 5 –10 birds. Samples were analyzed in duplicate and significant differences ($p<0.05$) are denoted with different letters.

Table 8.6 Plasma Amino Acid Profile of Broiler chicks fed 1.4% Lysine for 10 Days

	Plasma Amino Acid Values at			
Amino Acids	**Day 0[1]**	**Day 3**	**Day 6**	**Day 10**
Aspartic Acid	49.5 ± 9.7	40.9 ± 4.7	50.2 ± 6.2	59.6 ± 6.8
Glutamic Acid	111.9 ± 11.5	131.5 ± 20.3	144.1 ± 13.5	136.4 ± 10.4
Serine	260.2 ± 24.8	249.3 ± 16.4	284.2 ± 18.0	276.5 ± 24.9
Histidine	104.4 ± 17.8	124.5 ± 37.1	92.4 ± 14.2	104.8 ± 14.1
Glycine	338.4 ± 57.0	251.6 ± 34.4	287.8 ± 46.2	294.1 ± 52.5
Threonine	385.6 ± 43.1^{a}	591.9 ± 72.7^{b}	594.7 ± 95.9^{b}	640.8 ± 100.0^{b}
Arginine	214.7 ± 22.4	171.1 ± 10.0	149.3 ± 8.2	162.4 ± 10.9
Alanine	294.4 ± 16.6	405.4 ± 32.7	326.2 ± 45.6	294.5 ± 31.9
Tyrosine	49.5 ± 0.2	48.3 ± 1.1	49.2 ± 0.7	47.9 ± 0.6
Methionine	61.7 ± 7.1	85.7 ± 9.2	87.7 ± 6.8	83.6 ± 7.0
Valine	138.4 ± 17.7	118.8 ± 9.9	119.4 ± 6.9	126.6 ± 4.7
Phenylalanine	100.5 ± 14.8	87.1 ± 3.3	86.2 ± 3.8	93.2 ± 3.5
Isoleucine	71.6 ± 8.5	69.7 ± 5.5	67.9 ± 4.2	70.9 ± 4.4
Leucine	150.7 ± 16.4	220.3 ± 20.8	209.0 ± 15.6	194.6 ± 24.3
Lysine	91.4 ± 13.7^{a}	221.9 ± 28.0^{bc}	285.2 ± 35.5^{c}	117.4 ± 30.5^{ab}
Saccharopine	3.0 ± 0.1^{a}	3.4 ± 0.2^{a}	3.9 ± 0.2^{b}	3.2 ± 0.3^{a}
Aminoadipic acid	3.2 ± 0.1^{a}	6.6 ± 0.6^{b}	6.5 ± 0.2^{b}	4.6 ± 0.5^{c}

[1] Data are presented as Mean±SEM for n = 5 –10 birds. Samples were analyzed in duplicate and significant differences ($p<0.05$) are denoted with different letters.

Table 8.7 Plasma Amino Acid Profile of Broiler chicks fed 1.6% Lysine for 10 Days

	Plasma Amino Acid Values at			
Amino Acids	**Day 0**[1]	**Day 3**	**Day 6**	**Day 10**
Aspartic Acid	48.8 ± 10.7	33.9 ± 4.1	34.5 ± 4.8	48.9 ± 9.7
Glutamic Acid	109.5 ± 9.9	131.4 ± 7.3	134.8 ± 8.6	120.8 ± 10.0
Serine	258.2 ± 10.3	285.1 ± 15.2	257.4 ± 9.9	286.6 ± 21.7
Histidine	77.3 ± 10.2	85.1 ± 7.2	83.4 ± 6.5	92.0 ± 8.9
Glycine	326.6 ± 34.7	276.6 ± 26.6	303.2 ± 36.5	300.4 ± 32.1
Threonine	333.8 ± 28.0^{a}	596.6 ± 77.1^{b}	598.8 ± 63.1^{b}	572.2 ± 48.6^{b}
Arginine	191.7 ± 11.2	167.7 ± 21.8	143.3 ± 15.6	172.7 ± 32.6
Alanine	295.1 ± 17.3	379.2 ± 29.7	349.7 ± 19.1	367.0 ± 28.1
Tyrosine	49.2 ± 0.3	49.1 ± 0.5	49.9 ± 0.2	51.6 ± 1.3
Methionine	60.8 ± 10.3	89.7 ± 8.1	78.7 ± 4.6	76.4 ± 7.7
Valine	134.6 ± 8.2	129.7 ± 11.8	114.5 ± 3.1	133.8 ± 18.1
Phenylalanine	92.3 ± 7.1	94.9 ± 7.8	83.7 ± 3.3	98.4 ± 12.6
Isoleucine	63.7 ± 3.9	72.4 ± 6.1	65.9 ± 1.7	71.5 ± 8.8
Leucine	153.8 ± 34.4	222.3 ± 31.4	188.6 ± 15.7	230.3 ± 38.3
Lysine	53.2 ± 8.3^{a}	203.8 ± 60.4^{b}	278.4 ± 68.7^{b}	221.6 ± 72.6^{b}
Saccharopine	3.8 ± 0.7^{a}	3.5 ± 0.1^{a}	3.4 ± 0.1^{a}	2.7 ± 0.3^{b}
Aminoadipic acid	3.8 ± 0.6^{a}	4.7 ± 1.0^{a}	3.9 ± 0.9^{ab}	3.6 ± 0.5^{b}

[1]Data are presented as Mean±SEM for n = 5 –10 birds. Samples were analyzed in duplicate and significant differences ($p<0.05$) are denoted with different letters.

8.5.4 Tissue Localization of LKR/SDH Protein

8.5.4.1 Western Blot Analysis of LKR in Pig Tissues

Samples of mitochondria isolated from tissues of developing pigs were stored at -20°C until analysis. Tissue samples (15μg) were diluted 1:4 in a buffer containing 30% (v/v) glycerol, 10% (w/v) SDS, 10% (v/v) β-mercaptoethanol, in 25 mM Tris-HCl (pH 6.8) and heated for 1 minute in a boiling water bath. Samples were mixed with 0.1% (w/v) bromophenol blue prior to loading.

Electrophoresis was performed on a 10% (w/v) polyacrylamide mini-gel (Protean II, Bio-Rad Laboratories, Mississauga, ON, Canada) for 90-120 min at 100 V. Separated proteins were transferred to a nitrocellulose membrane (overnight, 40 mA), using a semi-dry technique (Hoefer, Multitemp III, Amersham Pharmacia Biotech, Montreal, Canada). Membranes were stained with Ponceau-S (Sigma-Aldrich) to confirm equal loading. They were subsequently destained, blocked in a buffer containing 5% (w/v) skim milk powder, 0.05% (v/v) Tween-20 and TBS followed by incubation with the primary antibody.

Epitope-purified primary polyclonal rabbit anti-AASS (courtesy of Dr. K. Blemings, University of Western Virginia, USA) was applied for 3 hours at room temperature in blocking solution (1:2500). Membranes were washed (2 x 10 minutes) in blocking solution and incubated with the secondary antibody, anti-rabbit IgG (Vector Laboratories, 1:5000 in blocking solution) for 30 min. Membranes were then washed 3 x 5 minutes and finally 1 x 5 minutes with TBS. Immunoreactivity was visualized with the ECL Plus chemiluminescence substrate (horseradish peroxidase immunodetection; Amersham

Pharmacia Biotech). Membranes were incubated for one minute in detection reagents, then drained and sealed in plastic. Membranes were exposed for 1-4 minutes and photographed using the Fuji Film Image Reader (version 1.4E) and Intelligent Dark Box.

Figure 8.13 Tissue Localization of LKR/SDH Enzyme Protein

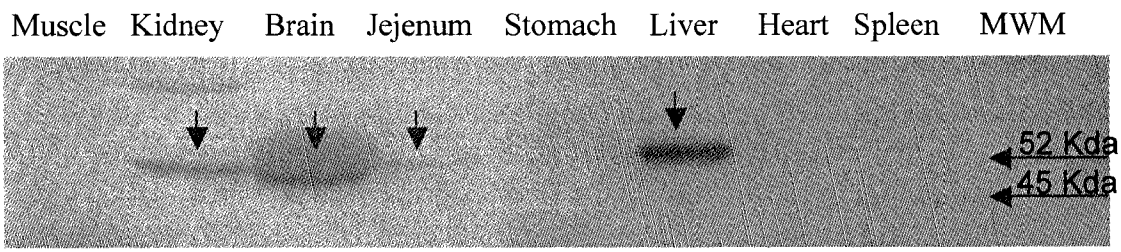


Table 8.8 Tissue Activity of Lysine α-Ketoglutarate Reductase in Developing Pigs

	Day 0		Day 3		Day 7		Day 14		Day 21		Day 28		Day 84	
	nmol/min• tissue	% of Total	nmol/min• tissue	% of Total	nmol/min• tissue	% of Total	nmol/min• tissue	% of Total	nmol/min• tissue	% of Total	nmol/min• tissue	% of Total	nmol/min• tissue	% of Total
Tissue														
Liver	0.84	3.62	3.32	32.3	5.52	45.79	13.58	76.15	9.52	66.68	11.70	57.64	56.89	69.65
Kidney	0.13	0.56	0.12	1.17	0.11	0.91	0.093	0.52	0.148	1.04	0.389	1.92	0.344	0.42
IEC	0.44	1.89	1.23	12.0	1.36	11.28	1.16	6.50	0.45	3.15	3.08	15.17	1.11	1.36
Muscle	19	81.8	5.4	52.5	3.47	28.78	2.96	16.60	4.16	29.14	5.13	25.27	23.2	28.41
Heart	0.04	0.17	0.073	0.71	0.015	0.12	0.04	0.22	ND	---	ND	---	0.13	0.16
Brain	2.78	11.97	0.14	1.36	1.58	13.1								