RESEARCH REPORT
ENVIRONMENT

Title: Utilization of Crystalline Amino Acids by the Gut in Growing Pigs – **NPB # 03-030**

Investigator: Nathalie L. Trottier

Institution: Michigan State University

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Abstract: The objective of this study was to determine if partial replacement of protein-bound AA with crystalline AA (CAA) increases portal blood appearance of AA. Six barrows (30 kg ± 0.5 BW) were assigned to three diets in a repeated Latin Square design. Diets consisted of a 16.9% CP (Control: C), and a 14.5 (Medium: M) and 12.5 % CP (Low: L) containing CAA. The M diet contained L-lys, L-thr, and DL-met, and the L diet contained L-lys, L-thr, DL-met, L-trp, and L-cys to meet true ileal digestible requirements. Feed was given twice daily providing 2.6 times ME required for maintenance. A catheter was placed in the portal vein and blood samples were collected at times (t) -30, 30, 60, 90, 120, 150, 180, 210, and 240 min relative to feeding. Portal lys concentration (µmol/L) at t30 was higher ($P < 0.001$) in pigs fed L (347.38 ± 18.24) and M (296.4 ± 18.38) compared to C (210.32 ± 18.24). At t60, compared to C (268.42 ± 18.24), lys was higher ($P < 0.01$) in pigs fed L (362.01 ±18.23) but similar in pigs fed M (286.35 ±18.36). Post t60, lys did not differ between L, M and C. Portal thr concentration at t30 was higher ($P < 0.05$) in L (311.37 ±17.13) than in M (257.61 ± 17.23) and C (229.09 ±17.13). At t60, portal thr in L (333.08 ±17.13) was higher ($P < 0.05$) compared to M (263.19 ±17.23) and C (284.96 ± 17.13). Portal thr between M and C did not differ at any time point. Portal met concentrations at t30 and t60 were higher ($P<0.05$) in pigs fed L (102.01 ±7.37 and 104.82 ±7.37, respectively) compared to C (72.19 ±7.37 and 85.40 ± 7.37, respectively) and did not differ between M (87.70 ±7.41 and 84.37 ± 7.41, respectively) and C. Portal trp at t30 was similar in L (87.35 ±4.61) compared to C (81.49 ± 4.61) and M (77.02 ±4.64), but at t60 was lower ($P < 0.05$) in M (78.39 ±4.64) compared to C (94.17 ± 4.61). At t60, portal trp was not different between L (88.60 ±4.60) and C. Partial replacement of protein-bound AA with CAA increases AA concentrations in portal blood, implying that CAA are absorbed more rapidly than protein-bound AA even in diets formulated on true ileal digestible basis.

Introduction: Excessive production of nitrogen (N) from livestock production systems has place a strain on the environment. Methods to reduce N losses from farm animals will become necessary to maintain animal production. The **long-term objective** of this project is to determine the feasibility of the use of crystalline amino acids (AA) in diet formulations to reduce N generated by farm animals thereby maintaining animal agriculture as an environmentally conscious industry.

Formulating diets using natural feeds to meet requirements for the first limiting AA results in large excesses of dispensable and indispensable AA. Amino acids fed in excess of requirements are not utilized by the animal: they are deaminated, and the N is voided in the urine in the form of urea. The carbon atoms are either oxidized and voided in the air as CO_2 or used into body fat or glycogen synthesis. Because of the ubiquitous presence of the enzyme urease, urinary urea is degraded rapidly into ammonia, which can de-ionize and volatilize at pH 5 and above. So far, the most effective method to reduce N losses in the urine is via reduction of dietary crude protein (CP) concentration and supplementation of limiting crystalline AA, thereby reducing a large portion of the dispensable and non-limiting indispensable AA. On average, for each one percentage unit reduction in CP, N excretion is reduced by 8.4%. Reduction in CP concentration of 4 unit percentage decreases ammonia emission by also half (Canh et al., 1998). Although low protein-AA supplemented diets ensures that no indispensable AA are limiting or in excess, their ability to optimize growth, feed intake, feed efficiency and carcass composition relative to animals fed diets containing higher protein concentration is inconsistent. Most studies have tested dietary CP reduction of 2 to 4% with crystalline AA inclusion and reported not reduction in growth performances and carcass traits of growing and finishing pigs (Boisen et al., 1991; Schoenherr and Schmidt, 1991; Spiekers et al., 1991; Lopez et al., 1994; Kerr et al., 1995; Maxwell, 1995; Tuitoek et al., 1997ab; Knowles et al., 1998; Friesen et al., 1999; Liu et al., 1999). We have recently shown that reduction of dietary CP of 6 percentage units minimizes N losses in urine and ammonia emission (Otto et al., 2003ab). However, we and others have shown that when dietary CP concentration is reduced by more than 4 percentage units, N retention decreases (Pierce et al., 1994; Kendall et al., 1999; Zervas and Zijlstra, 2000; Otto et al., 2003a; Pérez Laspiur et al., 2002), and feed efficiency and average daily gain are reduced (Tuitoek et al., 1997; Liu et al., 1999).

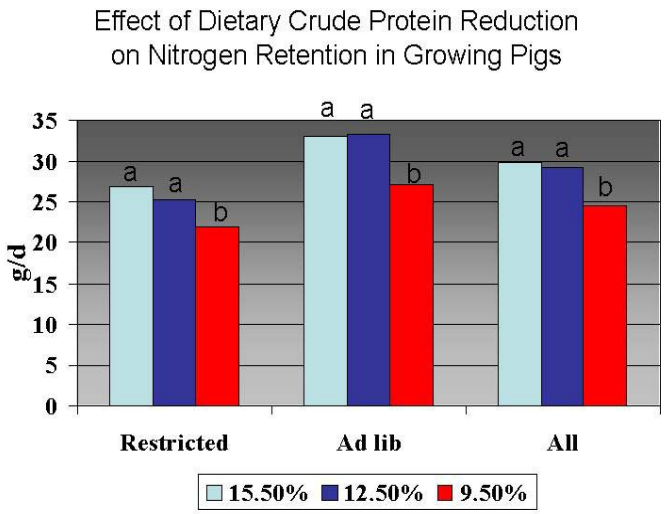
The reasons for a decrease in performance responses have not been investigated in the growing pig. Crystalline AA are rapidly absorbed across the gastro-intestinal tract and available for metabolic purposes (Leibholz et al., 1986; Sato et al., 1987; Izquierdo et al., 1988; Buraczewska and Swiech, 2000). On the other hand, protein-bound AA are released in the gastro-intestinal lumen following digestive processes, at which rate varies among feed ingredients (Melnick et al., 1946, Rolls et al., 1972). The differential absorption rate of crystalline and bound-AA may decrease AA utilization for whole body protein synthesis (Yen et al., 1991) and for gut tissue. This 'imbalance' could lead to the oxidation of the rapidly absorbed crystalline AA(s) as well as the later absorbed AA(s) derived from intact proteins (Buraczewska et al., 1980; Batterham and Bayley, 1989; Rerat, 1995). In these studies, however, inclusion levels of crystalline AA were lower than currently recommended (NRC, 1998). We have recently found (see earlier related research section) that pigs fed diets containing low CP concentration and crystalline AA have a lower protein to RNA ratio in intestinal mucosa tissue, indicating lower cellular activity in that tissue. Those results, combined with other research demonstrating an altered post-absorptive amino acid profile in low-protein AA supplemented diets, led us to formulate the current hypothesis for this proposed work. **We hypothesized that AA of synthetic origin (crystalline) are rapidly absorbed across the intestinal wall compared to those of protein-bound**

origin, and are not available to gut mucosa tissue for normal protein synthetic functions. We acknowledge that the alteration in post-gut amino acid profile may be an additional mechanism by which whole body protein accretion, as measured by N retention, is decreased.

2. Earlier related research by PI

Funded by National Pork Producers Council in 1997, 1999, and 2002, we have worked in the area of dietary crude protein reduction and growth performance of pigs, N excretion, ammonia and odor emission, and gut metabolism. We have shown that dietary protein concentration can be reduced by 3 percentage units in 50-kg growing pigs (from 15 to 12% protein), without compromising N retention, thus providing approximately 20% reduction in total N output, the majority being of urinary origin; further reduction to 6 percentage unit (from 15 to 9% CP) decreases N retention (**see Figure 1**) (Otto, E. R., M. Yokoyama, P. K. Ku, N. K. Ames, and N. L. Trottier. 2003. Nitrogen balance and amino acid digestibility in growing pigs fed diets reduced in protein concentration. *Journal of Animal Science 81:1743-1753*). Our work also demonstrated that reducing dietary protein from 15 to 9% and providing synthetic amino acids to meet digestible amino acid requirements minimized NH_3 emission from swine manure but did not improve odor quality (Otto, E. R., M. Yokoyama, R. D. von Bernuth, T. van Kempen, and N. L. Trottier. 2003. Ammonia, volatile fatty acids, phenolics and odor offensiveness in manure from growing pigs fed diets reduced in protein concentration. *Journal of Animal Science 81:1754-1763)*. Recent preliminary data also shows that urine pH from pigs fed 9% protein compared to 12 or 15% protein diets is reduced to close to 5, pH at which ammonia volatilization is prevented (Presented at the 2002 American Society of Animal Science Midwest Meetings, Des Moines, Iowa: Pérez Laspiur, J., C. Wickens, L. Recker, J. Moore, P. K. Ku, and N. L. Trottier. 2002. Effect of crude protein reduction and dietary fiber on nitrogen retention and excretion in the growing pig. J. Anim. Sci. 80(Suppl. 1):131(Abstr.). Recently, the PI has found that pigs fed diets containing low CP concentration and crystalline AA have a lower protein to RNA ratio in intestinal mucosa tissue, indicating lower cellular activity (**see Figure 2, manuscript submitted to The Journal of Nutrition**).

Figure 1

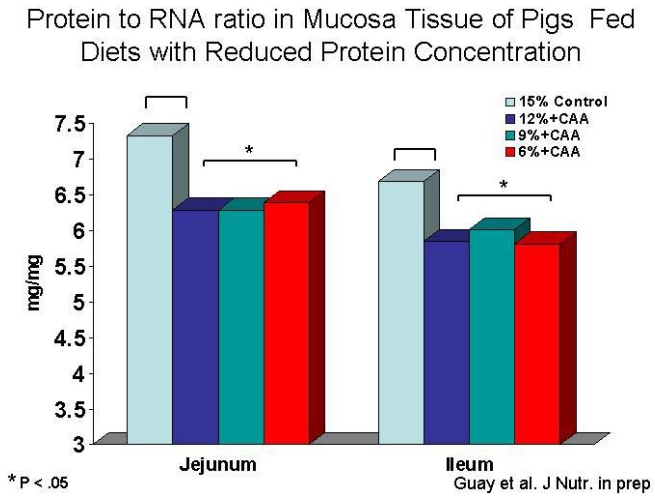


Project objective:

Overall research question: Is AA utilization by the gut lower in pigs receiving low CP diet with crystalline AA supplementation compared to that in pigs receiving diet containing intact proteins?

Specific aim: To determine if AA from synthetic origin are absorbed more rapidly than AA from intact protein sources in growing pigs.

Figure 2



Materials & Methods:

The Michigan State University All-University Committee on Animal Use and Care and the USDA Meat Research Center approved the use of the animals in this study.

Animals and Surgery

Animals. A total of six barrows (Yorkshire X Landrace) with an initial average BW of 30 kg ± 0.5, were housed individually in metabolism cages in two rooms with three cages per room, at a controlled temperature of 22º C. The metabolism cages were 81 x 46 x 122 cm in size and were equipped with woven-wire flooring and automatic nipple drinker. Cages were connected to individual 1.2 x 1.2 m pens to allow the animal free movement during periods between feeding and sample collection. Animals were offered feed twice daily at 0800 and 2000 and were trained to consume the entire meal within 15 minutes. Pigs were weighed weekly and the amount of feed offered calculated to meet 2.6 times the metabolizable energy required for metabolic body size (110 x $BW^{.75}$).

Surgery. Prior to surgery, animals were deprived of feed for approximately 24 hours. For surgery, anesthesia was initiated and maintained with isoflurane at a dose of 2%. Catheters were placed in the portal and ileal veins according to the procedure of Yen and Killefer (1987) and Yen (1991). The catheters were exteriorized dorsal and anterior to the abdominal incision and maintained in place with sutures and a bandage body suit. Pigs were allowed to recover in their pen and feed intake and body temperature were monitored daily. Pigs received antibiotics intramuscularly for 3 days, once a day (Excenel).

Experimental Design and Diets

Following surgical recovery (approximately five days) animals were allocated to three diets according to a repeated Latin Square arrangement of treatments. The diets consisted of a Control containing 16.9% CP, a Medium CP diet containing 14.5% CP and a Low CP diet containing12.5% CP (analyzed, as-fed). The Medium CP diet contained crystalline L- lysine•HCL, DL- methionine and L-threonine. The Low CP diet contained crystalline L-lysine•HCL, DL-methionine, L-threonine, L-tryptophan and L-cysteine. Crystalline amino acids were added to meet the true digestible ileal amino

acid requirement for the growing pig (NRC, 1998). Ingredient and nutrient composition of diets are given in Table 1.

Blood Sampling Protocol

Blood samples (5 mL) were collected from the portal catheter every 30 minutes, beginning 30 minutes prior to feeding, for a total of 4 hours. To maintain blood volume during the blood collection period, a saline solution (.9% NaCl) was infused through the ileal vein catheter with the use of a peristaltic pump to maintain blood volume. Samples were centrifuged within approximately 10 minutes from collection, at 4º C and 3, 300 x *g* for 10 minutes. Plasma was removed and frozen at -20º C.

Sample Analysis

Plasma. Samples were thawed at room temperature. Amino acids were analyzed by reverse-phase high pressure liquid chromatography (HPLC) (Alliance 2690, Waters Corp., Mildford, MA). Plasma glucose was analyzed using a colorimetric method according to the manufacturer recommendations (Wako Chemicals USA, Inc. Richmond, VA). Control samples (Accumark Controls, Sigma Diagnostics, St.Louis, MO) were included to each plate analyzed, and absorbance read at 505 nm (Spectra MAX 340, Molecular Devices Corporation, Sunnyvale, CA).

Diets. Feed ingredients were mixed using a horizontal ribbon mixer. A basal diet was prepared to which the ingredients for each of the three respective experimental diets were added. The Control diet was prepared first, followed by the Medium and Low CP diet. Feed samples were finely ground (Cyclotec 1093 Sample Mill, Foss Tecator, Hoeganaes, Sweden). Feed nitrogen was determined using a Carbon Hydrogen Nitrogen gas analyzer (Fp 2000, Laboratory Equipments Corporation, St. Joseph, MI) using EDTA as a calibration standard and urea as a control. Samples of feed were hydrolyzed in 6 *N* HCl at 110°C for 24 h before analysis by reverse-phase HPLC. Norleucine was added as an internal standard prior to hydrolysis. Determination of tryptophan and sulfur amino acid concentrations was performed at the Missouri Agricultural Experiment Station Chemical Laboratories (University of Missouri-Columbia). Briefly, tryptophan concentration was determined by colorimetric assay following enzymatic digestion and sulfur amino acids were determined according to AOAC (2000).

Statistical Analysis

Data were analyzed using the Mixed Procedure of SAS/STAT (Version 6.12, SAS Institute Inc., Cary, NC). Individual pig was considered as the experimental unit and sampling time as the repeated effect. The model included the random effect of pig(square) and fixed effects of square, diet, sampling time, sampling day, and interaction between time and diet. Differences in least squares means were considered to be significant at $P < 0.05$ or to tend to significance at $P < 0.1$.

Table 1. Ingredient and nutrient composition of experimental diets (as-fed basis)

Item	Dietary protein %		
	16.9 Control	14.5 Medium	12.5 Low
Ingredient, %			
Corn, dent yellow	71.26	71.27	71.27
Soybean meal, solvent	23.78	18.62	14.42
Corn starch	0.56	5.45	9.38
Corn oil	1.80	1.80	1.80
Dicalcium phosphate	1.15	1.15	1.15
Limestone	0.72	0.72	0.72
Salt, NaCl	0.24	0.24	0.24
MSU Vit. Premix	0.24	0.24	0.24
MSU Min. Premix	0.24	0.24	0.24
HCL·L-Lysine	-	0.14	0.25
L-Threonine	-	0.05	0.12
DL-Methionine	-	0.04	0.07
L-Tryptophan	-	-	0.02
L-Cysteine	-	-	0.01
Nutrient (calculated)			
DE, kcal/kg	3522.18	3537.70	3548.12
ME, kcal/kg	3367.24	3398.02	3420.8
Ca, %	0.61	0.59	0.58
Pt, %	0.58	0.70	0.79
NDF, %	10.0	9.32	8.76
Fat, %	4.94	4.87	4.82

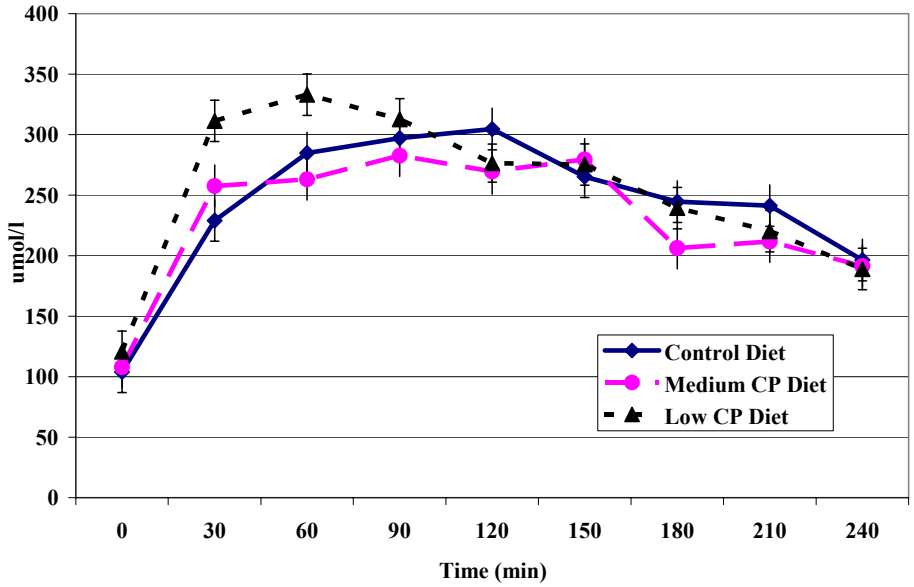
Table 2. Amino Acid Composition of Experimental Diets (%)

Item	Dietary Protein, %		
	16.9 Control	14.5 Medium	12.5 Low
Total indispensable, % analyzed			
Histidine	0.57	0.49	0.43
Isoleucine	0.83	0.69	0.60
Leucine	1.82	1.60	1.45
Lysine	0.94	0.88	0.85
Methionine	0.31	0.30	0.29
Cysteine	0.32	0.27	0.26
Phenylalanine	0.93	0.78	0.69
Threonine	0.64	0.57	0.54
Tryptophan	0.18	0.15	0.15
Valine	0.91	0.77	0.67
True digestible indispendable, % based on calculated			
Arginine	1.00	0.83	0.70
Histidine	0.42	0.36	0.31
Isoleucine	0.63	0.53	0.45
Leucine	1.42	1.25	1.11
Lysine	0.78	0.79	0.78
Methionine	0.25	0.26	0.27
Cysteine	0.26	0.23	0.22
Phenylalanine	0.75	0.64	0.55
Tyrosine	0.55	0.46	0.39
Threonine	0.54	0.51	0.52
Tryptophan	0.17	0.14	0.14
Valine	0.71	0.61	0.52

Results: Mean portal concentration of amino acids in pigs following feeding reduced crude protein-diets are shown in figures 3 to 7 for lysine, threonine, methionine, tryptophan and cysteine, respectively. Portal lysine concentration (µmol/L) at time 30 minutes following feeding was higher ($P < 0.001$) in pigs fed Low CP diet (347.38 ± 18.24) and Medium CP diet (296.4 ± 18.38) compared to that of pigs fed the Control diet (210.32 ± 18.24). At time 60 minutes, compared to Control diet (268.42 ± 18.24), lysine concentration was higher ($P < 0.01$) in pigs fed Low CP diet (362.01 ±18.23), but similar in pigs fed Medium CP diet (286.35 ±18.36). After time 60, lysine did not differ between Low, Medium and Control.

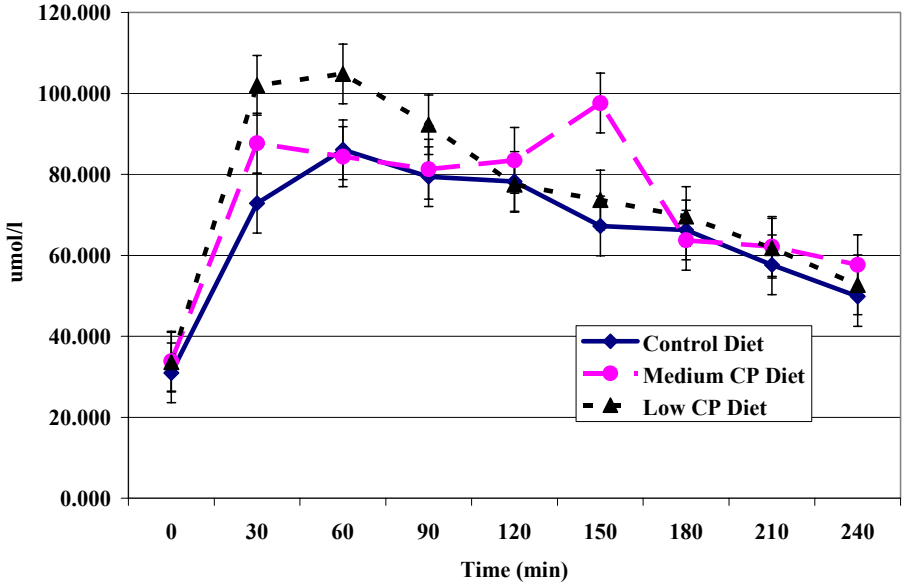
Portal threonine concentration at time 30 minutes was higher ($P< 0.05$) in pigs fed Low CP diet (311.37 ±17.13) than that in pigs fed the Medium CP diet (257.61 ± 17.23) and Control diet (229.09 ±17.13). At time 60 minutes, portal threonine in pigs fed Low CP diet (333.08 ±17.13) was higher ($P < 0.05$) compared to that in pigs fed Medium CP diet (263.19 ±17.23) and Control diet (284.96 ± 17.13). Portal threonine between pigs fed Medium CP diet and Control diet did not differ at any time point.

Figure 4. Portal Threonine Plasma Concentration



Portal methionine concentrations at time 30 and time 60 were higher ($P<0.05$) in pigs fed Low CP diet (102.01 ±7.37 and 104.82 ±7.37, respectively) compared to that of pigs fed Control (72.19 ±7.37 and 85.40 ± 7.37, respectively), and did not differ between pigs fed Medium CP diet (87.70 ±7.41 and 84.37 ± 7.41, respectively) and Control diet.

Figure 5. Portal Methionine Plasma Concentration



Portal tryptophan at time 30 in pigs fed Low CP diet (87.35 ±4.61) was similar to that of pigs fed Control (81.49 ± 4.61) and Medium CP diet (77.02 ±4.64), but at time 60 was lower ($P < 0.05$) in pigs fed Medium CP diet (78.39 ±4.64) compared to Control diet (94.17 ± 4.61). At time 60, portal tryptophan was not different between Low CP diet (88.60 ±4.60) and Control.

Figure 6. Portal Tryptophan Plasma Concentration

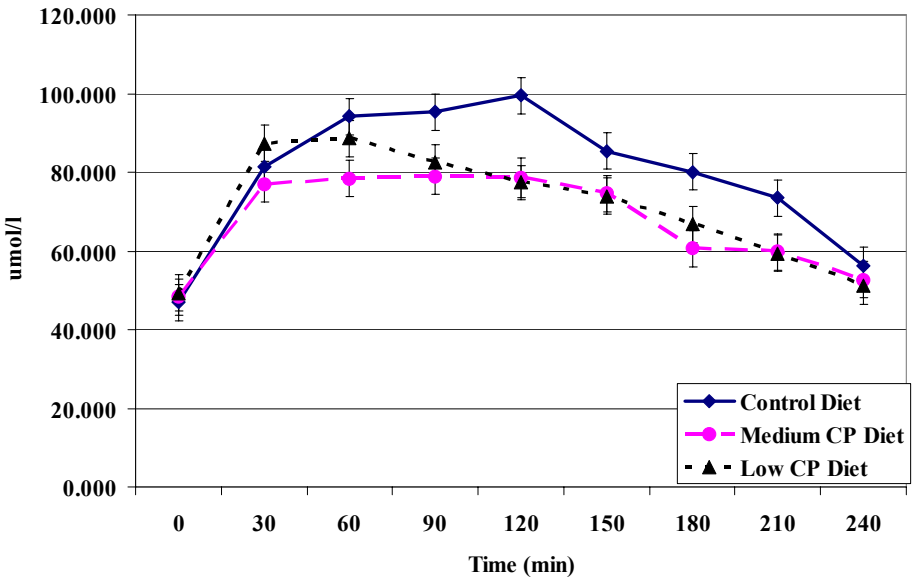
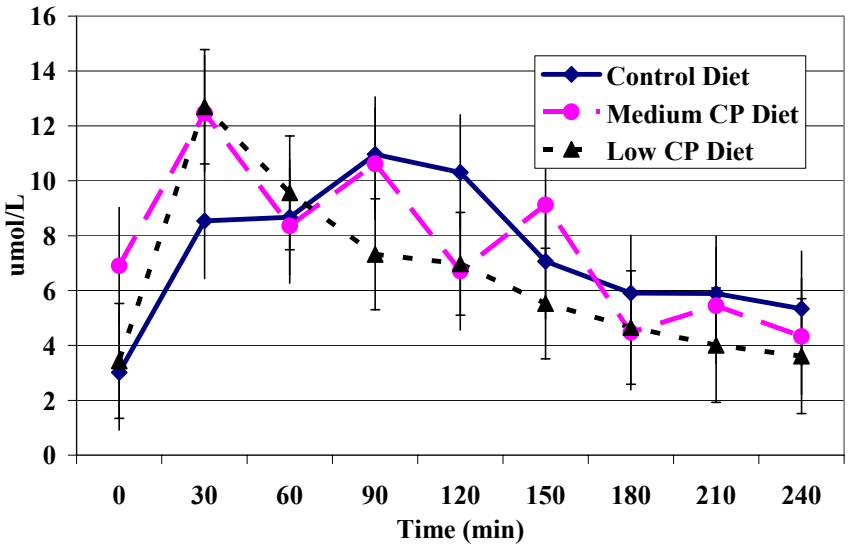


Figure 7. Portal Cysteine plasma concentration



Portal cysteine concentration did not differ at any time point between pigs fed the Low CP, Medium CP or Control diet.

Discussion: Changes in portal amino acid concentration occurred within the first hour following feeding, in particular for lysine, threonine and methionine. Amino acid concentration increased more rapidly in pigs fed the Medium and Low CP, and was mainly observed in pigs fed the Low CP diet. For instance, in pigs fed the Medium CP diet, no change in amino acid concentration resulting from crystalline amino acid inclusion was found, except for lysine 30, minutes following feeding. Partial replacement of protein-bound amino acids with crystalline increases amino acid concentrations in portal blood, implying that crystalline are absorbed more rapidly than protein-bound amino acid even in diets formulated on true ileal digestible basis. However, with moderate reduction in CP concentration from 16 to 14%, threonine and methionine are not absorbed at a more rapid rate, and lysine increased rate of absorption is only seen at 30 minutes post-feeding. Concurrently, other studies report no change in growth performance in pigs fed diets moderately reduced in crude protein (2 to 4%) (Boisen et al., 1991; Schoenherr and Schmidt, 1991; Spiekers et al., 1991; Lopez et al., 1994; Kerr et al., 1995; Maxwell, 1995; Tuitoek et al., 1997ab; Knowles et al., 1998; Friesen et al., 1999; Liu et al., 1999; Otto et al. 2003a). On the other hand, in this study, in pigs fed the Low CP diet, lysine, threonine and methionine concentration in portal blood indicated rapid absorption of these amino acids by the intestinal tract. In parallel, in pigs fed diets severely reduced in crude protein (4% and more), several studies have reported decrease in whole body protein accretion (Pierce et al., 1994; Kendall et al., 1999; Zervas and Zijlstra, 2000; Pérez Laspiur et al., 2002; Otto et al.,

2003a), and reduction in feed efficiency and average daily gain (Tuitoek et al., 1997; Liu et al., 1999). As mentioned earlier, the differential absorption rate of crystalline and protein bound-amino may decrease amino acid utilization for whole body protein synthesis (Yen et al., 1991). This 'imbalance' could lead to the oxidation of the rapidly absorbed crystalline amino acids as well as the later absorbed amino acids derived from intact proteins (Buraczewska et al., 1980; Batterham and Bayley, 1989; Rerat, 1995).

Based on these notions, results from this study indicate that amino acid utilization may be reduced in pigs fed diets with crude protein reduced by 4 percentage units. This may have important implication regarding the dietary formulation. While diets may contain truly equally digestible amino acids, this does not ensure equal availability of these amino acids to the animal for peripheral tissue utilization. This project focused on identifying mechanisms limiting the inclusion of crystalline AA in swine diets. Part 1 results of this study has generated important information to help design strategies to optimize the use of crystalline amino acids in swine diets. Strategies resulting in decreased rate of absorption of crystalline amino acids are essential to determine whether crystalline amino acids can be included in diets reduced in crude protein to minimize ammonia emission.

Lay Interpretation: Conventional diets containing corn and soybean meal fed to growing pigs can be reduced in protein when a portion of the protein can be replaced by crystalline amino acids. These amino acids allow the animal to digest dietary proteins more efficiently, with decreasing nitrogen excretion and ammonia emission into the air. However, the level of inclusion of these amino acids is limited because the rate of absorption by the gut is increased relative to the other amino acids found in intact feeds. Strategies to decrease the rate of absorption of crystalline amino acids may be an approach to allow more inclusion of crystalline amino acids in growing pig diets, thus minimizing environmental pollution.

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