

## **Chapter 1. Review of Literature**

### **Protein**

Protein is an essential component of living cells. Protein contains nitrogen and may also contain sulfur and phosphorus. The building blocks of protein are amino acids and type, sequence and number of amino acid combinations make proteins distinctive. Proteins serve several functions. Actin and myosin are contractile proteins that allow locomotion. Collagen and elastin are examples of structural proteins, and albumin and hemoglobin are examples of transport proteins. Hormones, enzymes and antibodies are also some of the many types of proteins (Munro, 1964).

Dietary protein comes from animal or plant sources. Animal sources of protein include meat (beef, chicken, pork, fish etc.), dairy products (milk, cheese etc.) and eggs. Other sources of protein from animal sources that are used for animal feeds are typically by-products such as meat and bone meal (from slaughterhouse scraps), milk by-products (whey, dried skim milk etc.) and fishmeal (from poor quality or scrap fish). Unfortunately, these sources of protein are generally not palatable for most animals, particularly the horse. Plant sources of protein include beans and peas as well as by-products from oil production such as soybean meal, peanut meal, sunflower meal, cottonseed meal etc. Palatability of these products vary and combined with relative cost, soybean meal has become the protein supplement of choice in horse feeds (Church and Pond, 1988). Non-protein nitrogen (NPN) sources such as urea would also provide the animal with nitrogen, which may be used to synthesize non-essential amino acids (Reitnour and Salisbury, 1976).

Amino acids are divided into two general categories: essential and non-essential. Non-essential amino acids are those that can be synthesized by the body (liver or microbial synthesis) in adequate amounts, that is, at a sufficient rate, to satisfy the needs

of the animal. Essential amino acids can be synthesized by the body but not in sufficient amounts (or rate of production) to satisfy the needs of the animal. All amino acids are required for protein synthesis. The correct supply and variety of amino acids must be present at the same time in order for protein synthesis to be complete (Munro, 1964). Both essential and non-essential amino acids can be supplied by the diet however, a source of nitrogen such as ammonia in the hindgut would be able to be used to meet the needs for non-essential amino acids (Mason, 1984).

### **Protein Digestion and Absorption**

Digestion of protein in the horse varies depending on the type of protein and its location of digestion. Protein in the feed enters the mouth and will be moistened by the saliva. After being swallowed, proteins enter the stomach where hydrochloric acid (HCL) is secreted making the stomach an acidic environment. When the pH of the stomach falls below 5, pepsin is converted to pepsinogen, which cleaves some internal peptide bonds. Although some peptide digestion takes place in the stomach, most protein digestion takes place in the jejunum. The pancreas secretes many proteases such as trypsin, chymotrypsin, carboxypeptidase A & B as well as elastase. These break the peptides into 3 main groups before entering the brush border: di- and tri-peptides, larger peptides (greater than 4 amino acids) and free amino acids (Johnson, 1997). The brush border has carrier systems for free amino acids as well as di- and tri-peptides. Amino acid absorption involves six systems (1) neutral for amino acids with aromatic and aliphatic side chains (phen, tyr, tryp, iso, leu, val, ser, thr), (2) basic (lys and arg), (3) phen (phen and met), (4) acidic (asp and glu), (5) Y<sup>+</sup> and (6) imino for proline. Many of these carrier systems are sodium dependent and require energy. The rate of absorption of amino acids depends on the length of the side chain and whether or not it is charged. Non-polar side chains such as those of leucine, glutamine etc. are absorbed rapidly. Carriers for the di- and tri-peptides are H<sup>+</sup> dependent. This prevents competition between small peptides and amino acids for carriers for absorption (Ganapath et al., 1994). The

efficiency of absorption of small peptides is better in many instances than with a mixture of synthetic amino acids (Hara et al., 1984). Along the brush border also lie peptidases that break down any small peptides that are allowed to pass into the gut wall and are broken down into di- and tri-peptides (Johnson, 1997).

Outlined above is the typical course of action for enzymatic digestion of protein in the foregut of the horse. Any protein that escapes digestion in the foregut may undergo fermentation by bacteria in the hindgut. The resulting end-product is ammonia. This ammonia can be absorbed into the general circulation where it may go to the liver and have one of several fates. It may be converted into urea and be excreted in the urine. Ammonia may also be used to synthesize non-essential amino acids which would require a source of nitrogen or it may be used to transaminate glutamic acid to glutamate which will diffuse into the gut and be used by enterocytes (Mason et al., 1984).

Amino acids that enter the general circulation have many fates. Amino acids will become part of the amino acid pool found in the blood stream, liver and muscle. The amino acids' most important function is to be incorporated into structural proteins (i.e. muscle) and blood proteins such as albumin. Those not being incorporated into protein are degraded mainly in the liver (also branch chain amino acids in the muscle and others can be degraded by the kidney) by separating the amino group and the carbon skeleton (deamination). The carbon skeletons can be transaminated to synthesize non-essential amino acids, be converted to a Krebs' cycle intermediates yielding energy or be converted into glucose or fat. The amino group is converted into urea and excreted in the urine as a waste product (Meijer et al., 1990). Proteins are labile, that is, constantly turning over. There is no storage of amino acids per se simply for storage as opposed to adipose tissue that is storage of fat for fuel.

Site of protein digestion may affect the resulting amino acids in the "pool" that are available for various functions in the body. Studies in horses have shown that the

efficiency of use for soybean meal (which would be digested in the foregut resulting in amino acids) was greater than that of urea (which would be a source of non-protein nitrogen very similar to ammonia that would result from fermentation of protein). I required more nitrogen to stay in nitrogen balance with urea (590 mg absorbed N/W<sup>.75</sup>) than with SBM (365 mg absorbed N/W<sup>.75</sup>). This may be due to limiting essential amino acids that result from using urea instead of SBM, which is a high quality protein (Hintz and Schryver, 1972). Hay protein digestion was only 37% prececaly (Gibbs et al., 1988) while mixed rations of 50:50 concentrate: hay resulted in prececal protein digestibilities of 70% (Potter et al., 1992). Thus the type of feedstuff may affect the site of digestion, protein digestion and hence the amino acid pool.

Fat has been shown in swine to affect amino acid digestibility. Addition of 12% canola oil to the diet of growing swine resulted in increases in ileal digestibilities but not total tract digestibilities (Li and Sauer, 1994). This increase in digestibility may be due to a slower gastric emptying observed with high fat diets thus exposing the brush border to amino acids for longer periods of time. Although the fat supplementation did not improve total tract digestibilities, it would increase the amounts of amino acids available in the small intestine where the main site of protein absorption takes place.

Dietary cation-anion difference in the diet, which affects acid-base status, may also affect the use of amino acids. In swine, the addition of CI to the diet depressed growth regardless of protein adequacy. Addition of sodium bicarbonate improved growth on a lysine deficient diet but did not affect performance on the lysine adequate diet. The use of lysine in these experiments may have been improved especially on the deficient diet (Austic et al., 1995).

Hindgut bacteria are capable of synthesizing amino acids however, their availability from the hindgut is very limited (Mason, 1984). Research has shown little to no absorption of amino acids from the hindgut of the horse. One study found less than

2% recovery of lysine, arginine and histidine from incubations of mucosa tissue from the ventral colon (Bochroder et al., 1994).

### **Nitrogen Excretion**

Intake of protein is often much greater than the body actually needs. Since protein is not stored, excess is degraded and removed from the body. Losses of nitrogen from the body occur in the feces, urine, sweat, milk and hair or skin loss. Losses of nitrogen in the feces are made of undigested protein and endogenous protein (sloughing of cells from the digestive tract) as well as nitrogen in secretions in the gastrointestinal tract. The main loss of nitrogen from the body (removal) is through the urine. Amino acids are degraded mainly in the liver with some degradation in the kidney and skeletal muscle. The first step in amino acid degradation is the removal of the amino group from the amino acid (deamination). Removal of the amino group results in ammonia production that can be toxic. Ammonia is converted to urea via the ornithine cycle in the liver (Meijer et al., 1990). Urea readily diffuses across tissues. Urea is filtered by the kidney and excreted in the urine however, urea may also pass into the gut. Bacteria in the gut can break down urea into ammonia which can be absorbed into the bloodstream and used for the production of non-essential amino acids by the body (Mason, 1984). Uric acid is also a nitrogen excretory product. Uric acid is a by-product of purine breakdown. In situations in which adenine monophosphate (AMP) is used to produce adenosine triphosphate (ATP), inosine monophosphate (IMP) and ammonia result from the breakdown of AMP. Uric acid is a breakdown product of IMP that is excreted in the urine (Cardinet, 1989).

### **Exercise and protein digestibility**

Moderate exercise delayed the transit time of the liquid phase of digesta (those nutrients that would be soluble and available for absorption) and increased transit time of

the solid phase of the digesta (Orton et al., 1985). Thus longer retention of the liquid phase in the gastrointestinal tract resulted in an increase in digestibility. A study with 2-year olds found equal average daily gains for horses fed 6% CP compared to horses fed 12% CP provided they were exercised. Exercise increased feed intake of horses on the 6% CP diets and also improved utilization of the crude protein (Orton et al., 1986).

In another study, a diet that contained 14% additional fat was fed to exercising horses (Wort et al., 1987). Exercise increased the digestibility of protein as well as ADF while tending to increase the digestibility of NDF. Fat decreased the digestibility of NDF but increased the protein digestibility. This increase in protein digestibility is consistent with a study in pigs that observed an increase in ileal digestibility of amino acids on higher fat diets. Slower gastric emptying due to higher fat is suspected to be the cause of the increased digestibility (Li and Sauer, 1994).

### **Protein Quality**

A high quality protein is one with a good variety and supply of amino acids especially the essential amino acids. Animal proteins generally are excellent sources of protein since they reflect the end product of amino acid use (tissue) and are high in lysine (considered the first limiting amino acid) however, they are not very palatable to the horse. Vegetable proteins generally have lower quality but are better accepted by the horse. Soybean meal has become the standard of choice for horse feeds due to its relatively high quality and acceptability (Church and Pond, 1988).

The concept of a limiting amino acid refers to the amino acid that is in short supply or missing in relation to needs. All amino acids in a protein product are needed at the same moment for its synthesis. If one is missing, it becomes limiting for its synthesis. Lysine has been shown to be limiting in many feedstuffs used in horse feeds and has been shown to improve growth when supplemented in the diet (Ott et al., 1981). Swine

research has developed the use of ideal protein in which the amount of an amino acid needed for optimum growth was determined based on nitrogen balance as well as tissue composition. Ratios of the amino acids, compared to lysine set as the standard, were developed to evaluate the amino acid levels needed for ideal protein (Wang and Fuller, 1989). Using these ratios, it appears that methionine, tryptophan and threonine are most likely the next limiting amino acids in horse feeds. Tissue analysis of muscle and milk from the horse has allowed development of amino acid ratios to lysine for the horse as was done in swine. Ratios developed in comparison to lysine (100) are 28, 61 and 52 for methionine, threonine and tryptophan (Bryden, 1991). Methionine has been tested as a limiting amino acid but not shown encouraging results or even was observed to suppress growth (Borton et al., 1971). The level of methionine is also low in mare's milk and it is believed that methionine is not a limiting amino acid for the horse (Saastamoinen, 1996). Threonine has been shown to have improved growth and girth gains without any difference in fat deposition compared to unsupplemented diets as well as lowered blood urea nitrogen levels (Graham et al., 1994). This evidence supports the hypothesis that threonine is the second limiting amino acid for horses fed typical grass forage and concentrate diets.

The overall value of protein is a function of protein quality as well as quantity. Inadequate intake of a high quality protein will not overcome the lack of adequate amounts of amino acids and N sources for protein synthesis (Eggum, 1970).

### **Protein Deficiency/Toxicity**

Protein intake that does not meet the needs of the animal can result in deficiency. Protein turnover in the body has been shown to adjust to the supply change (Waterlow, 1986). Protein degradation slows followed by a slower protein synthesis rate. The main tissues affected by protein deficiency are skin, GI tract, liver and muscle. Signs of protein deficiency would therefore affect these tissues and reflect changes in the protein

synthesis or degradation in the body. Decreased levels of albumin, total protein as well as other protein transporters such as retinol binding protein and transferrin are observed in a state of protein deficiency. Poor skin and coat are generally the first external signs of a protein deficiency followed by muscle wasting, weakness, disorientation, etc. (Gibson, 1990).

Protein toxicity probably does not really exist due to the body's ability to remove nitrogen from the body. There is no storage of protein in the body therefore the body has a large capacity to remove excess protein (or amino acids) from the body. Levels of enzymes in the ornithine cycle are rarely surpassed showing that the body has the ability to synthesize large amounts of urea (Meijer et al., 1990). However, high levels of protein do tax the body in a variety of ways. Degradation of excess amino acids does require energy and will decrease available energy for other functions. Adult horses handle excess protein better than young growing horses. A high intake level of protein in adult horses results in increased urea excretion (urination). Increased urination will increase the water requirement to balance losses of water in the urine. Higher levels of ammonia can result in the urine that for stabled horses can irritate the respiratory tract. This irritation could compromise breathing ability for the athletic horse (Meyer, 1987). Growing horses can have more adverse affects from excess protein. Aside from increased urination, excess protein can be converted to fat, resulting in increased body weight. This places stress on the bones which can result in epiphysitis as well as developmental orthopedic disease. These damaging problems can permanently affect growth and athletic potential of the horse.

### **Protein/Energy Relationship**

Energy level affects protein metabolism. Inadequate energy intake results in increased nitrogen excretion. Adding energy to a diet results in an increase in nitrogen retention (Munro, 1964). Presumably, the additional loss of nitrogen on an energy deficient diet is due to the use of protein for energy. Therefore, determining needs for

protein requires adequate dietary energy to prevent overestimation of protein requirements. Growing horses fed a restricted diet that was adequate in protein but deficient in energy had gains similar to horses fed low protein and energy diets indicating that the limited growth of those horses was due to decreased energy availability and not protein intake (Ott and Asquith, 1986).

### **Assessment of protein status**

Assessment of protein status should begin with an overall evaluation of the animal's health and condition. The first step should be assessing weight and body condition score. Changes in body protein due to insufficient protein intake are often first detected in muscle (although not outward), skin and then liver. When the deficiency affects the liver, this may be when blood proteins would be affected. Clinical signs of protein deficiency would include poor skin and coat, weight loss (muscle wasting), weakness, disorientation, etc. (Kronfeld, 1998a)

*Nitrogen balance.* Nitrogen balance is determined by subtracting total nitrogen lost from the body (sum of fecal nitrogen, urinary nitrogen, sweat, hair and skin nitrogen) from total nitrogen intake from the diet. When nitrogen balance is negative, not enough nitrogen is being taken in to meet needs (balance losses) while a positive nitrogen balance shows there is additional nitrogen to support additional growth etc. Zero nitrogen balance means needs are being met exactly. For growth to occur, positive nitrogen balance must exist (Gibson, 1990).

A drawback to the nitrogen balance method is the need to collect all feces and urine to measure nitrogen lost from the body. Nitrogen intake is often overestimated and nitrogen loss underestimated. Nitrogen recycling in the body (as well as amino acid recycling) can also affect the results. When not receiving adequate levels of protein, the body seems to adjust over several weeks to the new level of protein. Studies have shown

that laying hens and dogs fed low and high protein diets both achieve nitrogen balance over the course of 4 weeks (Wannemacher et al., 1966; Muramatsu, 1990). The body becomes more efficient at urea recycling as well as recycling amino acids. Protein turnover adjusts to the level supported by the diet. Seventy to eighty percent of the body's amino acids are recycled and incorporated back into tissue protein (Baker, 1991). Interpreting response data from nitrogen balance data alone can result in inaccurate interpretation of protein status.

Measuring nitrogen losses from the body of the horse found levels of 2.4 g DP/kg BW<sup>.75</sup> to replace the losses however, 3.4 g DP/kg B<sup>.75</sup> was recommended for maintaining protein reserves (Meyer, 1983). Simply replacing N losses would not maintain liver and muscle protein levels, therefore higher intake levels were recommended.

*Blood variables.* Many protein metabolites can be measured in the blood to help evaluate protein status. These include total protein, albumin, globulins, urea, creatinine, ammonia and uric acid. Transferrin, an iron transporter, as well as retinol binding protein can also be measured. Blood urea nitrogen has been found to correlate well to level of protein in the diet and biological value of protein (Eggum, 1970). These two factors need to be controlled when making comparisons between diets. Comparisons cannot be made on two different qualities of protein based on blood urea measurements alone. Blood urea nitrogen can be a measure of amino acid catabolism when amino acids are broken down for purposes other than protein synthesis. When amino acids are in excess, catabolism of that amino acid increases thus causing an increase in urea.

Total protein and albumin respond more slowly than urea to changes in dietary protein. Studies have shown that 3 weeks on a protein free diet in rats were necessary to cause a decrease in plasma albumin levels. These proteins do not generally respond as quickly as urea to different levels of protein intake but will reflect a deficiency over time.

Since total protein is made of albumin as well as globulin, other factors beyond protein adequacy such as infection and stress can affect the level of total protein. Total protein and albumin should be measured to monitor the overall health of the animal, along with transferrin and retinol-binding protein, but not relied on heavily for the determination of protein status by themselves (Young et al., 1990).

Creatinine is a product of muscle activity resulting from breakdown of creatine phosphate. At rest, it is a good reflection of muscle mass. Creatinine production and excretion are considered relatively constant. They are often used to standardize other metabolite measures such as urea by expressing the concentration in a ratio to creatinine. This ratio will take into account any changes in plasma volume. Activity beyond normal daily activity such as forced exercise will increase the level of creatinine seen in plasma and urine and should be considered when interpreting data (Gibson, 1990).

*Urine measures.* Measuring products of protein metabolism in urine can provide a picture of protein catabolism in the body. Urine volume and concentration can be affected by hydration status and varies depending on the individual. Because of differences in concentration, creatinine is often also measured in order to form a ratio with other metabolites (Gibson, 1990).

Increasing levels of urea are evidence of increased amino acid catabolism. Other nitrogen products such as ammonia and uric acid can also provide evidence of amino acids in excess of needs.

Another component of urine used to assess protein status is 3-methyl-histidine (3MH). This product is produced during breakdown of contractile proteins in muscle (actin and myosin). Histidine is methylated when it is incorporated into the contractile proteins and upon their breakdown the 3MH cannot be re-incorporated into any other proteins. Therefore, its measurement is specific evidence of muscle protein degradation.

If protein (or a specific amino acid) was lacking or deficient, the body may degrade skeletal muscle to meet this need and would be reflected in an increase in 3MH in urine (Gibson, 1990).

Exercise confounded the results from 3MH because exercise increased the activity of muscle. With increased muscle activity, 3MH will be increased in the urine following exercise but does not necessarily represent abnormal muscle protein breakdown (Hickson and Hinkelmann, 1985).

*Amino acid analysis of plasma.* Monitoring specific levels of amino acids can help determine requirements. Monitoring the change in a specific amino acid may help determine its use in the body as well as any depletion of a deficient amino acid (Young et al., 1990). Increases in alanine and glutamine can represent increased oxidation and transamination of amino acids in muscle (mostly from branch chain amino acids). Increased oxidation of BCAA may occur with a decreased energy supply (Young and Marchini, 1990). Increases of some of the essential amino acids may represent increased muscle turnover, although 3MH appears to be a better measure for this.

*Amino acid isotopes.* Using isotopes to mark either the  $\alpha$ -amino nitrogen or the terminal carboxyl group carbon of an amino acid or both can be used to determine the fate of that particular amino acid (Lazaris-Brunner et al., 1998). Observing an increase in labeled amino nitrogen in urine would represent incorporation of this amino acid's amino group into urea, demonstrating an excess of the amino acid. An increase in labeled carbon in fat or CO<sub>2</sub>, representing increased oxidation, will also show excess levels of the amino acid. The advantage of this method allows monitoring of a specific amino acid from the diet.

*Muscle and Liver Biopsies.* Animals, and presumably horses, will adjust to different levels of protein achieving nitrogen balance. How this is done on a lower level

of protein than is considered ideal must be accomplished by using some of the “protein reserves” in the body. These would include liver, muscle and skin protein.

It would therefore be ideal, to use the level of reserves in the determination of protein status. Dogs have been shown to adjust to a low level of protein and achieve nitrogen balance within four weeks on the experimental diet but, when liver and muscle biopsies were taken, it was apparent that the protein reserves had been depleted compared to controls on a higher level of protein. Dogs were fed levels of nitrogen between .15 and .6 g N/kg BW and in all cases nitrogen balance was achieved within four weeks. Optimal reserves of protein were seen with 7 g N/kcal for younger dogs and 10 g N/kcal for older dogs (Wannemacher and McCoy, 1966). Practicality of taking muscle and liver biopsies is low in the horse. Muscle biopsies would be more common than liver biopsies however, its use for protein status evaluation is rare. It is more commonly used to determine muscle glycogen levels.

*Protein Status Summary.* Along with a health evaluation and nitrogen retention measurements, blood samples should be taken. Minimum analysis should include urea and creatinine. Total protein, albumin and even transferrin or retinol-binding protein are useful in monitoring the overall health of the horse. If affordable, amino acid analysis of plasma including 3MH should be done.

Urine collection will help estimate catabolism of excess amino acids through excretion of urea. This should be expressed in a ratio to creatinine to take into account any dilutional effects. Use of blood and urine measurements should give an acceptable representation of use and catabolism of amino acids in the diet.

## **Protein Requirements**

Assessment of a protein status along with current feeding practices is not a matter

of finding one level that prevents signs of deficiency but rather a level that optimizes performance whether that be growth, exercise or maintenance. That level will vary depending on the individual and environment in many cases. Therefore, an optimum range should be recommended. In human nutrition, RDA's are 2 standard deviations above average that prevented signs of deficiency in 50% of the population. Ranges with two standard deviations will statistically cover 95% of the population.

Current dietary requirements for horses are considered near minimums (NRC, 1989) however, recent reports on sensitivity analysis of diets has explored the use of ranges in balancing diets for horses (Kronfeld, 1998b). Following the plan of human RDA's, equine allowances should be at least 1.3 times the minimum requirements. Research has shown that several nutrients (vitamin A, copper and zinc) are needed a twice the NRC recommended level (Hoffman and Kronfeld, 1998). Analysis of available data for protein levels for growing horses, found 16% CP to be optimum for weight gain in young horses (Figure 1).

### **Protein for the exercising horse.**

Protein needs of horses during exercise have received little attention in comparison to other nutrients such as carbohydrates and fat as energy sources. During conditioning with strenuous exercise, muscle hypertrophy occurs. Hypertrophy is mainly due to an increase in protein in the muscle fibers (Horton and Terjung, 1988). With an increase in muscle mass, one would expect an increased need for protein. Meyer (1987) identified three reasons for additional needs of protein with exercise; they included an increased level of endogenous fecal nitrogen losses due to increased dry matter intake with exercise, increased nitrogen lost in sweat and an increase in the assimilation of muscle tissue seen with conditioning.

Nitrogen balance studies have shown increased nitrogen retention due to

increased work loads that could not be accounted for in urine or feces (Freeman et al., 1986 and Freeman et al., 1988). Mature Quarter Horses were fed at a level to maintain body weight over the course of nine 14-day periods. Exercise levels were increased over the course of periods 2 through 5 and reduced from period 6 through period 8. Period 8 and 9 had no exercise. Nitrogen intake increased as workload increased due to DM intake increasing. The diets resulted in a protein level of 8.3% CP up to a level of 9% CP. Nitrogen retention increased during the exercise periods (Period 1 - 11.1 g N/d vs. 41.9g N/d in Period 5) and remained elevated after exercise had ceased in periods 8 (45.5 g N/d) and 9 (29.2 g N/d). This suggests that a greater amount of protein was needed to maintain additional lean tissue acquired as a result of exercise training compared to period one which was a non-exercise period before training (Freeman et al., 1988). Another study also showed increased nitrogen retention during a conditioning period (18 g N/d pre trial vs. 34.5 g N/d post- conditioning) and maintained during a period of consistent exercise following conditioning (29.4 g N/d). A significant decrease in body fat was also observed after exercise training. With no differences in weights compared to pre-training weights, this suggests that the weight was replaced by lean tissue. This is also supported through increased levels of RNA following exercise training, which is consistent with an increase in protein synthesis seen with muscle hypertrophy (Freeman et al., 1986). Neither of these studies took into account nitrogen lost in sweat as part of nitrogen balance. Significant amounts of sweat can be lost depending on the intensity of exercise (Hodgson et al., 1993) and the amount of urea in sweat actually increases during endurance exercise (Kerr et al., 1983). One study observed that water balance could not be reached even taking into account differences in urine output suggesting significant sweat loss during exercise (Freeman et al., 1986). With average nitrogen content of sweat being between 1-1.5 g/kg sweat (Meyer, 1987), nitrogen balance in the previously described studies still would have shown nitrogen unaccounted for and assumed to be retained for muscle mass accumulation or maintenance of additional lean tissue. These studies demonstrate an additional need for protein due to increased muscle mass, maintenance of new muscle mass, repair of muscles damaged during exercise and

nitrogen lost in sweat.

Mature horses in another study were fed 3 levels of protein (5.5, 7 and 8.5% CP) at 3 work intensities. Serum total protein and albumin were not affected by diet. A four day fasting period following exercise showed an elevated level of plasma urea for horses fed the highest protein level and had increased excretion of urea and ammonia indicating better stores of protein than the other groups. Exercise had no effect on protein stores. All levels of protein were deemed adequate, however, the highest level of protein provided better protein reserves (Patterson et al., 1985)

During exercise, protein synthesis decreases while protein breakdown increases. Researchers have observed increased levels of branch chain amino acids (leucine, isoleucine and valine) in muscle and decreases in plasma. Muscle can oxidize the branch chain amino acids for energy. As deamination occurs, pyruvate can accept the amino group to form alanine. Alanine can then diffuse into the blood stream where it can be converted into glucose in the liver. Glutamate also plays a role in exercise metabolism by removing the ammonia formed in muscle during exercise. The ammonia group is accepted by glutamate to form glutamine. These metabolic occurrences are supported in studies with horses by increased levels of alanine and decreased levels of glutamate in muscle (Miller-Graber et al., 1990). Using amino acids for energy in muscle is achieved by the oxidation of the branch-chained amino acids (leucine, isoleucine and valine). This fact is supported in a study that analyzed muscle biopsies taken immediately after an exercise bout. The study found a significant increase in leucine (.62 mmol/kg vs. .71), alanine (1.63 mmol/kg vs. 3.17) and a decrease in glutamate (4.27 mmol/kg vs. 1.80) in muscle. They also observed an increase in muscle lysine levels (.44 mmol/kg vs. .67) which may suggest increased muscle breakdown during exercise as seen in other species (Miller-Graber et al., 1990).

Support for alterations in amino acid metabolism also exists with data from

plasma. Increased oxidation of branch chain amino acids was demonstrated by increased plasma levels of alanine (pre: 19 mmol/kg vs. post: 36.3 mmol/kg) and glutamine (pre: 15 mmol/kg vs. post: 27.7 mmol/kg) (Poso et al., 1991). Increases in the oxidation of branch chain amino acid may result in decreased plasma levels as seen in another study in which decreased levels of leucine (pre: 21.7 umol/100ml vs. post: 16.7), isoleucine (pre: 11.5 umol/100 ml vs. post: 8.6) and valine (pre: 35.9 umol/100 ml vs. post: 27.8) were observed following a 2 week exercise period and continued decreasing following a one week recovery period compared to pre exercise values (12.8 leu, 5.7 iso and 20.2 va umol/100 ml respectively). They also observed significant decreases in plasma lysine following exercise (pre: 9.9 umol/100 ml vs. 7) and recovery periods (5.8 umol/100 ml) suggesting that dietary levels of lysine were insufficient for muscle repair and/or hypertrophy that occurs with exercise. In this study, horses were fed 1,300 g protein/day which is the recommended level for intensely exercised horses. Plasma total protein levels decreased significantly from pre-exercise values (7.92 g/100 ml pre-exercise vs. 6.04 g/100 ml post recovery) again suggesting that dietary protein level was not adequate (McKeever et al., 1986). Increases in alanine may also demonstrate the need for gluconeogenesis to maintain blood glucose levels during prolonged exercise. Furthermore, significant increases in muscle levels of several essential amino acids (Miller-Graber et al., 1990 and Poso et al., 1991) indicate protein catabolism during exercise, making amino acids available for energy. Additionally, lowered levels of several essential amino acids in plasma have suggested a period of increased protein synthesis commonly seen during the days of recovery following training (McKeever et al., 1986).

Although the previous discussion appears to suggest a need for additional protein for exercise, not all research has shown this. One study used 2 year old horses fed either low protein (6% CP) or high protein (12% CP) diets and each dietary group was split into an exercise group (E) and a non-exercise group (NE) (Orton et al., 1986). Exercise increased the rate of gain for horses on low protein (ADG: .6 kg NE vs. 1.2 kg E) but not

on high protein (1.1 kg NE vs. 1.2 kg E). Exercise stimulated increased intake for horses on the low protein diet (7.7 kg/d E vs. 6.6 kg/d NE) and allowed those horses to make more efficient use of protein for gain (.46 kg protein/d low protein diet and .9 kg protein/d high protein displaying similar ADG). These horses gained weight and thus provided no evidence for additional protein for exercise. As mentioned in the previous section, mature exercising horses have been fed levels of protein as low as 5.5% CP without any apparent adverse effects (Patterson et al., 1985).

Some research has also shown no detrimental effect of high protein on exercise performance. Several researchers have fed levels as high as 18% CP and found no detrimental effects over the lower protein diet (9% CP and 12.9% CP respectively) (Miller and Lawrence, 1988; Miller-Graber et al., 1991). Their conclusions were based on a lack of difference between heart rates and lactate levels. Lower lactate levels were found during some recovery points for horses fed high protein suggesting that high protein may influence lactate and pyruvate metabolism however, further research in this area is needed (Pagan et al., 1987; Miller-Graber et al., 1991).

Although the debate continues on how much protein exercising horses really need, keeping protein at a minimum during exercise has advantages for several reasons. High protein increases the water requirement through the increase in urea production (from excess amino acids). Thus urination increases complicate water balance already compromised due to increased sweat loss with exercise. Increased urination can increase the level of ammonia in the horse's stall. Ammonia fumes can cause respiratory problems for horses confined to stalls (Meyer, 1987). High protein also increases the heat increment that could potentially interfere with the horse's cooling mechanism in hot climates (Kronfeld et al., 1996).

## **Dietar Cation-Anion Difference (DCAD)**

The influence of diet on urine acidity or alkalinity was one of the first observations linking functions of the animal body to chemistry ( Lavoisier, cited in Needham, 1970). Certain foods were noted to contain acid or alkaline salts, and were used clinically to alter urine pH, hence to dissolve urinary stones or disinfect the urinary tract. Effects of diet on acid-base status have broad impacts on public health, for example, reducing the risk and severity of postmenopausal osteoporosis and senescent renal insufficiency (Frassetto et al., 1998). Influence of diet on acid-base status has also been recognized as important in poultry production (Mongin, 1981), livestock production (West et al., 1991, 1992), and equine performance (Poplewell et al., 1993). In general, diets that decrease chronic acidity of the body also increase efficiency of functions such as growth, milk or egg production, and exercise (Mongin, 1981). An exception is dietary acidification to reduce the risk of parturient paresis in dairy cows (Horst et al., 1997).

The overall influence of diet on acid-base balance is traditionally calculated as the algebraic sum of positively and negatively charged fixed cations and anions:  $(Ca^{+2} + Mg^{+2} + Na^{+} + K^{+}) - (Cl^{-} + S^{2-} + P^{1-8-})$ . This sum is called the dietary cation-anion difference (DCAD, mEq/kg DM), dietary cation-anion balance (DCAB), cation-anion balance (CAB), dietary electrolyte balance (dEB or EB), dietary undetermined anion (dUA), or base excess (BE).

The DCAD influences cation and anion levels in the bloodstream. Sodium is absorbed across the gut wall in exchange for hydrogen ions while chloride is commonly absorbed in exchange for bicarbonate depending on balance of sodium and chloride in the diet (Block, 1994; Johnson, 1997). Thus DCAD can affect acid-base status of the animal by altering hydrogen ion and bicarbonate levels as well as by altering plasma levels of cations and anions. A cationic diet may elicit metabolic alkalosis by decreasing hydrogen ion concentration or increasing cation levels and an anionic diet may induce acidosis by

decreasing bicarbonate levels or increasing anion levels.

The DCAD correlates well to the comprehensive physico-chemical description of acid-base by Stewart (1981). In this system, hydrogen ion concentration as well as bicarbonate levels are dependent variables that are determined by three independent variables: strong ion difference (SID),  $p\text{CO}_2$  and total weak acids. Strong ion difference is defined as  $(\text{Na}^+ + \text{K}^+) - (\text{Cl}^- + \text{lactate})$  which is essentially completely dissociated cations minus anions. Therefore, DCAD affects the components of SID and inevitably affect acid-base balance (Stewart, 1981).

*Chickens.* Mongin (1981) integrated DCAD and whole body acid-base balance, and promoted the use of DCAD to improve productivity. Only the monovalent ions were used since the author observed that the other cations and anions were fixed in diets due to well-known requirements for the other ions. Studies with growing chickens revealed that blood pH and  $\text{HCO}_3^-$  were linearly related to DCAD ( $\text{Na}^+ + \text{K}^+ - \text{Cl}^-$ ), and that growth was maximized at 250 mEq/kg dry matter (DM). Several other researchers have confirmed increasing pH and  $\text{HCO}_3^-$  in conjunction with increasing DCAD. This relationship prevailed regardless of the cation or anion used to manipulate DCAD (Cohen and Hurwitz, 1974; Hamilton and Thompson, 1980; Keshavarz and Austic, 1990 and Ruiz-Lopez and Austic, 1993). Lower weight gains, egg production and feed intakes were found with a lower DCAD (Keshavarz and Austic, 1990). Body weight, egg production and feed intake were optimized with a sodium plus potassium to chloride ratio of 1.92-2.83 (Hamilton and Thompson, 1980).

*Dairy Cattle.* Studies have related increasing plasma pH and  $\text{HCO}_3^-$  to increasing DCAD in lactating, pregnant and dry cows (Tucker et al., 1988, 1991a, b; West et al., 1991, 1992; Wang and Beede, 1992). Manipulations were made using calcium chloride, sulfur, ammonium chloride, ammonium sulfate, potassium and sodium bicarbonate. Results were consistent regardless of the mineral manipulated to alter DCAD or the

mineral source used. This supports the hypothesis that results were due to changes in DCAD not just a certain mineral alteration. Milk production and feed intake increased with increasing DCAD (Tucker et al., 1988; West et al., 1991, 1992). Urinary calcium excretion increased with decreasing DCAD (Tucker et al., 1991b; West et al., 1992) and may provide evidence of increased bone mineral turnover that has been postulated to decrease the incidence of milk fever.

Studies related cation-anion difference in blood (the effective SID) to increasing DCAD (Tucker et al., 1988; West et al., 1991, 1992). Since increasing DCAD had either no effect (Tucker et al., 1988; West et al., 1992) or increased (Tucker et al., 1991a; West et al., 1991)  $p\text{CO}_2$  with increasing DCAD, the resulting increase in pH would be explained by increased SID instead of  $p\text{CO}_2$  in the system described by Stewart (1981).

*Other Species.* Similar responses in acid-base balance have been documented in beef cattle (Ross et al., 1994), swine (Patience and Wolynetz, 1990) and lambs (Fauchon et al., 1995), all associated with increased feed intake and average daily gain with increasing DCAD.

*Horses.* Horses have had increasing serum pH and  $\text{HCO}_3^-$  with an increase in DCAD, as demonstrated in other species. These effects have been shown in sedentary adult horses (Baker et al., 1992, 1998), growing horses (Wall et al., 1995) as well as exercising horses (Stutz et al., 1992; Popplewell et al., 1993) with a DCAD above 200 mEq/kg DM. Manipulations of DCAD were achieved using calcium chloride, potassium citrate, magnesium sulfate, ammonium chloride as well as potassium and sodium bicarbonate and did not differ in their results. Acidogenic effects of chloride as well as sulfur were observed in horses (Baker et al., 1998).

Exercise induces acidosis mainly through an increase in serum  $p\text{CO}_2$  in horses during incremental exercise or repeated sprints (Kronfeld et al., 1998a). Acidosis

contributes to fatigue during exercise by decreasing the force of muscle contraction and inhibiting glycolysis. Slower elapsed times over 1600 m were found in horses fed diets with low DCAD of 10 mEq/kg DM compared to a high DCAD of      mEq/kg DM (Poplewell et al., 1993). Increasing DCAD can increase serum SID, which will increase serum pH and  $\text{HCO}_3^-$  and may delay fatigue. Altering DCAD can be achieved by adding cations or lowering anions. Addition of sodium bicarbonate to replace sodium chloride in the diet increases cations and decreases anions (sodium and chloride, respectively). Higher serum pH and  $\text{HCO}_3^-$  were observed in exercising horses fed higher DCAD diets (Stutz et al., 1992; Poplewell et al., 1993). Higher  $\text{pCO}_2$  was observed on the cationic diets (Baker et al., 1992; Stutz et al., 1992; Poplewell et al., 1993), once again allowing increased pH to be due to increased SID. The danger of removing chloride from the die is due to impaired ability to replenish losses in sweat especially for horses exercising hard in the heat. Other alternatives to widening the DCAB may be beneficial.

Increased urinary calcium levels with decreasing DCAD were observed in sedentary horses (Baker et al., 1993 and 1998), growing horses (Cooper et al., 1995; Wall et al., 1997), and in exercising horses (Wall et al., 1993) with DCAD levels below 100 mEq/kg DM. This could potentially affect skeletal development in the growing horse and athletic performance in the exercising horse.

Diets for horses contain concentrates which have a typical DCAD range between 80-120 mEq/kg DM and forages that have a typical DCAD range between 400-600 mEq/kg DM (Baker et al., 1992). As concentrates in the equine diet replace hay to increase energy density of the diet for athletic performance, the DCAD would be lowered possibly inducing acidosis that may be detrimental to performance.

Dietary alterations that may avoid lowering DCAD in diets for athletic horses would include fat supplementation and lowering dietary protein. The addition of fat to the diet will increase the energy density of the diet without adding anions (as well as

cations) and hence not alter DCAD. Protein contains sulfur and phosphorus that are used to calculate DCAD. Restricting protein risks deficiency and can decrease the level of anions and widen DCAD (Kronfeld et al., 1998b).

### **Acid-Base Status**

Increasing  $[H^+]$  contributes to fatigue by altering proteins which affects contractile proteins, sarcoplasmic reticulum function as well as enzyme kinetics (Mainwood and Renaud, 1985). Acid-base status has commonly been evaluated using the traditional bicarbonate buffering system. This system has limitations and therefore, a more comprehensive system was developed by Stewart (1981). Although this system appears to be better, it is not without weakness (Cameron, 1989).

*Bicarbonate System.* The bicarbonate buffering system is defined by the Henderson-Hasselbach equation. This equation describes pH as a function of bicarbonate and carbon dioxide along with its dissociation constant. The equation is as follows:

$$pH = pK'_a + \log \left( \frac{[HCO_3^-]}{d \cdot pCO_2} \right)$$

where  $pK'_a$  is the dissociation constant of carbonic acid (6.1 at 37° C) and  $d$  is a combination of an equilibrium constant and the solubility of  $CO_2$ . Measurements of pH and  $pCO_2$  are made, and bicarbonate is calculated indirectly from pH and  $pCO_2$  (Cameron, 1989).

*Advantages.* The advantage of the bicarbonate system is that pH and  $pCO_2$  are relatively easy to measure. The Henderson-Hasselbach equation is physico-chemically correct. This system has been used quite extensively to explain changes in acid-base. The assumption that  $pCO_2$  is under respiratory control while  $[HCO_3^-]$  is under metabolic control allows for explanations of changes in acid-base to be categorized as either

respiratory or metabolic in nature. Researchers have also argued that the respiratory and metabolic components of the system allow the bicarbonate buffering capacity to increase despite the pK of 6.1 which is lower than the pH of blood at 7.4 (Kronfeld, 1996).

*Limitations.* The Henderson-Hasselbach equation however, cannot always explain the behavior of changes in pH. The log relationship between  $p\text{CO}_2$  and pH as described by the bicarbonate system is linear. However, studies have demonstrated that the linear relationship is displaced by changes in protein, sodium or chloride (Constable, 1997). This shows that factors other than pK,  $p\text{CO}_2$  and  $[\text{HCO}_3^-]$  affect hydrogen ion concentration. The Henderson-Hasselbach equation is unable to explain these changes.

The dissociation constant of the bicarbonate system is 6.1 that is far removed from the pH of blood at 7.4 resulting in a weak buffering at physiological pH levels. The dissociation constant is also affected by temperature that would change its buffering capacity. When  $\text{CO}_2$  produced during oxidation enters the blood stream, it will diffuse into red blood cells where it will be hydrated with water and form carbonic acid. Carbonic acid dissociates into  $\text{H}^+$  and  $\text{HCO}_3^-$ . The hydrogen ion combines with hemoglobin while  $\text{HCO}_3^-$  moves out of the cell causing chloride to move into the cell (chloride shift)(Carlson, 1995). This process is reversed in the lungs. As such, it becomes apparent that  $\text{CO}_2$  is affected by respiratory as well as metabolic functions and as it increases, it will also increase both  $[\text{H}^+]$  and  $[\text{HCO}_3^-]$ . Therefore, any interpretation such as the Henderson-Hasselbach equation that implies that  $[\text{HCO}_3^-]$  partially determines  $[\text{H}^+]$  is physiologically incorrect (Kronfeld et al., 1998b).

The effect of  $p\text{CO}_2$  on both  $[\text{H}^+]$  and  $[\text{HCO}_3^-]$  causes traditional terminology of respiratory and metabolic acidosis/alkalosis as well as the interpretations of the Henderson-Hasselbach equation to become incorrect. Respiratory acidosis and alkalosis refers to an increase or decrease, respectively, in  $p\text{CO}_2$  whereas metabolic acidosis and alkalosis refer to a decrease or increase, respectively, in bicarbonate. Since bicarbonate is

dependent on  $p\text{CO}_2$  this makes the separation of this terminology incorrect and implies that changes in  $[\text{HCO}_3^-]$  are strictly metabolic which is not correct (Kronfeld et al., 1998b).

As mentioned earlier, changes in sodium and chloride were shown to affect the linear relationship between  $\text{CO}_2$  and pH. This suggests that strong ions such as  $\text{Na}^+$  and  $\text{Cl}^-$  affect pH. During strenuous exercise changes in plasma strong ion concentrations occur including increases in  $\text{Na}^+$ ,  $\text{K}^+$  as well as lactate ( $\text{La}^-$ ) and often a decrease in  $\text{Cl}^-$  (Carlson, 1995). These changes are not accounted for in the bicarbonate system. Also, during exercise, plasma volume changes with movement of water into cells thus changing plasma volume and protein concentration. The bicarbonate system also does not account for these changes in water or protein concentration (Constable, 1997).

Therefore, the limitations of the bicarbonate system include the implied causation between  $[\text{H}^+]$  and  $[\text{HCO}_3^-]$  and the assumption that  $[\text{HCO}_3^-]$  is under strictly metabolic control. The bicarbonate system is also unable to account for changes in strong ions, protein changes and water shifts that have been shown to affect pH.

*Stewart System.* The Stewart system is referred to as a comprehensive system because it accounts for all of the factors that affect  $[\text{H}^+]$  and  $[\text{HCO}_3^-]$ . In this system,  $[\text{H}^+]$  and  $[\text{HCO}_3^-]$  are shown to be dependent variables for which behavior can be explained through a series of equations. These equations involve the physico-chemical analysis of four isolated solutions containing 1) pure water, 2) strong ions, 3) weak electrolytes and 4)  $\text{CO}_2$  (Stewart, 1981).

Results produce six equations that when solved simultaneously, reveal three independent variables that dictate changes in dependent variables ( $\text{H}^+$  and  $\text{HCO}_3^-$ ). The three independent variables are  $p\text{CO}_2$ , strong ion difference (SID) and total weak acids (Atot). Strong ion difference is calculated as the algebraic sum of  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$  and  $\text{La}^-$ .

Total weak acids are mainly proteins and phosphate, estimated from plasma albumin or total protein sometimes in combination with phosphate (Stewart, 1981).

The equations involve 1) dissociation of water and its equilibrium constant, 2) complete dissociation of strong ions to maintain electrical neutrality, 3) partial dissociation of weak electrolytes and 4) equilibrium constant allowing for the dissociated and non-dissociated forms, 5) behavior of CO<sub>2</sub> in its various forms (dissolved CO<sub>2</sub>, carbonic acid and bicarbonate) and 6) dissociation of those forms of CO<sub>2</sub>. All the equations must conform to the laws of dissociation equilibrium and maintenance of electrical neutrality as well as the conservation of mass (Stewart, 1981).

*Advantages.* The main advantage of the Stewart system is first and foremost that it is comprehensive, so reveals information not evident in the bicarbonate buffer (Stewart, 1981). It takes into account changes in pH and [HCO<sub>3</sub><sup>-</sup>] due to strong ions, pCO<sub>2</sub> as well as changes in protein concentration. The equations make the system physio-chemically correct as well as physiologically correct. As pCO<sub>2</sub> or Atot increases, [H<sup>+</sup>] increases. If SID is increased however, [H<sup>+</sup>] decreases. Likewise, an increase in pCO<sub>2</sub> or SID will increase [HCO<sub>3</sub><sup>-</sup>] while an increase in Atot will cause a decrease in [HCO<sub>3</sub><sup>-</sup>].

This system will also reveal alterations in [H<sup>+</sup>] or [HCO<sub>3</sub><sup>-</sup>] due to changes in water concentration that would lead to concentration alkalosis and dilutional acidosis. For example, when water shifts from plasma to interstitial fluid and, perhaps, cells during strenuous exercise decreasing plasma volume, this causes an increase in plasma sodium. The concentration of sodium seen with a change in plasma volume would in theory produce an increase in [H<sup>+</sup>] due to the concentration of plasma. However, instead of [H<sup>+</sup>] increasing, it decreases. This decrease is due to the fact that increases in [Na<sup>+</sup>] are greater than increases in [Cl<sup>-</sup>], so SID increases and [H<sup>+</sup>] decreases. In reality however, changes during exercise do not involve an increase in sodium exclusively. What is generally seen

results in increases in sodium, potassium and lactate as well as a decrease in plasma chloride (due to the chloride shift) resulting in no change in SID (Carlson, 1995).

The Stewart system also allows for correct use of respiratory versus metabolic acidosis and alkalosis terminology. Arterial  $p\text{CO}_2$  is a good measure of respiratory adequacy while venous  $p\text{CO}_2$  is a measure of production by muscle. Strong ion difference is a good measure of metabolic status since it is not affected by  $\text{CO}_2$ .

Therefore, overall advantages of the Stewart system involve a comprehensive examination of effects of  $p\text{CO}_2$ , SID and  $A_{\text{tot}}$  on the dependent variables,  $\text{H}^+$  and  $\text{HCO}_3^-$ . This system also accounts for any changes in water shifts. It reveals changes in opposing variables that cancel out and are not evident in the bicarbonate buffer system.

*Limitations.* Although the Stewart system appears to be superior to the bicarbonate system, it is not without limitations. The main problem with the Stewart system is in estimation of  $A_{\text{tot}}$ . Total weak acids consist mainly of protein and phosphate and are estimated by either albumin or total protein (sometimes in combination with phosphate). The problem arises because composition of blood proteins varies between species. Recent work that simplified the strong ion model of Stewart, found  $A_{\text{tot}}$  to be  $15.0 \pm 3.1$  mEq/L for the horse. The author gave an equation to estimate  $A_{\text{tot}}$  by using total protein ( $2.24 \times \text{TP}$  in g/L) with normal phosphate levels (Constable, 1997).

Another weakness in the system is the lack of inclusion of all strong cations and anions in SID may contribute to an error in this variable. There is debate over how to calculate SID and which cations and anions should be included. Undetermined anions that may affect SID include beta-hydroxybutyrate, pyruvate or citrate that are not currently used in calculating SID (Constable, 1997).

*Effect of Fat Adaptation on Acid-Base.* Fat adaptation, which is the feeding of a

high fat diet in conjunction with training, may take up to 12 weeks to be complete. Use of a high fat diet during training results in preferential oxidation of fat over glucose for energy. Oxidizing fat compared to glucose, results in less CO<sub>2</sub> production (fat oxidation results in 1 mole of CO<sub>2</sub> for every 8 ATP generated while glucose oxidation results in 1 mole of CO<sub>2</sub> for every 6 ATP generated on an equal O<sub>2</sub> basis because fat contains less oxygen than glucose). Less CO<sub>2</sub> production will result in less H<sup>+</sup> accumulation. Accumulation of H<sup>+</sup> contributes to fatigue by altering proteins. Negative effects are observed on enzyme kinetics, contractile proteins and sarcoplasmic reticulum function which can all contribute to fatigue (Mainwood and Renaud, 1985).

Studies have also observed changes in lactate accumulation with fat adaptation which will affect SID and thus acid-base status. Aerobic exercise and fat adaptation have been shown to spare muscle glycogen (Greiwe et al., 1989) which will result in less lactate accumulation. Preferential fat oxidation will increase citrate levels that inhibit phosphofructokinase. This will cause an accumulation of glucose-6-phosphate and decrease muscle glycogen breakdown. As glucose-6-phosphate accumulates, hexokinase will be inhibited which will decrease blood glucose uptake (Randle, 1986).

Anaerobic exercise, however, has shown varying results with several studies demonstrating increased lactate concentrations for fat adapted horses during sprints (Oldham et al., 1990) or no difference in lactate response (Taylor et al., 1995a). This response may be due to the inhibitory effect of acetyl-CoA accumulation that occurs during fat oxidation which will inhibit pyruvate dehydrogenase and shunt pyruvate to lactate instead of into the Krebs's cycle (Randle, 1986). Changes in lactate responses with fat adaptation can affect SID which in turn can affect [H<sup>+</sup>] and [HCO<sub>3</sub><sup>-</sup>] and hence acid-base status.

Therefore, the main influences of fat adaptation on acid-base include decreased CO<sub>2</sub> levels and changes in lactate (and thus SID) depending on the type of exercise which

will affect  $H^+$  and  $HCO_3^-$ .

Dietary protein can also affect acid-base. Protein contains sulfur and phosphorus which upon oxidation yields sulfate and phosphate which are acidogenic (Patience, 1990). Sulfur and phosphorus also contribute to the calculation of DCAD and will lower the level of DCAD. Lowering DCAD will increase  $[H^+]$  and decrease  $[HCO_3^-]$ .

### **Changes in acid-base during exercise.**

In horses during exercise, venous  $pCO_2$  goes up due to production of  $CO_2$  during oxidation in muscle. This  $CO_2$  diffuses into plasma and is quickly taken into the erythrocyte. Here,  $CO_2$  is hydrated with water to form  $H^+$  and  $HCO_3^-$ . Bicarbonate diffuses into plasma in exchange for  $Cl^-$  thus  $Cl^-$  concentration decreases in plasma since more is taken into the RBC in exchange for  $HCO_3^-$ . Plasma  $Na^+$  increases due to changes in plasma water. The main shifts in water are from plasma into the interstitial space. Some water is believed to move into muscle. There is also belief that some sodium moves into muscle as well, probably in exchange for  $H^+$ . This may be a way to help remove free  $H^+$  from muscle and delay fatigue (Carlson, 1995). Plasma potassium also increases during exercise as a result of  $K^+$  moving out of contracting muscle. The level of  $K^+$  is related to exercise intensity and is correlated with lactate (Harris and Snow, 1988). Potassium is believed to be a vasodilator and may diffuse out of muscle to increase blood flow to muscle. This increase in plasma  $K^+$  results in a concurrent decrease in muscle  $K^+$ . A decrease in muscle  $K^+$  decreases the activity of the  $Na^+/K^+$  voltage receptors and makes the muscle fiber less sensitive to neural stimulus (Hyypya and Poso, 1998).

During ATP regeneration in muscle, at high intensity, lactate is formed as a by-product of anaerobic metabolism. Lactate readily diffuses out of muscle and enters plasma. Lactate will also enter erythrocytes that act as sinks for lactate. Accumulation o

lactate is a function of production as well as clearance. At low exercise intensities such as endurance exercise, lactate clearance keeps up with production and thus no accumulation takes place. During high intensity exercise, production increases exponentially while clearance reaches an asymptote and thus accumulation of lactate occurs (Kronfeld et al., 1998c). Lactate threshold is defined as the point in which accumulation is observed. In humans this has been determined to be approximately 4 mmol/L. Training will increase the lactate threshold. Training also increases the amount of fat oxidation thus sparing glycogen and decreasing lactate accumulation. Lactate can also be used as a fuel by slow twitch muscle fibers and heart as well as be converted to glucose by the liver to help maintain blood glucose (Brooks, 1988).

Arterial  $pO_2$  and  $pCO_2$  are representative of respiratory function. During exercise  $pO_2$  will be maintained if ventilation increases adequately, otherwise a decrease will result (Rose et al., 1983). In Arabians, hypoxemia is rarely seen (Taylor et al., 1995b) compared to other horses. If ventilation increases enough,  $pCO_2$  will actually decrease and will only increase if respiration does not increase adequately.

Several studies have shown that the main influence on the increase in  $[H^+]$  during exercise is due to  $pCO_2$  (Lindinger et al, 1992; Pieschel et al., 1992; Kronfeld et al., 1998a). Strong ion difference has a smaller effect on  $[H^+]$  but has a larger effect on  $[HCO_3^-]$  (Ferrante, 1994).

### **Protein and acid-base**

Proteins are made of amino acids. Amino acids can be positively or negatively charged. They can act as weak acids, in the case of albumin, or buffers in the case of histidine. Histidine contains imidazole side chains that attract protons. Substances in plasma such as histidine and  $\alpha$ -amino groups of amino acids that have  $pK_a$  values between 5.9 and 8.9 act as buffers at physiological pH (Constable, 1997). Carnosine

found in muscle fibers acts as a buffer and contains high levels of histidine which probably contributes to its effectiveness as a buffer (Lawrence, 1992).

Also, released during amino acid oxidation are  $\text{SO}_4^-$  and  $\text{H}_2\text{PO}_4^-$  due to sulfur and phosphorus contained in amino acids such as methionine and cysteine. Oxidation of 1 mole of sulfur containing amino acids (methionine and cysteine) results in production of 2 moles of endogenous acid. Oxidation of dibasic and phosphorylated amino acids also accounts for the endogenous acid load (Patience, 1990). Endogenous acid production from protein ingestion, oxidation and ureagenesis is estimated to be the sum of two times the quantities of ingested sulfur amino acids plus phosphorylated amino acids (MacKensie, 1986).

Lowering the amount of protein may also widen DCAB by lowering P and S in the diet however, just because they are in the diet does not mean they will be oxidized and contribute to the acid load. Having to remove fewer excesses of amino acids through the urea cycle also saves energy. In one study, human subjects fed either high (120g CP/d) or low (60 g CP/d) protein found the additional endogenous acid production was entirely attributable to the sulfur amino acid content of the diet (Trilok and Draper, 1989). Recently, a study found that the main contributors to renal net acid production (RNAE) was positively correlated with protein content ( $r^2 = .62$ ) and negatively correlated with potassium content of the diet (Frassetto et al., 1998).

### **Fat Supplementation/Adaptation**

The athletic horse has high-energy demands that cannot be met by the traditional substrate of forages that are high in fiber. Therefore, more energy dense products such as grains are incorporated into the horses' diet. Grains are high in sugars and starches. These substrates have a limited digestive capacity to the horse due to the design of their digestive system. Efficiency of digestion of starch is very high in the horse, however

levels that exceed .4% of the horse's body weight per meal have shown significantly greater amounts of starch entering the hindgut thus risking digestive disturbances (Potter, 1992).

An overload of sugar/starch may cause some undigested starch to enter the hindgut where it will be fermented and produce lactic acid. This increase in lactate production destroys lactate-utilizing bacteria in the gut and allows lactate to accumulate. This acidic environment will cause the pH in the gut to change which will alter the microbial population of the hindgut. A cellulitic bacteria begin to die off they release endotoxins which are absorbed into the blood stream. Endotoxemia has been linked to founder and laminitis. The fermentation of starch also leads to production of excessive gas. Decreased gut pH and gas production causes distension of the gut which will cause abdominal pain (colic) as well as the development of diarrhea (Clarke, 1990).

Another energy dense substrate that has shown promise for the exercising horse is fat. Levels of fat up to 20% have been fed to exercising horses up to 34 weeks without adverse effects. The horse appears to be very efficient with 5-15% dietary fat without adverse effects on fermentation (Potter et al., 1992). Higher levels of fat are not efficiently digested by the horse and thus exceed the capacity of the foregut and enter the hindgut. There is no useful digestion of fat in the hindgut (none that benefits the horse) and excess fat may interfere with microbial fermentation as it does in ruminants, and cause loose stools. Also, high levels of fat have not been very palatable to the horse. Success in feeding fat to horses has been observed with corn oil and rice bran. Horses on high fat diets have shown a glucose sparing effect, less spontaneous activity and less metabolic heat. There is also less water retained in the gut but with less metabolic heat, this is not detrimental to the horse and reduces fecal output (Kronfeld et al., 1996).

Fat adaptation combined with exercise takes about 12 weeks (Kronfeld et al., 1998c). Benefits include increased muscle glycogen, improved stamina as well as

improved sprint racing times (Harkins et al., 1992). There is an increase in the oxidative capacity of the horse with training that includes the increase in capillarization of the muscle fibers, increased conversion of type IIb fibers to type IIa fibers which are capable of aerobic metabolism. A higher number of mitochondria increases aerobic capacity. The lactate threshold also is increased which decreases glycogen use and may delay fatigue (Hodgson, 1986). If these benefits are combined with an increase in a desirable substrate such as free fatty acids (FFA) then performance should be improved.

Sources of feedstuffs may affect the use of glucose or fat during exercise. A study in which alfalfa hay or corn was fed prior to exercise saw a greater increase in FF when fed the alfalfa diet compared to the corn feeding (Zimmerman et al., 1992). The corn diet was much higher in soluble carbohydrate (78.8% CHO-H vs. 43% for alfalfa) but alfalfa was only 1.6% starch compared to 66.4% starch in the corn. Thus the glycemic response from the meal of corn increased insulin release which would inhibit mobilization of fat and thus interfere with the use of FFA during exercise.

*Use of high fat feeds.* Use of high fat feeds has become increasingly popular and has shown many benefits for the exercising horse. Care should be taken to introduce a change in diet slowly to prevent digestive upsets. The horse's body weight and body condition score should also be monitored to prevent obesity from the increase in energy density from additional fat.

Acceptance of fat in the diet has been shown to be very high when using corn oil and even lecithin/corn oil blends (Holland et al., 1998a). Animal fat is not generally palatable to the horse. Amounts of fat, in the form of oil, vary from ¼ cup twice daily to 1 cup twice daily have been fed in practice without problems. Also, a balanced ration with added fat should be fed (such as a commercial grain product) rather than top-dressing with fat. This will allow minerals and vitamins to be balanced in the diet if fed according to the directions. Top-dressing fat onto an already balanced ration will dilute

the concentrations of essential nutrients and risk imbalances.

*Increased Energy Density of Diet/Decreased Amount of Starch.* Fat provides 2.25 times the amount of digestible energy per pound compared to starch. Therefore, use of fat can increase the energy density of the diet. One study observed that 15% less feed was needed to maintain constant body weight in exercising horses when fed a diet that was 8% fat (Hintz et al., 1978). With an increase in energy density of the diet, less feed will be needed to meet nutritional needs. Less feed will result in smaller meals, which can improve utilization of the feed as well as reduce the risk of colic. Increasing the fat level in the diet will also decrease the starch level in the diet.

*Behavior.* Horses fed high fat diets have been shown to be calmer with less spontaneous activity and have less reaction to external stimuli. This is in contrast to horses fed high carbohydrate diets that have been reported to be “hot” and more difficult to handle (Holland et al., 1998b).

*Heat and Water Balance.* High fat diets produce less heat of fermentation allowing the horse to make more efficient use of the metabolizable energy portion of the diet (Scott et al., 1993). This results in more net energy available for work. Less heat of fermentation also decreases heat production in the body and will be an advantage for the horse exercising in hot, humid conditions. Increasing body temperature contributes to fatigue and is exacerbated in the heat (Kronfeld et al., 1998b).

Increasing energy density of the diet may decrease the voluntary consumption of hay or pasture by the horse resulting in less fiber intake. Fiber binds water in the hindgut, which increases water weight and fecal weight due to increased fecal water. Lowering the fiber level in the hindgut by using a high fat feed will lower the amount of water in the hindgut and thus weight in the digestive tract. Lowering weight will benefit the horse whether the horse is racing or participating in an endurance race. Lowering the water in

the gut may not seem like an advantage at first, however, lowering gut water b decreasing the fiber in the gut will also decrease the amount of fecal water lost which may benefit the horse when exercising in the heat (Kronfeld et al., 1996).

*Improved Fatty Acid Oxidation.* Feeding high fat diets increases the availability of fatty acids to the muscle for use as fuel. Combined with interval training which increases the oxidative capacity of the horse (increased blood flow with improved oxygenation, increased mitochondria and fat oxidation enzymes), fat will be the preferred substrate during exercise. This is particularly true during aerobic exercise at low to moderate intensity. Studies observed horses fed high fat diets having increased levels of fatty acids in the bloodstream prior to exercise and during recovery (Harkins et al., 1992). Lower lactate levels were also reported which is evidence of increased fatty acid oxidation. Fatty acid oxidation also produces less CO<sub>2</sub>, which will improve acid-base status of the horse (less hydrogen ion accumulation) and well as minimize respirator stress to remove CO<sub>2</sub> from the body. Increased fatty acid oxidation will improve stamina at low to moderate intensity exercise and delay fatigue (Eaton et al., 1995).

*Glycogen Sparing.* Use of high fat feeds has been shown to increase the use of fat during exercise. This will decrease the use of glucose or glycogen. Increased fa oxidation results in an increase in citrate levels in the Krebs' cycle. Citrate inhibits phosphofructokinase in glycolysis that results in an accumulation of glucose-6-phosphate. Hexokinase is inhibited by high levels of glucose-6-phosphate that will inhibit the breakdown of glycogen and decrease the uptake of blood glucose. Horses fed high fa diets containing up to 12% fat have shown increased muscle glycogen levels as well as increased levels of blood glucose prior to exercise and during recovery (Hambleton et al., 1980).

Depending on type of exercise (aerobic vs. anaerobic), use of glycogen may or may not be different for horses fed high fat diets compared to traditional diets. During

aerobic exercise, muscle glycogen use appears to be less for horses fed a high-fat diet as represented by lower lactate levels (Griewe et al., 1989). Decreased use of muscle glycogen is accomplished by fat shutting of glycolysis and the use of muscle glycogen as described above. During anaerobic exercise, studies have shown either no difference in glycogen use or increased glycogen use for fat adapted horses reflected in no change or an increase in lactate levels (Taylor et al., 1995a; Harkins et al., 1992 respectively). In this case, acetyl-CoA accumulation (due to lack of oxygen to enter the Krebs's cycle) will inhibit pyruvate dehydrogenase that will prevent pyruvate from entering the Krebs's cycle. Pyruvate will convert to lactate instead. Increased formation of lactate may appear to be detrimental yet increased utilization of glycogen that generated lactate may have improved power output (Kronfeld et al., 1995).

*Protein Sparing.* Fat has been shown to spare protein when added to the diet. The result is transitory in that short term experiments have shown an increase in nitrogen excretion when carbohydrates were replaced by fat however, nitrogen balance was achieved with fat after four days (Munro, 1964). Also, addition of fat to the diet without totally replacing the carbohydrate resulted in increased nitrogen retention (Brennan et al., 1975). During periods of high energy demand when energy supply is marginal to deficient (trauma, starvation), fat has a beneficial protein-sparing effect through preferential oxidation of triglycerides (Donoghue, 1989). Exercise may also be an example of a high energy demand and may be influenced by a protein-sparing effect of fat.

Overall, it appears that combination of a high-fat diet and interval training elicit benefits to the horse regardless of its lifestyle or activity. Improvements in endurance as well as speed have been seen in horses fed high fat-diets. Using care in the addition of fat to the diet as described here can assist the horse in the adjustment to meal feeding due to increased energy and performance demands on the horse.

**Summary**

Fatigue is related to an increase in intracellular  $[H^+]$  as well as low blood glucose and decreased muscle glycogen. The combination of high fat and low protein may be the ideal combination to avoid these adverse affects on exercise. High fat helps spare glucose and glycogen thus maintaining blood glucose levels while restricting protein minimizes acidosis and widens the DCAD that improves acid-base balance.

Therefore, the following studies were undertaken to determine the effect o restricted dietary protein fortified with limiting amino acids on acid-base responses t repeated sprints in Arabians fed high fat diets. An evaluation of protein status was also made to determine if the lower level of protein was adequate for these exercising horses.

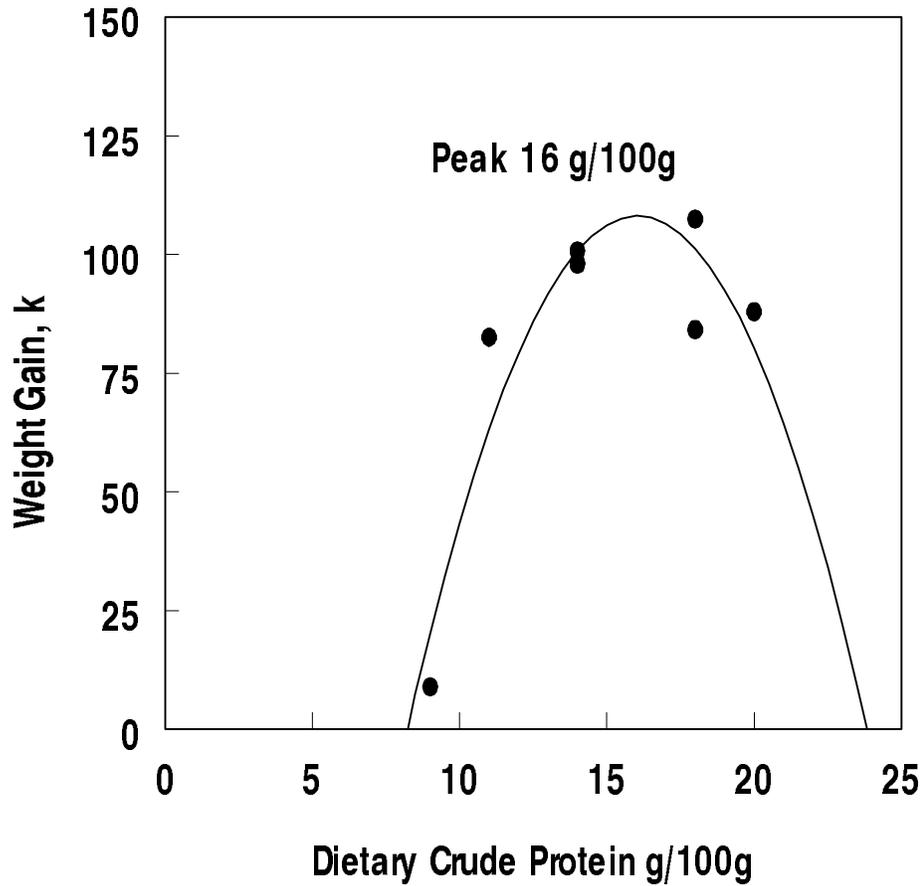


Figure 1. Optimum dietary protein for growing horses fit on a parabolic curve using data from three growth studies (Yoakum et al., 1978; Ott et al., 1979; Shryver et al., 1987).