Standardisation of methods for studies with laying hens was the objective of the present studies.

2. Current State of Knowledge – Literature Review

The present chapter introduces the different AA digestibility measurements and correction methods by considering the terminology improved during these studies. The advantages and disadvantages of them will be discussed and finally the references that studied these factors will be mentioned briefly. When these methods are studied, the different stages of digestibility, absorption and metabolism of ingested protein in animals as shown in Figure 1-1 are considered.

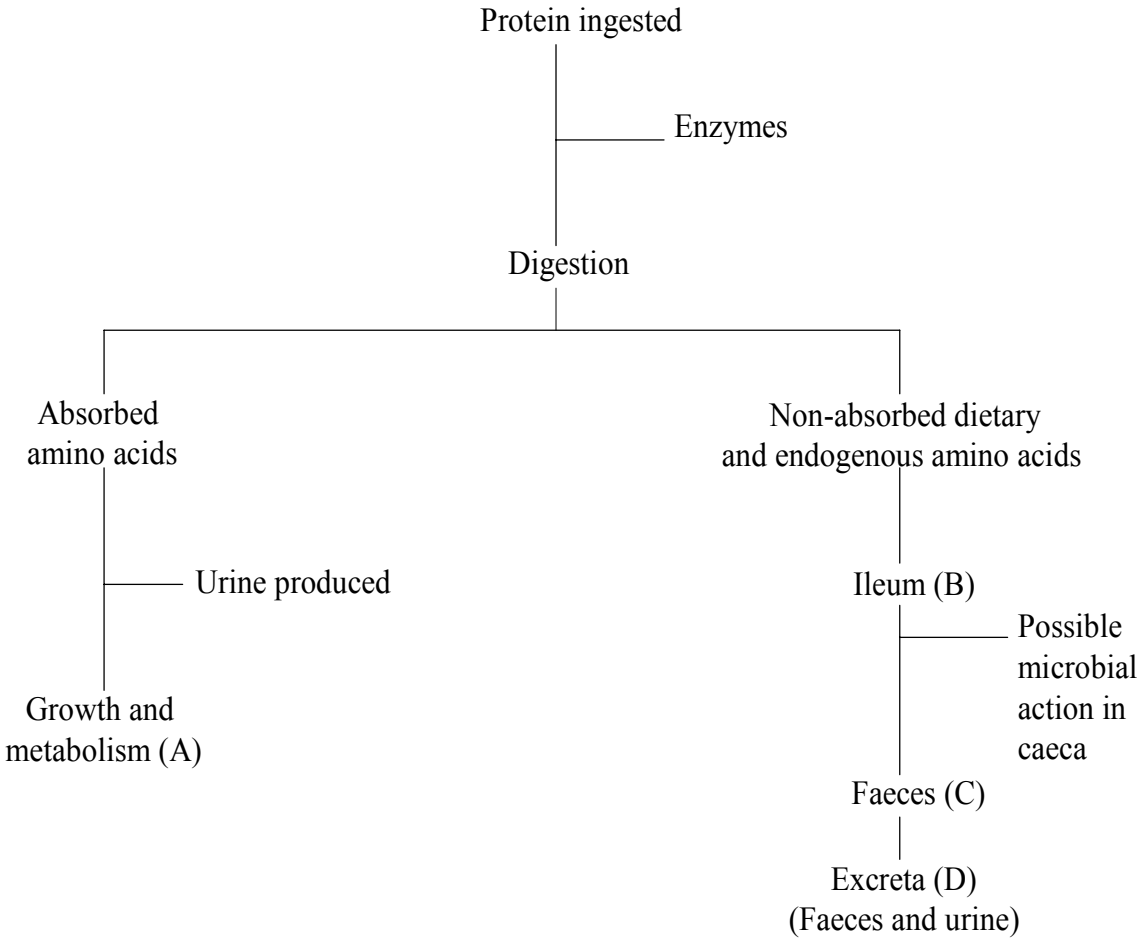

Figure 1-1. *Schematic diagram of measurements of AA availability (A), precaecal AA digestibility (B), faecal AA digestibility (C) and excreta AA digestibility (D) (Johnson, 1992)*

2.1. Total tract and precaecal digestibility

There have been numerous experiments in different sampling places for digestibility measurements. Figure 2-1 shows different parts of gastrointestinal tract (GIT) where such samplings can be made. Based on literature, the sampling places for digestibility measurements will be distributed within two main categories; Total tract (TT) and Precaecal (PC).

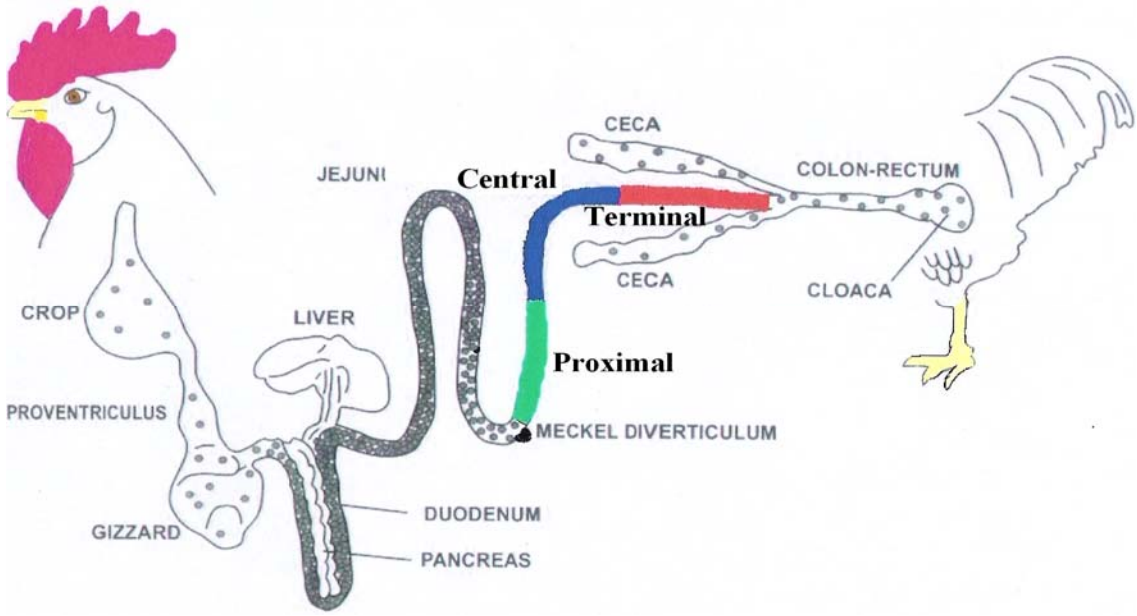

Figure 2-1. *Diagram showing gastrointestinal tract of poultry (Adapted and redrawn by author and quoted by Gauthier, 2005 from Herpol and Van Grembergen, 1967; Riis and Jokobsen, 1969; Hill, 1971; Simon and Versteeg, 1989)*

2.1.1. Total tract sampling

Total tract sampling means collecting all output of GIT in three ways: TT excreta collection in intact poultry, TT excreta collection in caecectomised poultry, TT faeces collection in caecectomised plus colostomised poultry.

2.1.1.1. Total tract excreta collection in intact poultry

Many published values currently available on digestible AAs for poultry are based on excreta analysis. This is because of its simplicity and because the assay can be carried out on large numbers without sacrificing the birds

(Angkanaporn *et al.*, 1997a; 1997b; Ravindran *et al.*, 1999). However in studies with excreta-based collection assay to determine AA digestibility, the values obtained for feedstuffs may have been over or underestimated (Norberg *et al.*, 2004). These samples are not very reliable, often due to the effects of hindgut micro-flora especially in the caeca that change the AA profile of digesta and widely diversifying digestibility results. A second disadvantage is the mixing of urine with faeces in poultry and forming together excreta.

2.1.1.2. Total tract excreta collection in caecectomised poultry

Caeca are the main sites of micro-flora activity in the hindgut. Because of this, caecectomy (surgically removing or ligation of caeca) has been proposed as a method for reducing micro-flora influence on AA digestibility measurement (Parsons, 1984). It has been suggested that bacteria may be able to synthesise AAs or utilise undigested AAs. By using guanine + cytosine profiling and 16S rDNA sequencing techniques 140 different genera and 640 different species of bacteria in the chicken GIT were found (Apajalahti *et al.,* 2004). The bulk of bacteria are distal to the ileum, which means that compounds supporting their growth have to escape host absorption (Apajalahti *et al.*, 2004).

Many studies indicate that the caecectomised bird may be a better model for estimating AA digestibility than the conventional bird. Parsons (1984) concluded that intestinal micro-flora had less influence on AA excretion by caecectomised hens than on that by conventional hens. Caecectomy has several advantages compared with ileal cannulation techniques which are used to measure AA digestibility. Caecectomy is a much simpler surgical procedure than is the implantation of ileal cannula. Caecectomised birds can be maintained much more easily than ileal cannulated birds; there are no problems associated with digesta passage or flow rate and there is no need for digesta markers since excreta can be collected quantitatively. Parsons (1986) showed that true digestibility values determined with caecectomised cockerels were lower than those determined with conventional cockerels and were in better agreement with chick availability values. True digestibilities of all sixteen measured AAs were lower for

caecectomised than for conventional cockerels, with the average difference being approximately 10 %. In other experiments there was also a tendency to overestimate digestibility of lower digestible feed ingredients when using intact cockerels (Dalibard and Paillard, 1995).

Other studies showed that the effect of the caeca in poultry is dependent on the feedstuff being measured. With feedstuffs such as meat and bone meal which may have a low AA digestibility the use of intact birds could result in an overestimation of AA digestibility. Therefore caecectomised birds should be used when measuring AA digestibility in poultry by excreta analysis. Johnson (1992) and Parsons *et al.* (1997) showed that AA digestibility (estimated by fasted roosters) of meat and bone meal values determined in caecectomised roosters were generally lower than those determined in conventional roosters. Ragland *et al.* (1999) in another study demonstrated that the effect of caecectomy is dependent on the feedstuff under consideration, and that the general effects of caecectomy are similar for ducks and chickens. It seems that the apparent AA digestibility (AAAD) of good quality protein sources may be determined using intact birds but the use of caecectomised birds to be preferred if the protein source is of poor digestibility (Angkanaporn *et al.*, 1997a). Johns *et al.* (1986b) also determined that the digestibility coefficients measured using caecectomised cockerels were lower than those determined with intact birds.

Son *et al.* (2000) showed that caecectomy had no significant effect on feed intake (FI) or body weight (BW) gain but caecectomy caused significantly higher moisture content in excreta. Karasawa *et al.* (1997) showed that ligation of the caecum significantly improved N balance and utilization by up to more than 2 times. The treatment significantly decreased uric acid excretion by 77 mg N/day and also total N excretion. The amount of faecal water excretion was increased by caecal ligation in colostomised chickens. It is concluded that the lower intestine plays a useful role in the water economy of chickens (Son and Karasawa, 2001).

Bacterial enumeration results, together with polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) profiles, showed that the composition of micro-flora in ileum of chickens was age dependent and

influenced by dietary fat source and antibiotic supplementation. An increased incidence of streptococci, enterobacteria, and *Clostridium perfringens* with age of the chickens was demonstrated. Lactobacilli and *C. perfringens* were the bacterial groups most strongly affected by the dietary treatments (Knarreborg *et al.*, 2002). However, by working with caecectomised birds the effect of urine on measurement is not omitted but some researchers have shown that the urinary AA contribution to total excreta AA is small and usually has a negligible effect on calculated digestibility values (Whittow, 2000). Also, Yamazaki (1983) found no differences between true AA digestibility (TAAD) values measured with colostomised hens and intact adult cockerels (quoted by Parsons, 1986).

2.1.1.3. Total tract faeces collection in caecectomised and colostomised poultry

Excreta analysis does not measure digestibility as classically defined but rather AA metabolisability, because faeces and urine are voided together in birds. Colostomy (making an artificial anus originating from the colon) is performed for removing the effect of urine from excreta in caecectomised poultry. Then it will be possible to collect TT faeces separately from urine (Ravindran *et al.,* 1999). Methods have been developed for separating faeces and urine in birds prior to excretion using such techniques as colostomy and exteriorisation of the ureters. However, the urinary contribution of AAs to excreta is generally not considered. The rationale being that, as the concentrations of AAs in urine is very small; it has negligible effect on digestibility estimates (Ravindran and Bryden, 1999).
In many avian species ureteral urine flows from the urodeum into the caeca passing through the colon and water absorption may occur in the colon and the caeca. The flux from the small intestine is also reported to fill the caeca in the chicken (Figure 2-2). Also dietary urea can be utilised through the caeca in chickens fed a low-protein diet. The amount of faecal water excretion was increased by caecal ligation in colostomised chickens. It was concluded that the lower intestine plays a useful role in the water economy of chickens (Son and Karasawa, 2001).

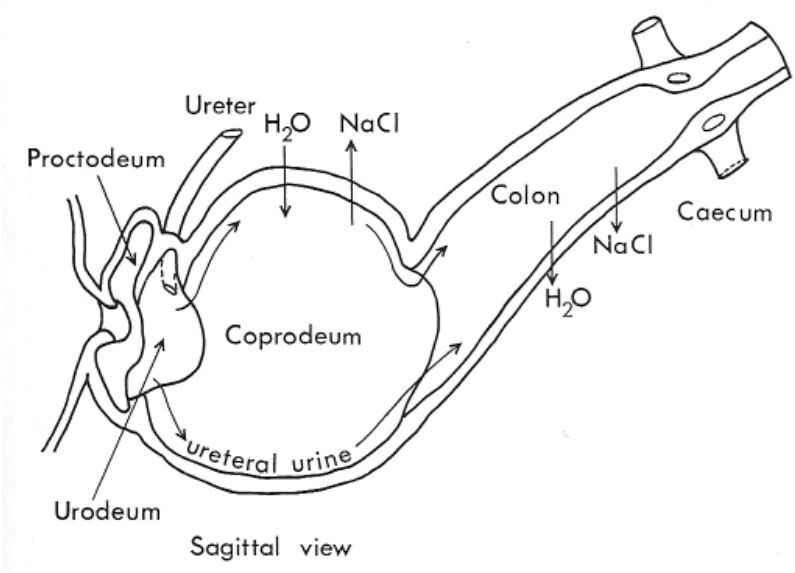

Figure 2-2. *Diagram of lower intestine of the domestic fowl, arrows indicate the retrograde flow of urine from urodeum to coprodeum, colon, and caeca, as well as possible directions for net fluxes of water and NaCl in coprodeum and colon (Whittow, 2000)*

2.1.2. Precaecal sampling

In terms of protein quality, the digestion of individual AAs up to the terminal ileum or PC digestibility (often referred to ileal digestibility) is gaining increasing attention in the feeding of both pigs and poultry (Ravindran and Bryden, 1999; Rodehutscord *et al.*, 2004). Payne *et al.* (1968) were the first to suggest that analysis of ileal contents rather than excreta may be a reliable method for assessing protein and AA digestibility in poultry (quoted by Ravindran *et al.*, 1999). It was because of the proteins may be degraded by hindgut micro-flora, and microbial cells may contribute to faecal protein output. These problems are largely avoided when digestibility measurements are based on PC digesta (Siriwan *et al.*, 1993; Ravindran *et al.*, 1999).

This method requires the collection of PC digesta after killing the birds or the use of cannulation. This means that digesta must be collected before reaching the ileo-caeca-colonic junction (ICCJ). With both approaches it is not possible to collect total digesta. Thus, diets must contain indigestible markers in order to calculate nutrient flow. Significant differences were

found between PC and excreta-based digestibility of certain AAs in some feed ingredients, with excreta values being usually higher than the PC values, indicating a net catabolism of AAs postileally. Kadim *et al.* (2002) reported that the degree of overestimation was often considerable, ranging from 8.9 % (digestibility of threonine in soybean meal) to 56 % (digestibility of aspartic acid in wheat). They concluded that digestibility values measured at the terminal ileum provide a more reliable measure of AA availability than those measured in the excreta.

In another study by Ten Doeschate *et al.* (1991) the AA digestibi1ity va1ues determined from i1ea1 digesta or faeces differed considerably. For eight AAs faeca1 digestibi1ity values were significant1y higher. Differences were observed between the digestibilities at faecal and PC level for most AAs. These differences are not for all AAs of the same magnitude and direction, so PC digestibility has to be determined for feed ingredients to assess their protein values.

The PC digestibility assay has two distinct advantages over that based on excreta analysis. Firstly the modifying action of the hindgut micro-flora on protein composition is avoided. Secondly, the complication arising from the combined voiding of faeces and urinary AAs and N is overcome. Moreover, it appears that AAs are not absorbed in the hindgut of the chicken in nutritionally significant quantities (Kadim *et al.*, 2002). The criticisms of the precision feeding assay can be overcome by determining PC digestibility. In this method, digesta are sampled from the distal part of the ileum and analysed. As a result, urine AA as a source of error and the modifying effects of hindgut microbial fermentation are eliminated (Ravindran and Bryden, 1999; Lemme *et al.*, 2004; Perttilä *et al.*, 2001b).

The amount of energy-yielding carbohydrates reaching the hindgut appears to determine whether net degradation or net synthesis of AAs will take place. When fermentable carbohydrates are limiting, the undigested nitrogenous substances will be deaminated by the microbes to ammonia and amines resulting in net disappearance of AAs. When fermentable carbohydrates are available, the microbes will utilise the ammonia and amines for the *de novo* synthesis of microbial proteins, resulting in net synthesis of AAs. It is noteworthy that the ileal-excreta digestibility

differences were rather large for poorly digestible feed ingredients such as feather meal, meat meal and meat-and-bone meal. This is to be expected because the lower the AA digestibilities at the ileum, the more undigested N will reach the hindgut, providing a substrate for microbial degradation resulting in large differences between PC and excreta digestibilities. In contrast, with the highly digestible feed ingredients such as fish meal and blood meal only modest differences were recorded (Ravindran *et al.,* 1999; Butts *et al.*, 2002).

2.1.2.1. Precaecal digesta collection after poultry slaughtering

The sampling of digesta from the terminal ileum after surgical modifications became standard in pigs, but is difficult to practise with young poultry. Instead, digesta is collected from a certain PC gut section immediately after slaughtering the birds. Using PC digestibility as a measure of protein quality implies a description of feedstuff potential. While working with cannula allows for a collection of digesta over a time period at the end of the ileum, sampling from slaughtered animals needs a certain sub-section of the terminal ileum in order to obtain a sufficiently large sample (Ravindran and Bryden, 1999; Kadim *et al.,* 2002; Rodehutscord and Mosenthin, 2005; Kluth *et al.*, 2005b). However, care must be taken to ensure that the bird is not severely startled or stressed during or just before killing to prevent the shedding or the lining of the gut mucosa (Short *et al.,* 1999).

Different opinions exist to how long the sampled ileum section in AA digestibility measurements should be. Kadim and Moughan (1997a) cited that the terminal 15 cm of the ileum was a suitable section for sampling ileal digesta from the broiler chicken. As expected, the length of ileum sampled had a significant effect on the proportion of ingested Cr recovered. It should be appreciated that the length of ileum chosen for sampling represents a proportion of the total ileum, the length of which is a function of the age and size of the birds.

Kluth *et al.* (2005b) reported that AAs still disappear from the small intestine of broilers posterior to Meckel's diverticulum (MD). It cannot be

differentiated as to what extent this is caused by absorption or secretion. However, digestibility studies aimed at measuring the potential of a protein source need a restriction in the sampled sub-section of the intestine. Kluth *et al.* (2005b) mentioned that the proximal third of the sub-section between MD and the ICCJ should not be sampled. It needs to be further studied whether the sub-section must be taken shorter as feed intake increases or it is dependent on poultry species.

2.1.2.2. Precaecal digesta collection after cannulation

A refinement is possible for collecting PC digesta without slaughtering the birds. Raharjo and Farell (1984) and Gurnsey and James (1985) outlined a procedure for inserting glass cannula into the terminal ileum of adult cockerels. A procedure for the ileostomisation of adult roosters has been described with the use of flexible silicon cannulas. Apparent PC digestibility coefficients for DM, crude protein (CP) and AAs in six diets, formulated with maize, wheat gluten, faba beans, lupins, soybean meal and casein as the main protein sources were determined in the ileostomised roosters fitted with silicon cannulas (Leeuwen *et al.,* 2000).

The simple T-cannulation procedure was used in some studies for collection of ileal digesta rather than the slaughter method, since sampling with the slaughter method could lead to a bias in results due to unrepresentative sample of digesta collected. Simple T-cannulation has been widely accepted by researchers as a means of sampling ileal digesta and at least for non-bulky diets and with frequent sampling, simple T-cannulation has been shown to be an acceptable technique (Donkoh and Moughan, 1994; Donkoh and Moughan, 1999).

The slaughter method needs many animals to collect enough digesta for analyses and to have a representative sample of the digesta over a longer period. Also the way of sampling is critical because from the dead intestine easily mucosa can be scraped off (Leeuwen *et al.,* 2000).

Killing the birds led to slightly lower PC digestibility values by carbon dioxide inhalation or bleeding than mechanical stunning and neck dislocation (Palander *et al.,* 2004a). Although the CO_2-stunning technique

is not recommended by these authors for collection of ileal digesta in the study of PC digestibility of AAs and N, this method may be suitable to measure the digestibility of organic matter or other dietary constituents of feedstuffs and diets (Prawirodigdo *et al.,* 1998).

2.2. True and apparent digestibility

The discrepancy between apparent (AAAD) and true AA digestibility (TAAD) arises when endogenous amino acid (EAA) secretions are taken into account or not (Dalibard and Paillard, 1995). With the true measure, correction is made for the flow of endogenous AAs. Angkanaporn *et al.* (1997b); Butts *et al.* (2002) and Kadim *et al.* (2002) reported that TAAD may provide more constant and meaningful data in AA absorption than AAAD. Fan and Sauer (2003) also reported that apparent PC digestibility values of CP and AA determined in barley samples are not reliable and should not be used in diet formulation for pigs but true PC digestibility of CP and AA determined from various barley samples should be used in diet formulation for pigs.

Endogenous AAs originate from digestive enzymes, mucoproteins, desquamated cells, AAs produced by cellular breakdown and albumin and other non-dietary but not strictly endogenous materials, such as bacteria and ingested hair (Donkoh and Moughan, 1999). In other words the AAs reaching the terminal ileum originate from different sources. Part comes from the dietary AAs that have not been absorbed and part from the AAs contained in the secreted endogenous protein. The latter have commonly been separated into two components, the 'basal' and the 'specific' EAA. Neither the amount nor the AA composition of the endogenous protein is constant. While the basal endogenous protein is commonly assumed to depend mainly on DM intake, the specific endogenous protein is affected by the amount and nature of the dietary protein under study like its digestibility, the fibre content, non-starch polysaccharide content and digesta viscosity and other anti-nutritional factors (Angkanaporn *et al.,* 1997b; Dänicke *et al.,* 2000; Souffrant, 2001). Hence, in feed evaluation studies the specific endogenous losses need to be considered for each feed

ingredient as they are part of that feed ingredient quality. Basal endogenous losses, in contrast, are not attributable to any feed ingredient. Within a complete evaluation system specific EAA are best considered as part of the animal's requirement, that is, a cost of feed consumption and passage. Consequently, in pigs, it has been suggested that the measured PC digestibility should be corrected by a certain factor in order to account for the contribution by the basal endogenous secretion, and then entitled 'true' digestibility (Sauer *et al.*, 2000).

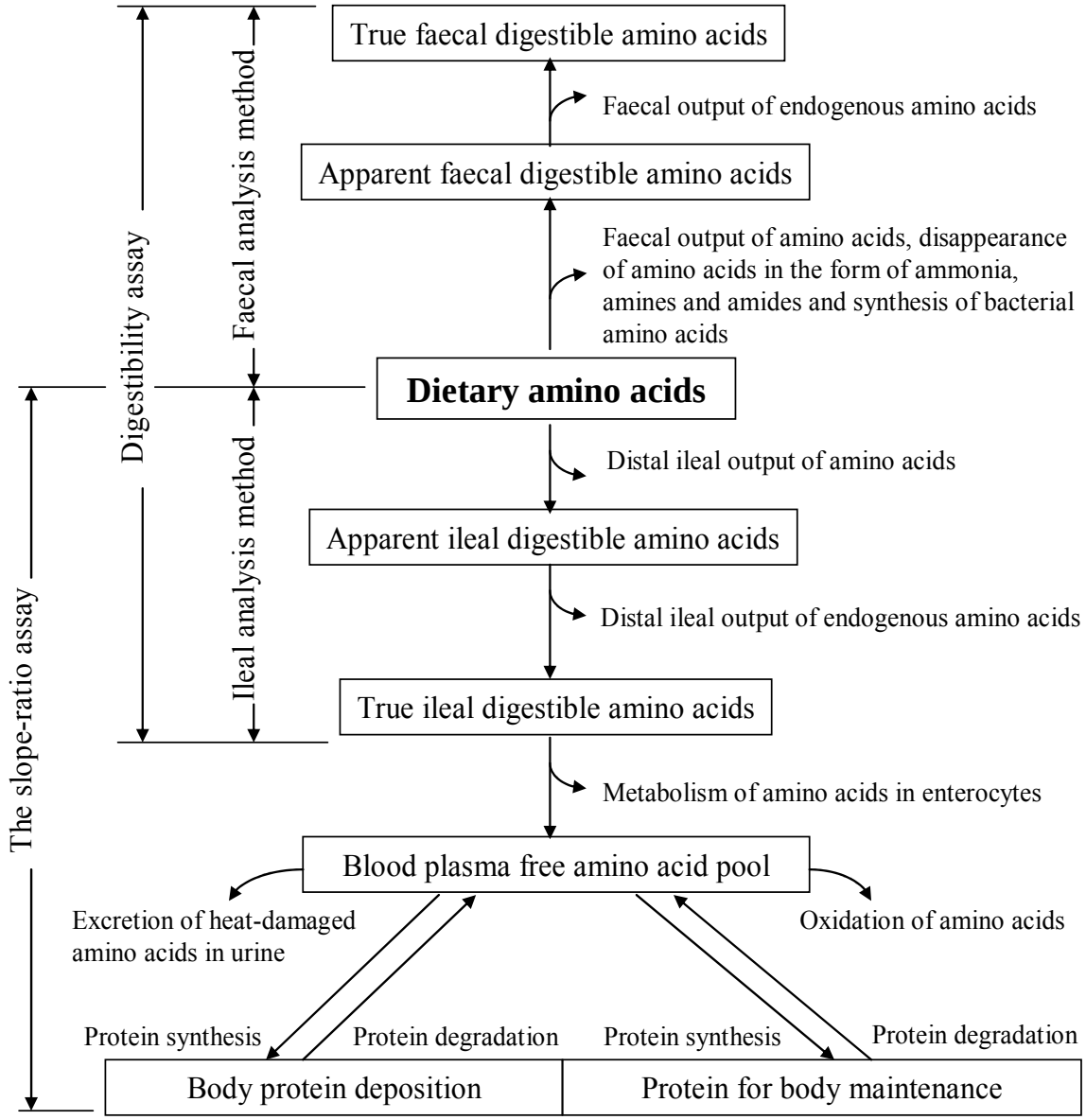

Figure 2-3. *Schematic representation of amino acid utilisation in growing pigs (Redrawn by author from Fan, 1994)*

Several factors are known to be partially responsible for the differences in the endogenous CP and AA outputs. These factors include determination methods and (or) techniques used, BW and physiological status, DM intake levels, dietary fibre levels and types, as reviewed by several authors (Donkoh and Moughan, 1999; Fan and Sauer, 2002; Fastinger and Mahan, 2003; Clarke and Wiseman, 2005).

The excreta and ileal assays described above determine 'apparent' values and do not account for EAA losses, which can have a variable effect on calculated digestibility coefficient. This effect is most pronounced when protein or AA intake is low. Apart from being influenced by dietary AA intakes, apparent digestibility values of individual feed ingredients are assumed to be additive when combined in diet formulations. The difference between standardised and AAAD ranged between 0 and 17 percentage points for cereal grains but only between 0 and 7 percentage points for plant protein sources and animal by-products (Lemme *et al.*, 2004). Theoretically, for a given AA, the apparent digestibility increases nonlinearly approaching a plateau as the quantity of AA intake increases because the proportion of endogenous excretion relative to total excretion decreases. As a result, when the ingested quantity of feed is very low, the calculated apparent digestibility underestimates the actual digestibility. By contrast, TAAD is not affected by the level of FI (Dalibard and Paillard, 1995).

2.3. Assay method

Various techniques have been evaluated to determine the output of EAAs. The classical approaches use N-free diets or fasted animals and regression analysis. However, N-free diets or fasting techniques have been criticised because during starvation or the absence of dietary protein, the body will be in negative N balance and the rate of whole-body protein synthesis will be reduced. In practical conditions amount of AAs in digesta can affect EAA secretion into the alimentary channel. For example, when AAs in digesta based on feed consumption increase, enzyme secretion increases also for

better digestion and then it is an error for measuring EAAs in N free diets (Parsons *et al.,* 1983; Adeola *et al.,* 1997).

The development of the peptide alimentation method (also known as enzymatically hydrolysed casein / ultra filtration method) by Moughan *et al.* (1990) overcomes some of the above limitations, enabling the measurement of EAA flow under more normal physiological conditions (quoted by Ravindran and Hendriks, 2004a). Peptide alimentation technique, a method for estimating ileal EAA flow, involves feeding the animal with peptides (from enzymatically hydrolysed casein) followed by ultra filtration of the ileal digesta. Although not subject to the criticisms of the traditional methods, this approach generates estimates applicable only to correction of ileal flows for protein sources, such as animal protein meals, which do not contain fibre and/or anti-nutritional factors. This technique may also underestimate endogenous flow because some endogenous free AAs and endogenous small peptides may be discarded in the low molecular weight fraction (Lemme *et al.,* 2004).

Another technique based on the guanidination of dietary proteins to distinguish between endogenous secretions and exogenous or dietary sources of AAs in intestinal digesta was proposed by Hagemeister and Erbersdobler in 1985 (quoted by Ravindran *et al.,* 1998). This technique is called Homoarginine approach, using homoarginine as a marker, to determine EAA secretions. Lysine residues in dietary proteins are transformed into homoarginine by guanidination which involves treatment with O-methyl isourea under alkaline conditions. After the labelled protein is fed, EAA losses are determined by comparing AA: homoarginine ratios in the diet and ileal digesta. Homoarginine is not found in normal feedstuffs. However, homoarginine is digested and absorbed in a manner similar to other AAs, but does not reappear in endogenous secretions into the gut. Two major problems were noted when continuous feeding of guanidinated proteins was attempted. Firstly, feeding diets containing guanidinated casein resulted in marked depressions in FI of chicks. Subsequent studies found that the reduced FI may reflect a direct effect of lysine deficiency and/or homoarginine on FI regulation. Secondly, preliminary observations indicated that low dietary electrolyte balance is a

problem in diets containing guanidinated proteins due to a chloride overload and that the diets need to be balanced for electrolytes to prevent the occurrence of watery excreta (Ravindran and Bryden, 1999).

The results showed that the ileal endogenous flows of N and AA are markedly enhanced by the presence of protein and peptides, above those determined following feeding of an N-free diet. It was concluded that the use of enzyme hydrolysed casein and homoarginine methods enables the measurement of ileal endogenous losses in chickens under normal physiological conditions (Ravindran *et al.*, 2004).

Isotope markers techniques have been involved as well. Numerous studies have been undertaken to measure EAAs using either stable (^{15}N) or radioactive isotopes (^{14}C, ^{35}S, ^{75}Se). Although attractive, this technique suffers from several constraints, because the ^{15}N enrichment of the endogenous secretions is not easy to determine. The inability to measure the recovery of all individual AAs in ileal digesta and the rapid precursor pool recycling are other drawbacks. Standardisation of conditions such as feeding frequency, diet type, and infusion protocol, rate of tracer infusion, sampling procedures, sample preparation and choice of precursor pool is necessary if reliable comparisons of data are to be made (Ravindran and Bryden, 1999).

The use of regression analysis where graded amounts of protein are given to animals also has been criticised. In this method, increasing levels of protein are fed and AA excretion is determined. The increased excretion of AAs, which may be from undigested feed and/or endogenous proteins, is assumed to be directly proportional to the increased intake. A regression equation is then used to calculate the AA excretion at zero protein intakes and this is considered to be an estimate of endogenous losses. This methodology, however, assumes that there are no changes in the amount of EAA secretions and that the increase of ileal AA flow is attributed entirely to increases in undigested feed proteins. Although the method overcomes the constraint of physiological abnormality, it incorrectly assumes that the flow of EAA does not vary with the amount of protein given. It has been shown that part of the increased ileal AA flow results from an increase in unabsorbed EAAs (Ravindran and Bryden, 1999).

Fan and Sauer (1997) reported that with regression technique, one can extrapolate the recovery of endogenous protein and each of the individual AA under relatively normal conditions of protein (AA) supply. They cited that linear relationships between dietary inputs and the ileal outputs of AA exist. Differences in ranges of graded dietary levels of AA affected the linear relationships and resulted in large differences in the estimation of the EAA levels. Therefore, the determination of a suitable range of graded dietary levels of AA is an important methodological aspect of the regression analysis technique. Furthermore, the results of their studies tend to suggest that the ileal outputs of AA, g/kg dry matter intake, can be linearly partitioned at different dietary levels of AA. The relative contributions of EAA, as percentages of dietary contents, curvilinearly decreased with increasing dietary contents of AA. The true PC digestibility values of AA appear to be independent of dietary AA contents. Adeola *et al.* (1997) also reported that regression analysis produced a higher estimation of ileal and faecal EAA excretion than feeding an N-free diet.

Attempts to measure EAA secretion leads, however, to highly variable results (Donkoh and Moughan, 1999) with poorly identified reasons for this variation. It is doubtful, therefore, whether the use of fixed values for EAAs to correct digestibility coefficients is a real improvement in the accuracy with which the quality of dietary protein is described (Angkanaporn *et al.,* 1997b; Rodehutscord *et al.,* 2004). However the regression analysis technique is potentially a very promising approach for digestibility estimation. The most advantageous point with a regression method is that AAD can be also measured without the need to measure EAA separately. In this method the relationship between AAs intake and AAs disappeared or unexcreted will be measured. The digestibility of each AA is the slope of this linear relationship between AA intake and AA disappeared (Short *et al.,* 1999; Fan and Sauer, 1995 and 2002; Rodehutscord *et al.,* 2004). The proportions of CP and AAs digested responded linearly to increased intake and the relationships between quantitative intake and digested amounts of AAs were described by simple or multiple linear regressions. The slope determined for each ingredient was taken as a measure of AA digestibility without the need for

consideration of basal EAA and CP secretions. Kluth *et al.* (2005a) reported that the multiple linear regression approach is a suitable method to measure AA digestibilities for feed ingredients. They interpreted that multiple linear regression approach measures AA digestibility of protein ingredients after excluding the effect of basal diet and basal EAAs on feed ingredients digestibility measurements. However in regression method it is not possible to exclude specific EAA from estimates but because specific EAA is the characteristic of each ingredient they should be considered as the cost of feed and should not necessarily be excluded (Rodehutscord *et al.,* 2004).

It can be concluded that approaches not depend on a separate determination of endogenous losses appear advantageous for the purpose of feed evaluation. Such an approach is the linear regression analysis which can be applied when at least two supplementary levels of the protein ingredient under study are used (Short *et al.,* 1999; Rodehutscord *et al.,* 2004; Kluth *et al.,* 2005a). Rodehutscord *et al.* (2004) reported the use of regression analysis for measuring the PC AA digestibility for rapeseed meal. The PC digestibility for AAs for field beans and peas was determined with linear regression analysis by Simon (2004) and Kluth *et al.* (2005a).

2.4. Factors affecting digestibility measurements

Several other factors have been studied in digestibility and EAA measurements. For example effect of feed intake (Zuprizal *et al.,* 1991; Furuya and Kaji, 1992; Butts *et al.*, 1993; Kadim and Moughan, 1997b; Hess and Seve, 1999; Stein *et al.,* 1999; Albin *et al.,* 2001; Stein *et al.,* 2001; Moter and Stein, 2004), feed processing (Zuprizal *et al.,* 1991; Amornthewaphat *et al.,* 2005), enzymes supplementation (Sebastian *et al.,* 1997; Hew *et al.,* 1999; Lap-Im *et al.,* 1999; Ravindran *et al.,* 2001; Perttilä *et al.,* 2001a; Rutherfurd *et al.,* 2002; Cowieson *et al.,* 2004; Rodehutscord *et al.,* 2004; Wang *et al.,* 2005), soluble non-starch polysaccharides (Dänicke *et al.,* 2000), markers (Jagger *et al.,* 1992; Kadim and Moughan, 1997a; Fan and Sauer, 2002; Fan and Sauer, 2003), feed particle size (Svihus and Hetland, 2001; Fastinger and Mahan, 2003), Poultry Species

(Huang *et al.,* 2000; Ravindran and Hendriks, 2004a; Ravindran and Hendriks, 2004b; Kluth and Rodehutscord, 2006), feeding regime (Kadim and Moughan, 1997a and 1997b; James *et al.,* 2002), anti nutritional factors (King *et al.,* 2000; Wiseman *et al.*, 2003), age of poultry (Wilson and Leibholz, 1981; Zuprizal *et al.,* 1992; Siriwan *et al.*, 1993; Sohn *et al.*, 1994; Whittow, 2000; Knarreborg *et al.,* 2002; Batal and Parsons, 2002a; Batal and Parsons, 2002b; Zelenka *et al.,* 2003; Lemme *et al.,* 2004; Palander *et al.*, 2004a; Ravindran and Hendriks, 2004b; Thomas and Ravindran, 2005; Huang *et al.* , 2005), dietary fat content (Li and Sauer, 1994; Dänicke *et al.,* 2000), grain volume weight (Perttilä *et al.,* 2001b), feed preservation method (Perttilä *et al.,* 2001a), plant varieties (Short *et al.,* 2000; Dowling *et al.,* 2002; Fan and Sauer, 2003; Kluth *et al.* , 2005a; Simon, 2004; Singh *et al.,* 2005) and dietary fibre (Raharjo and Farrel, 1984; Parsons, 1984; Souffrant, 2001) have been studied previously. These references imply a wide range of digestibility measurements and the factors affecting it by different methodology. The objectives of new studies are the development of a standard method which will be investigated in the next chapter.