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J Anim Sci 2006. 84:E79.

Measuring synthesis rates of nitrogen-containing polymers by using stable isotope tracers[1,2]

M. Z. Fan,*[3] L. I. Chiba,† P. D. Matzat,‡ X. Yang,* Y. L. Yin,*§ Y. Mine,# and H. H. Stein‖

*Department of Animal and Poultry Science, University of Guelph, ON, Canada N1G 2W1; †Department of Animal Sciences, Auburn University, Auburn, AL 36849-5415; ‡Elanco Animal Health, Greenfield, IN 46140; §Institute of Subtropical Agricultural Research, The Chinese Academy of Sciences, Changsha, P.O. Box 10, Hunan Province, China 410125; #Department of Food Science, University of Guelph, ON, Canada N1G 2W1; and ‖Department of Animal and Range Sciences, South Dakota State University, Brookings 57007

ABSTRACT: The major N-containing polymer compounds in the body include protein, RNA, and DNA. The endogenous gastrointestinal secretions as well as the portal-drained visceral and peripheral immune responses are basic physiological functions. Elevated endogenous secretions and immune activities, as affected by developmental stages, diets, and management factors, decrease the availability of dietary nutrients for peripheral muscle synthesis and deposition. Measurements of in vivo protein, RNA, and DNA synthesis rates associated with the viscera, peripheral immune cells, and skeletal muscles should, in principle, be the sensitive biochemical and cellular endpoints for studying factors affecting nonruminant nutrition, metabolism, and growth. The selection of stable isotope tracers for precursors, routes of tracer delivery, and mass spectrometric analyses of tracer enrichments are the major methodological considerations. To measure in vivo protein, RNA, and DNA synthesis rates, oral feeding with heavy water (^2H_2O), and continuous infusion of [U-^{13}C]glucose and [^{15}N]Gly intravenously for labeling the sugar moieties ribose and deoxyribose and de novo purine base synthesis have been established. Flooding doses of tracer Phe, for example, L-[ring-$^2H_5$]Phe, via the i.p. route are reliable and cost-effective for measuring in vivo protein synthesis rates, especially for the viscera in small nonruminants. Therefore, measurements of the major N-containing polymer synthesis rates in the viscera, the peripheral immune cells, and muscles through oral feeding with ^2H_2O and/or i.p. flooding doses of Phe tracers are the emerging tools for studying nonruminant nutrition, metabolism, and growth under research and field test conditions.

Key words: in vivo synthesis rate, nonruminant, stable isotope tracer

 J. Anim. Sci. 2006. 84(E. Suppl.):E79–E93

INTRODUCTION

The major N-containing polymer compounds in the animal body and its dietary ingredients include protein, RNA, and DNA, which, along with other forms of N compounds, are classically measured by the Kjeldahl procedure and defined as CP in animal nutrition research (e.g., AOAC, 1993). Research on improving the efficiency of CP use and metabolism in nonruminant nutrition has been a fascinating topic worldwide for 3 major reasons.

First, biosynthesis and degradation of these major N polymers are highly ATP energy-demanding processes in the body, and the partial energy efficiency for CP deposition (k_p) in animals is low; for example, k_p = 0.47 to 0.55 compared with the partial energy efficiency for the crude fat deposition (k_f) at k_f = 0.67 to 0.86 in growing pigs (ARC, 1981; Close et al., 1983; Williams et al., 1997b). Thus, strategies that can improve the efficiency of CP use would improve productivity and profit. Second, on a global scale, developing sustainable animal production for reducing pollution to the environment becomes a major issue (Mackie et al., 1998). Nitrogenous compounds are the major precursor for the biogen-

[1]Invited review. Presented at the "Stable Isotope Tracer Techniques for Nonruminant Research and Their Practical Applications" symposium held at the American Society of Animal Science Annual Meeting, Cincinnati, OH, July 24–28, 2005.

[2]Related research activities have been supported by grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada, and the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA)-University of Guelph Animal and Food Research Programs (to MZF) as well as by support (to YLY) from the National Basic Research Program of China (grant #2004CB117502) and the Natural Science Foundation of China (grant #30371038).

[3]Corresponding author: mfan@uoguelph.ca

Received August 31, 2005.
Accepted November 27, 2005.

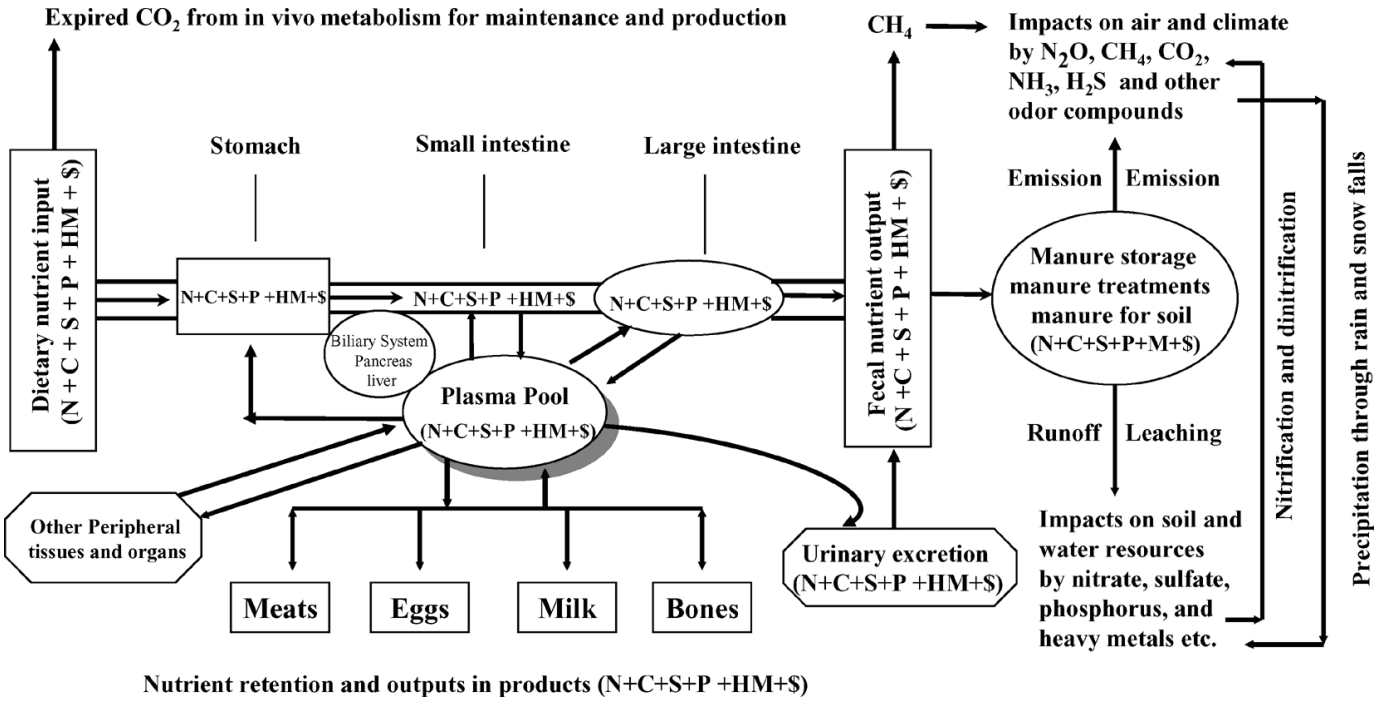

Figure 1. Schematic illustration of the relationships between dietary nitrogenous nutrient input, metabolism at the tissue and/or organ levels, outputs of products, manure excretion, and their potential impact on the environment in intensive nonruminant production systems. N = nitrogen compounds; C = carbon-containing compounds; S = sulfur-containing compounds; P = phosphorus compounds; HM = heavy metals; N_2O = nitrous oxide; CH_4 = methane; CO_2 = carbon dioxide; NH_3 = ammonia; H_2S = hydrogen sulfide and other volatile sulfides; and $ = economic impacts.

esis of the major pollutants and these include ammonia, greenhouse gases (CO_2, CH_4, and N_2O), volatile odor compounds, and nitrate, all of which contribute to acid rain, global warming, odor disturbance, and pollution of drinking water resources (Figure 1; Mackie et al., 1998; Rideout et al., 2004). Although environment-sensitive compounds are produced during animal feeding, the majority of the impact arises from the storage, treatment, and land application of manure as illustrated in Figure 1. Correspondingly, efficiency of N use is low for most of the duration of growth, for example, in pigs as summarized in Table 1. Consequently, a fundamental and effective strategy would be to cure the source by developing comprehensive genetic, nutritional, and management regimens for minimizing nutrient loading, optimizing their intermediary conversion, and reducing their expiration and manure excretion. Third, animal models, especially the pig, have been increasingly used to explore some clinical aspects of AA and protein metabolism and requirements for humans (Burrin and Reeds, 1997; Bertolo et al., 1998; Davis et al., 2002). In addition, animal and human growth is basically the process of hyperplasic and hypertrophic deposition of cellular protein, RNA, and DNA (Lawrence and Fowler, 1997; Perez and Reeds, 1998; Hellerstein, 2003). Therefore, understanding factors affecting in vivo cell proliferation, transcriptional RNA synthesis, as well as protein synthesis and degradation is important to understand growth regulation in both animals and humans with implications to human health management.

The objectives of this review are 1) to review up-to-date concepts and contents of the digestive and the postabsorptive use of dietary N polymer compounds in nonruminants and the major classical techniques developed for related studies; 2) to compile literature reports for supporting the use of the N polymer compound synthesis rates as sensitive and complementary endpoints for studying nonruminant nutrition, metabolism, and growth; and 3) to discuss major methodological aspects of measuring in vivo N polymer compound synthesis rates focusing on the use of stable isotope tracers.

QUANTIFYING DIGESTIVE AND POSTABSORPTIVE-USE PROCESSES

Use of dietary N compounds at the whole-body level can be further partitioned into more specific components at the digestive and postabsorptive processes in nonruminants as illustrated in Figure 2.

Digestive Use

Digestive use of dietary and endogenous N compounds includes the steps of gastric, exocrine, pancreatic, and intestinal mucosal enzymatic hydrolyses, microbial modifications of the luminal nitrogenous com-

Table 1. Postnatal changes in the efficiency of whole-body N use and metabolism in pigs

Item	Growth stages of pigs			
	Suckling[1]	Weanling[2]	Growing[2]	Finishing[2]
Dietary N levels,[3] g/kg of DMI of diet	48.0	42.0	32.0	22.0
Dietary N levels,[3] % of diet	4.8	4.2	3.2	2.1
The endogenous N loss,[4] g/kg of DMI of diet	1.2	1.7	2.6	2.6
The relative endogenous N loss,[5] % of dietary N	2.5	4.1	8.1	11.8
Average true AA or N digestibility,[6] % of dietary levels	91.3	85.8	90.9	90.9
Relative fecal loss of dietary N,[7] % of dietary N	8.7	14.2	9.1	9.1
Relative urinary loss of N,[8] % of dietary N	8.8	38.9	27.9	40.9
Total metabolic losses of N,[9] % of dietary N	11.3	43.0	36.0	52.7
Apparent efficiency of N retention,[10] % of dietary N	80.0	57.2	57.9	39.6
True efficiency of N retention,[11] % of dietary N	82.5	61.3	66.0	51.4

[1]Suckling pigs were fed sows' milk or a milk protein-based liquid formula.
[2]Weanling pigs (5 to 10 kg of BW) were fed corn and soybean meal (SBM)-based diets. Growing (20 to 50 kg of BW) and finishing (50 to 120 kg of BW) pigs were fed corn, and SBM-based diets.
[3]Dietary N level for suckling pigs adapted from Mavromichalis et al. (2001); and dietary N levels for the weanling, growing, and finishing pigs adapted by multiplying the dietary CP levels by the coefficient 6.25, as recommended by NRC (1998).
[4]The ileal endogenous N loss data adapted for the suckling pig from Mavromichalis et al. (2001), for the weanling pig from Fan (2003), and for the growing-finishing pig from Fan et al. (1995).
[5]The relative endogenous N losses for the pigs were calculated as: (reported ileal endogenous N loss [g/kg DMI diets]/dietary N levels [g/kg DMI diets]) × 100%.
[6]The average true ileal AA or N digestibility in sows' milk for the suckling pig adapted from Mavromichalis et al. (2001); and the average true ileal AA or N digestibility in SBM for the weanling and the growing-finishing pigs adapted from Caine et al. (1997), Mahan (1992), and Fan et al. (1995).
[7]The relative fecal loss of dietary N for the suckling, growing, and finishing pigs was obtained by subtracting the average true N or AA digestibility.
[8]The relative urinary loss of N for the suckling pig was adapted from Mavromichalis et al. (2001) and Bertolo et al. (1999), for the weanling pig from Mahan (1992), for the growing pig from Rideout and Fan (2004), and for the finishing pig from Mroz et al. (2002).
[9]Total metabolic losses of N were the sum of the endogenous N loss and the urinary N loss.
[10]The apparent efficiency of N retention for the suckling pig was adapted from Le Dividich et al. (1994), and Bertolo et al. (1999), for the weanling pig from Mahan (1992), for the growing pig from Rideout and Fan (2004), and for the finishing pig from Mroz et al. (2002).
[11]The true efficiency of N retention was calculated from the summarized apparent efficiency values and the endogenous N losses.

pounds in terms of microbial synthesis, and degradation of AA and nucleosides, and the absorption of the digestive end products across the luminal surface, as illustrated in Figures 3 and 4. From the physiological point of view, transport of the digestive end products from the intestinal lumen across the intestinal brush border or the apical membrane via the paracellular and transcellular routes marks the completion of the entire digestive-use process (Figures 3 and 4; Cheeseman, 1986).

From the nutritional point of view, the disappearance of the digestive end products from the intestinal luminal surface at the end of an appropriate intestinal segment is measured as digestibility. For both pigs and poultry, the ileal CP and AA digestibility are measured at the end of the small intestine due to the impact from microbial fermentation in the hindgut (Parsons, 1986; Sauer and Ozimek, 1986). Ingredient-specific differences in true ileal CP and AA digestibility are well recognized (Table 2). True ileal CP and AA digestibility, after correcting the gastrointestinal metabolic endogenous contributions, are recommended for diet formulations in both pigs and poultry due to improved sensitivity and reduced variability of the measurements (Parsons, 1986; Sauer et al., 2000).

The measurement of true ileal CP and AA digestibility in feed ingredients for pigs and poultry needs to consider 2 additional correction factors. These corrections are i) quantification of the contribution of microbial protein synthesis (Fuller and Reeds, 1998), and the microbial degradation of free AA in the lumen (Fan, 2003); and ii) the correction for the amount of heat-damaged indispensable AA, such as lysine, that is absorbable but not available for protein synthesis in cells (Rutherfurd et al., 1997).

Of all of the steps involved in the digestive use of nutrients from practical diets by nonruminants, intrinsic enzymatic hydrolysis and apical membrane transport are not likely to be the rate-limiting steps (Weiss et al., 1998). Thus, exogenous enzyme supplementations and various techniques for processing the texture of dietary ingredients have proven effective for improving digestive use of dietary CP and AA (Li et al., 1996; Huang et al., 1998; Hancock and Behnke, 2001).

Postabsorptive Use

The transport of the digestive end products across the intestinal apical membrane is the starting point of the postabsorptive-use process (Figures 2 and 3). The

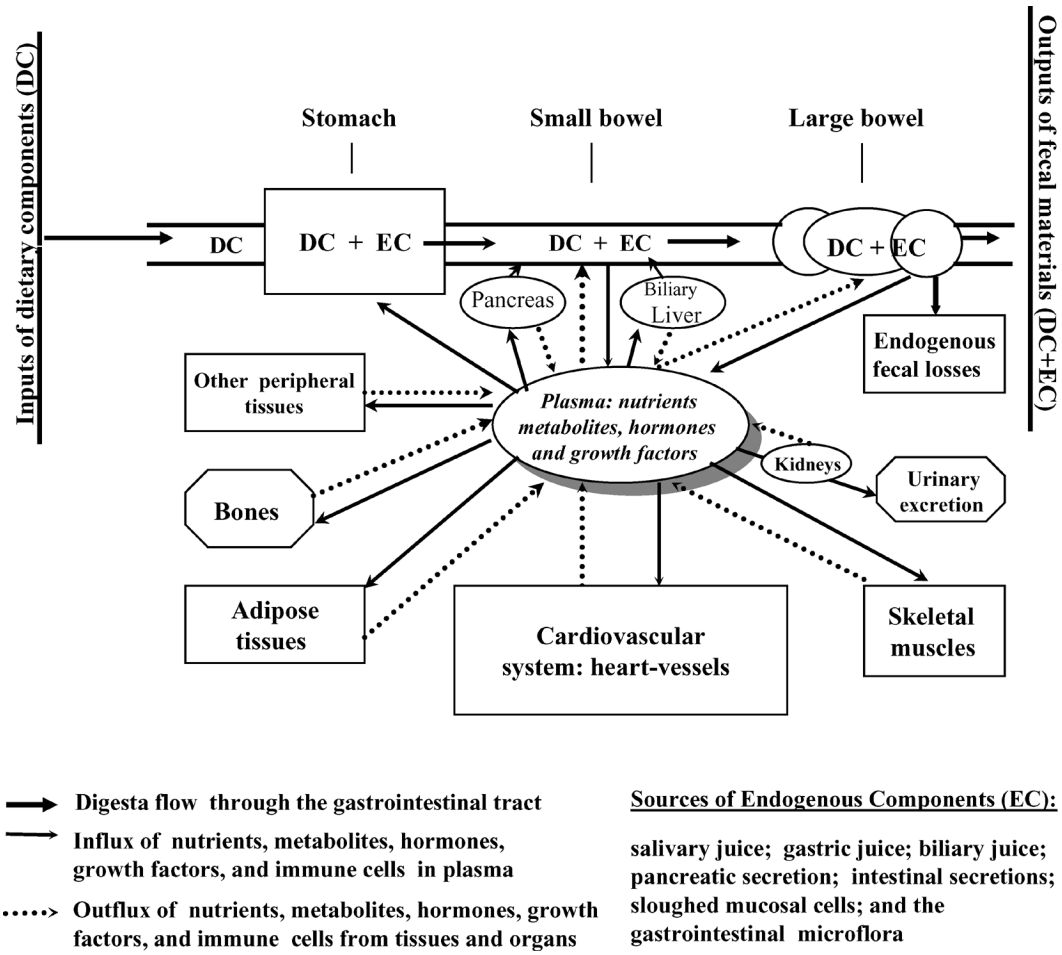

Figure 2. Digestive and postabsorptive use of nitrogenous compounds, and metabolic interactions between dietary components and various tissues and organs at the whole-body level in nonruminants.

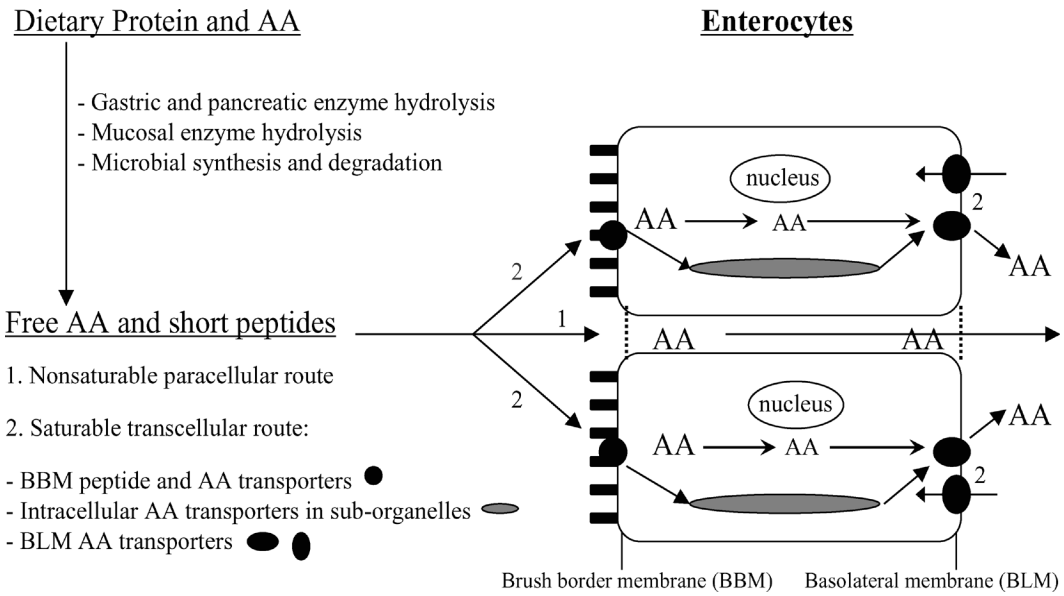

Figure 3. Schematic representation of the major steps involved in the digestive use of dietary proteins in nonruminants (adapted from Alpers, 1994).

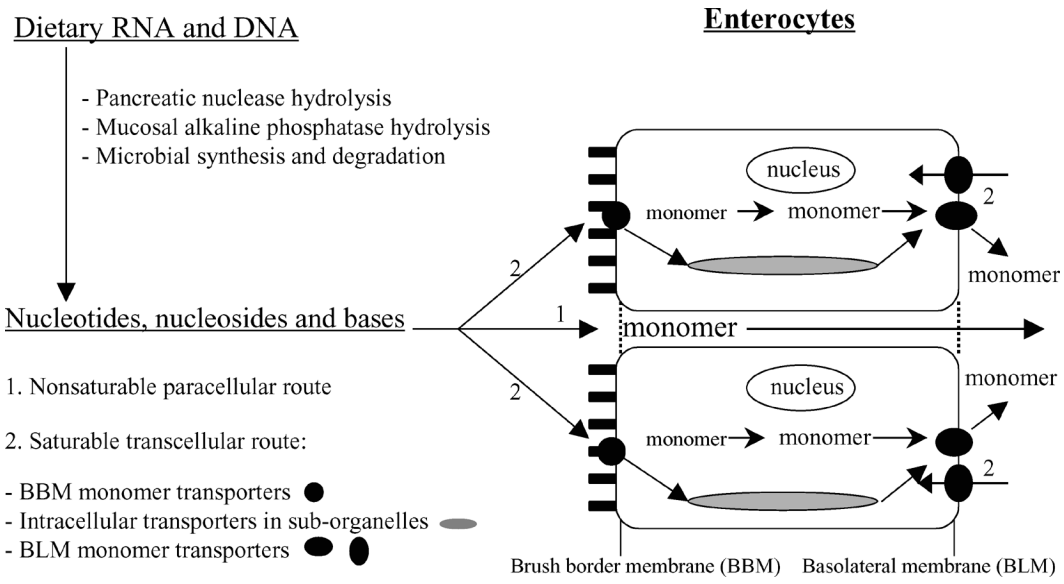

Figure 4. Schematic representation of the major steps involved in the digestive use of dietary nucleic acids, including RNA and DNA, in nonruminants (adapted from Carver and Walker, 1995).

postabsorptive use of absorbed N compounds includes the steps of movement through the intestinal epithelial apical membrane, use and metabolism inside the epithelia, transport across the epithelial basolateral membrane, subsequent movement through the interstitial fluid, and their use and metabolism by the other visceral organs and peripheral tissues (Figures 2, 3, and 4).

The concept of the first-pass portal-drained visceral use and metabolism of various nitrogenous digestive end products has evolved through several classical studies. Windmueller and Spaeth (1980) demonstrated that a large proportion of dietary dispensable AA, such as Gln, Glu, and Asn, were catabolized in the intestinal mucosa during the absorption process in rats. Rèrat (1981) also demonstrated the extensive metabolism of the dispensable AA during the intestinal absorption with the portal-arterial mass balance technique in pigs. Wu (1998) extensively investigated dispensable AA metabolism with isolated jejunal upper villus epithelial cells in vitro in the postnatal developing pig. Stoll et al. (1997, 1998a) showed the preferential use of dietary AA for hepatic protein synthesis in pigs. Bertolo et al. (1998) and Stoll et al. (1998b) independently demonstrated a large proportion of the indispensable AA use by the portal-drained viscera in pigs with tracer kinetic techniques. Inadequate supply of nutrients, especially AA, via the enteral route impaired intestinal growth in piglets (Burrin et al., 2000). In addition to AA, enteral sources of nucleotides are utilized for RNA and DNA syntheses in the gut mucosa (Lopez-Navarro et al., 1996; Burrin and Reeds, 1997). The concept of the first-pass visceral use of the nitrogenous digestive end products refers to the preferential use and metabolism of a large proportion of the enteral sources of AA and nucleosides for the visceral growth in providing fuels and precursors for biosynthesis (Burrin and Reeds, 1997; Wu, 1998). Therefore, the metabolic status of the viscera will determine the availability of the enteral sources of nitrogenous substrates for the synthesis of the N polymers in the peripheral tissues such as skeletal muscles and skin.

The knowledge of the first-priority visceral use of both the enteral source and the peripheral tissue reserves of the nitrogenous substrates for preserving pivotal functions carried out by the viscera of the organisms under various challenge conditions has been well demonstrated. Chronic systematic immune activation and infection result in the following effects: i) induction of inflammatory responses such as reduced feed intake and fever (Williams et al., 1997a); ii) increased whole-body protein synthesis and protein breakdown rates (Obled, 2003) with no significant changes in whole-body N and AA use efficiency (Williams et al., 1997a; Webel et al., 1998a,b); and iii) increased protein synthesis

Table 2. Comparison of true ileal CP and selected essential AA digestibility (%) among casein, soybean meal, and barley for growing pigs (mean ± SE)

	Feed ingredients		
Item	Casein[1]	Soybean meal[2]	Barley[3]
No. of observations	16	24	72
CP	97.0 ± 1.5	89.7 ± 1.2	78.3 ± 2.7
Lys	99.0 ± 0.6	90.7 ± 0.9	77.1 ± 5.3
Leu	98.0 ± 0.9	90.5 ± 0.8	88.7 ± 2.7
Iso	95.0 ± 2.7	92.1 ± 1.0	86.4 ± 2.6
Met	99.0 ± 0.7	93.6 ± 0.8	92.3 ± 3.3
Thr	94.0 ± 2.3	86.6 ± 1.2	80.6 ± 4.1
Val	96.0 ± 1.8	89.8 ± 0.9	88.3 ± 2.8

[1]Adapted from Jørgensen and Gabert (2001).
[2]Adapted from Fan et al. (1995).
[3]Adapted from Fan and Sauer (2002).

Table 3. The gastrointestinal endogenous losses of CP and selected essential AA associated with casein, soybean meal, and barley for growing pigs (mean ± SE)

Item	Feed ingredients		
	Casein[1]	Soybean meal[2]	Barley[3]
No. of observations	16	24	72
	g/kg of DMI		
CP	8.6 ± 3.0	16.5 ± 2.1	35.1 ± 3.0
Lys	0.27 ± 0.10	0.47 ± 0.10	1.57 ± 0.13
Met	0.11 ± 0.04	0.13 ± 0.02	0.75 ± 0.07
Thr	0.44 ± 0.20	0.69 ± 0.08	1.82 ± 0.11
	% of the NRC (1998) requirements		
CP	4.3 ± 1.5	8.3 ± 1.1	17.6 ± 1.5
Lys	2.9 ± 0.05	5.1 ± 1.1	17.1 ± 1.4
Met	4.6 ± 1.7	5.4 ± 0.8	31.3 ± 2.9
Thr	7.6 ± 3.5	11.9 ± 1.4	31.4 ± 1.9

[1]Adapted from Jørgensen and Gabert (2001).
[2]Adapted from Fan et al. (1995).
[3]Adapted from Fan and Sauer (2002).

rates in the viscera such as gut and liver for providing self-defense mechanisms such as producing acute-phase proteins at the expense of muscle protein synthesis (Klasing and Austic, 1984a,b; Mackenzie et al., 2003). Thus, under challenge conditions such as infections, both the enteral source and the peripheral reserved nitrogenous substrates are prioritized for enhanced use by the viscera for improving the opportunity of survival at the cost of productivity such as muscle growth.

The contributions of the gastrointestinal endogenous N secretions, recycling, and losses to whole-body N homeostasis have been recognized in nonruminants (Fuller and Reeds, 1998). The major components of the endogenous N losses are illustrated in Figure 2. Methods for measuring the endogenous N and AA losses have been reviewed (Nyachoti et al., 1997; Fan and Sauer, 2002). Under normal nutritional and physiological conditions, the endogenous N loss is not the largest single route of N inefficiency (Table 1). However, differences in ingredient-specific endogenous N loss are much more profound than the ingredient-specific variability in the true ileal CP and AA digestibility as shown in Tables 2 and 3. Pigs fed high fiber and antigenic protein components had considerably augmented endogenous N losses (Fan and Sauer, 2002; Gaudichon et al., 2002). Thus, distal ileal endogenous N loss can constitute a large part of postabsorptive metabolic N inefficiency.

The slope-ratio assay has been developed for directly measuring the availability of dietary limiting indispensable AA for the digestive and the postabsorptive use and growth in nonruminants (Batterham, 1992). However, this assay may be limited for its scope of applications due to the assay variability in response end points and difficulties in study designs (Sauer et al., 2000).

As summarized in Table 1, metabolic urinary N loss is the largest single route of whole-body inefficiency of N use including microbial catabolism, and catabolism loss in the viscera and the peripheral tissues. Several techniques are available for quantifying and assessing whole-body oxidative losses of N such as N balance (Mahan, 1992), comparative slaughter technique (Williams et al., 1997a), plasma urea N levels (Coma et al., 1995), whole-body AA oxidation (Matthews et al., 1980), and indicator AA oxidation (Bertolo et al., 1999). These approaches and endpoints are useful to reflect whole-body N metabolism status for AA requirement studies, and can be used to probe, to some extent, the intermediary N and AA metabolism.

MAJOR N POLYMER COMPOUND SYNTHESIS RATES AS SENSITIVE METABOLIC ENDPOINTS

A growing body of literature supports the notion that measurements of organ and tissue protein, RNA, and DNA synthesis rates are more informative than whole-body synthesis rate measurements in revealing N metabolic status and growth in nonruminants.

Whole-Body vs. Organ or Tissue Synthesis and Breakdown Rates

At the whole-body level, CP deposition ($CP_{Deposition}$) should be strictly defined as the difference between the whole-body synthesis ($CP_{Synthesis}$), degradation ($CP_{Degradation}$), and the endogenous CP loss ($CP_{Endogenous\ loss}$) as described by Eq. [1]:

$$CP_{Deposition} = CP_{Synthesis} - CP_{Degradation} - CP_{Endogenous\ loss}. \quad [1]$$

At an organ or tissue level, CP deposition ($TCP_{Deposition}$) should be strictly defined as the difference between synthesis ($TCP_{Synthesis}$), degradation ($TCP_{Degradation}$), and the sloughing or secreting CP loss mainly in the viscera ($TCP_{Sloughing\text{-}secreting\ loss}$) as described by Eq. [2]:

$$TCP_{Deposition} = TCP_{Synthesis} - TCP_{Degradation} - TCP_{Sloughing\text{-}secreting\ loss}. \quad [2]$$

Measurements of protein synthesis rates have been established at the whole-body (Waterlow, 1981; Wolfe, 1992; Rathmacher, 2000), and the organ or tissue levels (Wolfe, 1992; Rathmacher, 2000). However, direct measurements of the whole-body protein, RNA, and DNA degradation rates are more challenging. Quantitative measurements of organ or tissue protein degradation have been described, including the arterial-venous difference, tracer amino acid decay kinetics, and 3-methylhistidine excretion specific for muscles (Rathmacher, 2000). Qualitative analysis of organ or tissue protein breakdown status can also be conducted by measuring the 3 major proteolytic-pathway key enzyme expressions, including the lysosomal system, the ubiquitin-

proteasome pathway, and the Ca-dependent calpain system (Rathmacher, 2000; Coeffier et al., 2003).

For example, sepsis and consumption of soybean proteins dramatically increased both whole-body protein synthesis and degradation rates, leading to reduced whole-body N accretion rates (Klasing and Austic, 1984b; Schadereit et al., 1999). In contrast, dietary supplementation of antibiotics improved the whole-body N retention rate by exerting greater effects on the whole-body protein degradation rate than the synthesis rate (Roth et al., 1999). Therefore, the measurement of whole-body protein synthesis rates alone cannot define whole-body N metabolic status.

Postnatal Changes in Organ or Tissue Protein and DNA Synthetic Rates

The declining whole-body efficiency of N use (Table 1) is associated with a reduction in skeletal muscle protein synthesis rates (Reeds et al., 1993), and sustained or even elevated visceral organ protein synthesis rates especially for the pancreas, liver, and the small intestine (Burrin et al., 1991; Reeds et al., 1993). Intestinal epithelial life span, as reflected by cellular DNA synthetic rates, is also dramatically decreased during postnatal growth (Smith and Jarvis, 1978; Fan et al., 2001). Thus, postnatal growth is associated with increased endogenous N losses as summarized in Table 1.

Effects of Dietary Protein and Nucleotide Supply on Organ or Tissue Protein Synthetic Rates

Inadequate supply of dietary nutrients, especially protein, AA, and nucleotides, reduces intestinal, hepatic, and muscle protein synthesis rates (Lopez-Navarro et al., 1996; Burrin et al., 2000; Frank et al., 2005). Chronic low-protein intake impairs intestinal, hepatic, muscle, and skin protein synthesis rates (Wykes et al., 1996). Poor dietary protein quality also reduces the visceral and peripheral tissue protein synthesis rates (Tesseraud et al., 1996; Tujioka et al., 2004).

Effects of Dietary Fiber and Protein Sources on Organ or Tissue Protein Synthetic Rates and Cellular Proliferation

Dietary nonstarch polysaccharide component was likely responsible for the increased intestinal epithelial proliferation and protein synthesis rates (Southon et al., 1985). High fiber intake increased intestinal epithelial proliferation in pigs (Jin et al., 1994). Compared with casein, feeding rats with soy protein isolates reduced whole-body N retention rates by increasing whole-body protein degradation but not protein synthetic rate (Schadereit et al., 1999). On the other hand, replacing extruded soybean product with plasma protein enriched in immunoglobulin improved whole-body N efficiency by reducing the proliferation of small intestinal mucosal cells in early-weaned pigs (Jiang et al., 2000).

Effects of Infections on Organ or Tissue Protein Synthetic Rates and Cellular Proliferation

Intestinal inflammation increases the gut, hepatic, and plasma protein synthesis rates with reduced skeletal muscle protein synthesis rates (Obled, 2003). Systemic infection, as mimicked by injection of lipopolysaccharides, augments the visceral and plasma protein synthetic rates, and decreases muscle protein synthesis rates (Orellana et al., 2002; Obled, 2003). Sepsis and viral infection increases intestinal epithelial and T-lymphocyte turnover rates by increasing cellular DNA synthesis rates (Noguchi et al., 1997; Mohri et al., 2001).

To conceptually summarize these reports, it can be concluded that under challenge conditions, the visceral and the plasma protein synthesis rates are elevated at the expense of reduced muscle protein synthesis rates. Therefore, simultaneous measurements of the visceral protein and DNA synthesis rates, and the skeletal protein synthetic activity can provide a reliable end point for assessing animals' responses to nutritional and management conditions.

METHODOLOGICAL ASPECTS OF MEASURING THE MAJOR N POLYMER SYNTHESIS RATES

Methodology of measuring in vivo organ or tissue protein, RNA, and DNA synthesis rates has been reviewed previously (Wolfe, 1992; Perez and Reeds, 1998; Hellerstein, 2003). This section focuses on linking classical method studies with potential research applications to nonruminant farm animal species.

Protein Synthesis Rates

Major methodological aspects of measuring in vivo protein synthesis rates in organs and tissues include types of tracers, the selection of precursor pools for the analysis and kinetic calculations, the regimens of tracer delivery, and routes of tracer delivery (Reeds and Davis, 1999; Rathmacher, 2000), as illustrated in Figure 5.

Radioactive tracers are initially used for this purpose, and are usually ^{3}H- or ^{14}C-labeled dispensable and indispensable AA such as L-[^{14}C]Lys (Waterlow and Stephen, 1966) and L-[^{14}C]Gly (Garlick, 1969). The use of the radioactive tracers for measuring protein synthetic activity requires the liquid chromatographic fractionation of the tracer and tracee AA in the free and bound pool samples, and the subsequent analyses of radioactivity with a liquid scintillation counter (Garlick, 1969). The radioactive tracer approach is sensitive and suitable for in vitro work (Adeola et al., 1989) and in vivo work with laboratory animals and small farm animals such as piglets (Davis et al., 2002). However, radioactive tracers may be limited in their applications with

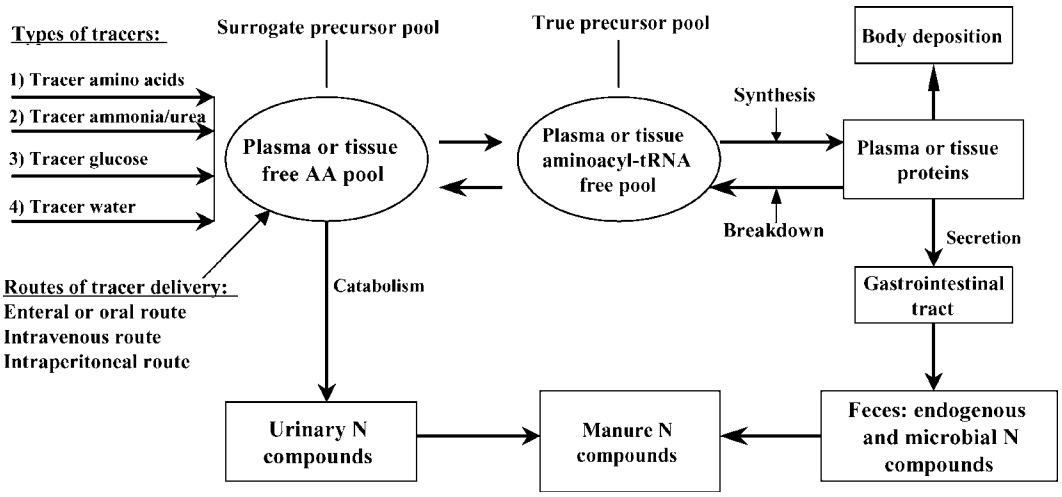

Figure 5. Schematic representation of the major methodological aspects affecting the measurement of fractional protein synthesis rates of organs and tissues in vivo, and their impacts on determining the efficiency of nitrogen use in nonruminants.

large-frame farm animals due to the difficulties in containing radioactive tracer wastes.

Stable isotope tracers, including non-AA tracers such as ^{15}N-ammonium, ^{15}N-urea, ^{13}C-glucose, $^{2}H_2O$, and $H_2{}^{18}O$ as well as ^{15}N-, ^{13}C-, and ^{2}H-AA tracers, are increasingly used in human and animal studies because of considerably reduced costs for desktop mass spectrometers and tracer materials (Wolfe, 1992; Hellerstein, 2003). Stable isotope tracers are convenient to study for all types of animals. However, these tracer detection sensitivities are relatively low compared with the radioactive tracers. Thus, comparatively larger amounts of tracer materials are needed to label test subjects. The choice of a tracer for a specific study will depend on the nature of the study such as test subjects, study duration, regimens, and routes of tracer delivery, budget for tracer materials, and the type of a mass spectrometer for tracer enrichment analysis. One of the major technical considerations when using stable isotope tracers is to obtain the molar percentage of tracer enrichments from monitored ion fragment values in the free and the bound pool samples. If one of the ^{15}N-, ^{13}C-, or ^{2}H-labeled AA tracers, for example, L-[$^{2}H_5$]Phe, is used, a calibration curve needs to be established by using a commercially available pure tracer product (Figure 6). If one of the non-AA tracers such as $^{2}H_2O$ and $H_2{}^{18}O$ is used for labeling animals, the mass isotopomer distribution analysis (MIDA) approach is used for converting the measured ion fragment values to tracer molar enrichments (Hellerstein, 2003).

The aminoacyl-tRNA (**tRNA-AA**) pool is the true precursor pool for protein synthesis (Figure 5). However, a large throughput analysis of tRNA-AA tracer enrichments in collected samples of targeting organs and tissues is challenging. The intracellular free AA pool is a convenient surrogate free pool to be analyzed for tracer enrichments and used for calculating protein synthesis rates (Figure 5) if tracers are delivered according to established protocols (Davis and Reeds, 2001). However, the organ or tissue surrogate free AA pool may not always be in equilibrium with the true tRNA-AA pool valid for probing in vivo protein synthetic activities.

Two major regimens of tracer delivery have been developed including the flooding dose technique and the continuous infusion or feeding technique (Rathmacher, 2000). The flooding dose technique is suitable in combination with a Phe tracer for laboratory and small-size farm animals especially when the use of a surgically implanted catheter can be avoided (Danicke et al., 2001, 2003; Bregendahl et al., 2004). A critical criterion for successfully applying the flooding dose technique is to ensure that the designed doses of tracer and tracee Phe are large enough to flood the plasma and all of the targeted organ or tissue free AA tracer pools to a similar level of tracer enrichment (Table 4). The major limitation for using this technique in large-frame farm animals is the cost of purchasing tracer Phe, especially stable isotope tracer Phe. Continuous infusion of AA tracers via various routes and oral feeding of non-AA tracers are potentially suitable for all types of animals with reasonable tracer costs. However, multiple plasma samples need to be collected to ensure a steady-state tracer enrichment has been achieved before the final organ and tissue harvesting (Rathmacher, 2000; Davis and Reeds, 2001; Hellerstein, 2003).

Four major routes are available for tracer delivery including oral feeding, an enteral catheter, an intravenous (i.v.) route, and an intraperitoneal (i.p.) route (Figure 5). Oral feeding of non-AA tracers such as $^{2}H_2O$ and $H_2{}^{18}O$, for reaching a steady-state tracer enrichment, is suitable for measuring organ and tissue protein synthesis rates for all types of nonruminants according to the continuous infusion approach. Delivery of AA tracers

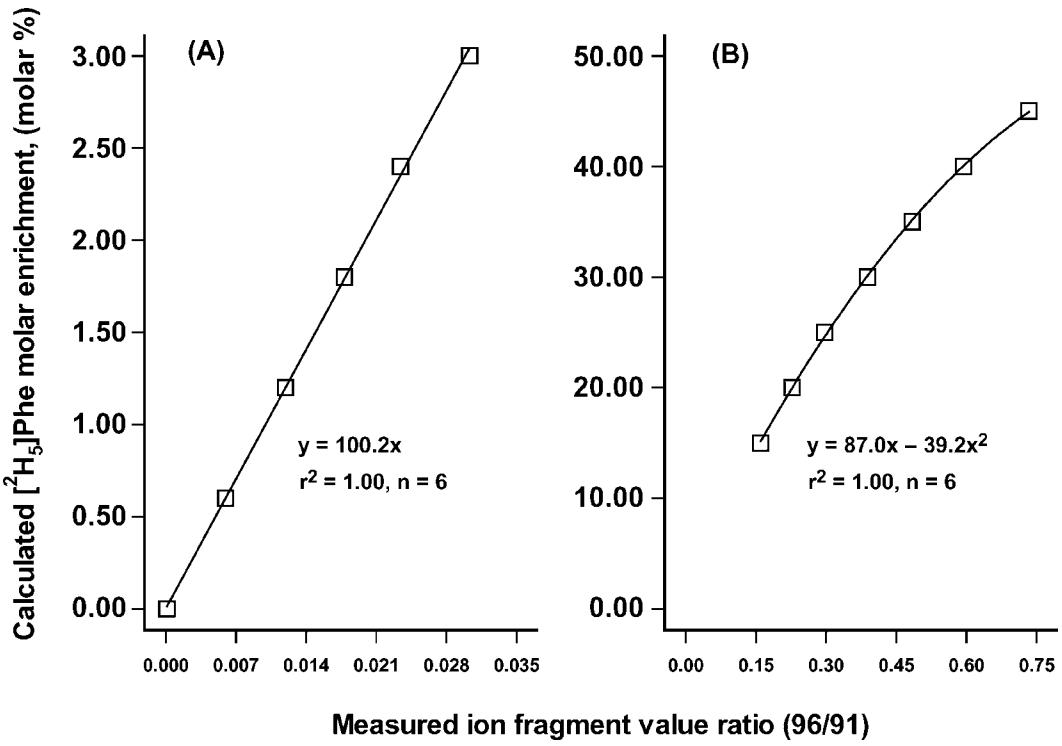

Figure 6. Representative calibration curves for the calculation of molar enrichments of stable isotope tracers with gas chromatography-mass spectrometry. Related analytical procedures were reported previously (Bregendahl et al., 2004). A) Linear calibration curve for calculating the tracer Phe molar enrichment in the bound pools. B) Curve for calculating the tracer Phe molar enrichment in the free pools after a flooding dose of Phe containing L-[$^2H_5$]Phe in studies with postnatal developing pigs. Data were observations of X. Yang and M. Z. Fan at the University of Guelph, ON, Canada.

via oral feeding and an enteral catheter according to a continuous infusion regimen may be costly for tracer materials because large doses of tracer AA may be needed due to the first-pass AA metabolism by the viscera. Delivery of tracer AA via an i.v. or i.p. catheter according to the flooding dose or a continuous infusion regimen is suitable for all types of nonruminants under controlled research conditions. Under such settings, test animals can be implanted with catheters for tracer infusion and blood sampling, and managed during a study. Delivery of tracer AA via the i.p. injection according to the flooding dose regimen can be conveniently applied for small animals under research and test field conditions; thus, offering a powerful tool for studying protein synthetic activities in nonruminants (Danicke et al., 2001; Bregendahl et al., 2004).

Table 4. Time-course of fractional protein synthesis rates (%/d) measured in plasma and tissues of fed weanling pigs after an i.p. injection of a flooding dose of Phe containing L-[ring-$^2H_5$]Phe[1]

	Time after the tracer injection, min						
Tissue	15	30	45	60	75	SEM[2]	*P*-value[3]
Plasma[4]	15.6	11.1	12.9	12.8	14.1	0.6	0.001
Proximal small intestine	63.8	79.3	74.2	69.7	80.7	5.8	0.272
Pancreas	109.7	93.8	105.1	80.9	87.5	13.9	0.620
Liver	33.4	48.9	45.5	44.8	43.2	5.9	0.539
Spleen	34.4	35.0	35.2	29.7	26.9	3.0	0.227
Heart[5]	14.4	13.4	12.2	11.5	11.1	0.3	0.047
Biceps muscle[4,5]	10.2	7.6	6.6	5.4	5.4	0.5	0.001

[1]From Bregendahl et al. (2004).
[2]The pooled standard error of the mean for the time effect (n = 5).
[3]Probability value for the main effect of time after injection.
[4]Quadratic effect of time after tracer injection ($P < 0.05$).
[5]Linear effect of time after tracer injection ($P < 0.05$).

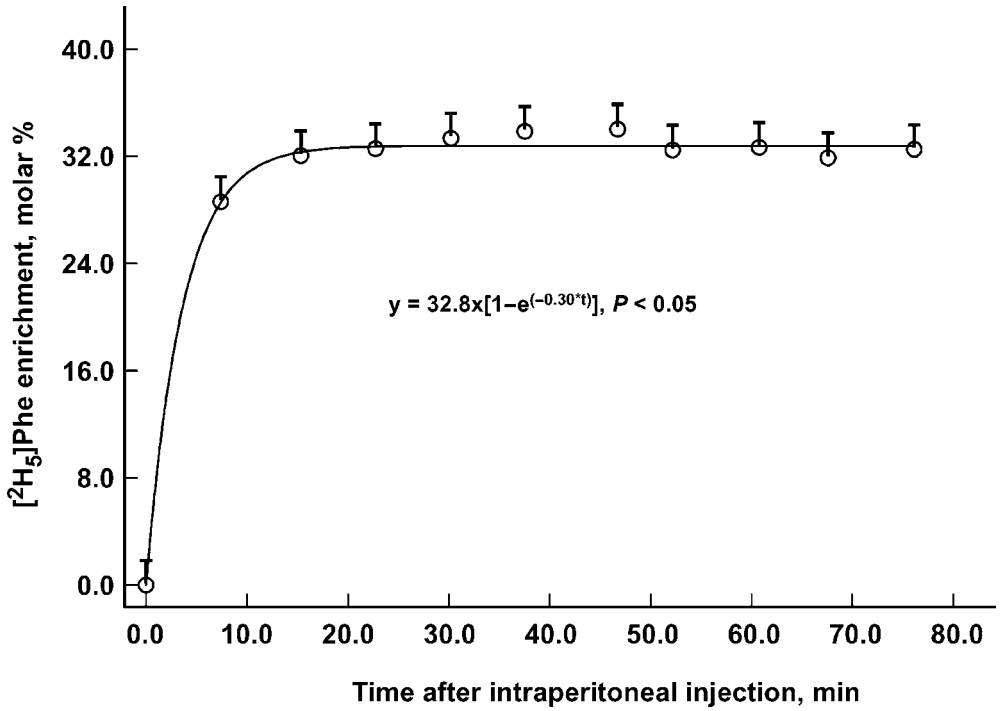

Figure 7. Quantitative relationship between isotopic enrichment of L-[ring-$^2H_5$]Phe in the plasma free pool after an intraperitoneal injection of a flooding dose of Phe in fed weanling pigs. Values are means ± pooled SE (n = 5; Bregendahl et al., 2004).

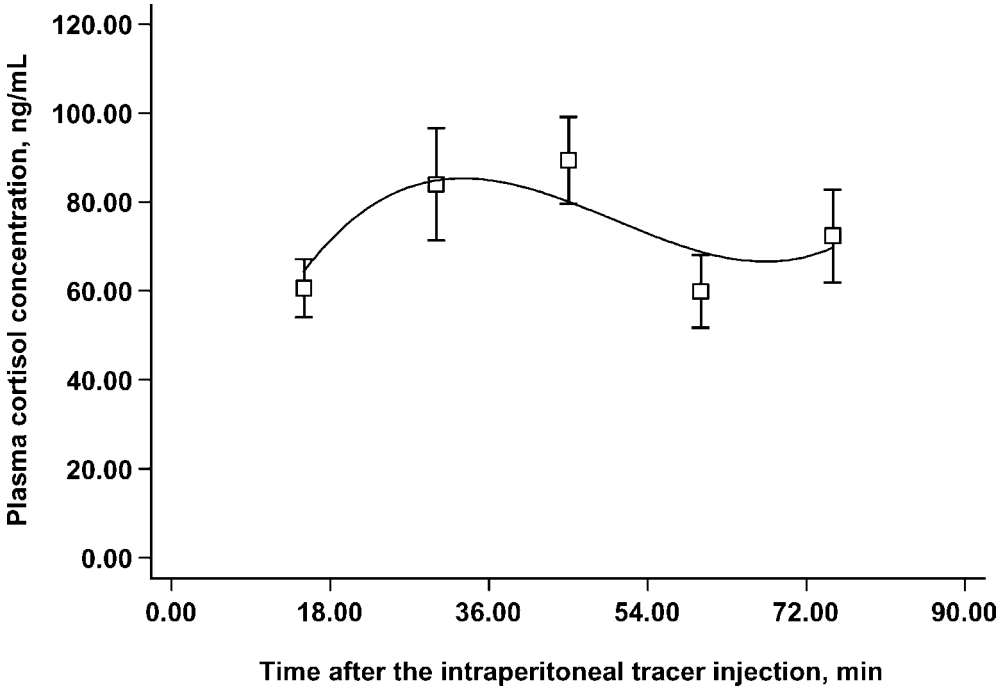

Figure 8. Responses of plasma concentrations of cortisol to time after an intraperitoneal injection of a flooding dose of Phe (1.50 mmol/kg of BW) in fed weanling pigs. Values are means ± SE (n = 5). Linear (P = 0.98), quadratic (P = 0.06), and cubic (P = 0.02) effects of time were examined by polynomial orthogonal contrasts. The change can be described by the function $y = (6.17 \pm 0.93)x - (0.14 \pm 0.04)x^2 + (0.00092 \pm 0.00033)x^3$, n = 25, r^2 = 0.93 (SAS NLIN procedure; SAS Inst., Inc., Cary, NC). Plasma cortisol levels were measured with ^{125}I-radioimmunoassay (Clinical Assays GammaCoat Cortisol ^{125}I-RIA Kit, Stillwater, MN) and were observations of X. Yang and M. Z. Fan at the University of Guelph, ON, Canada.

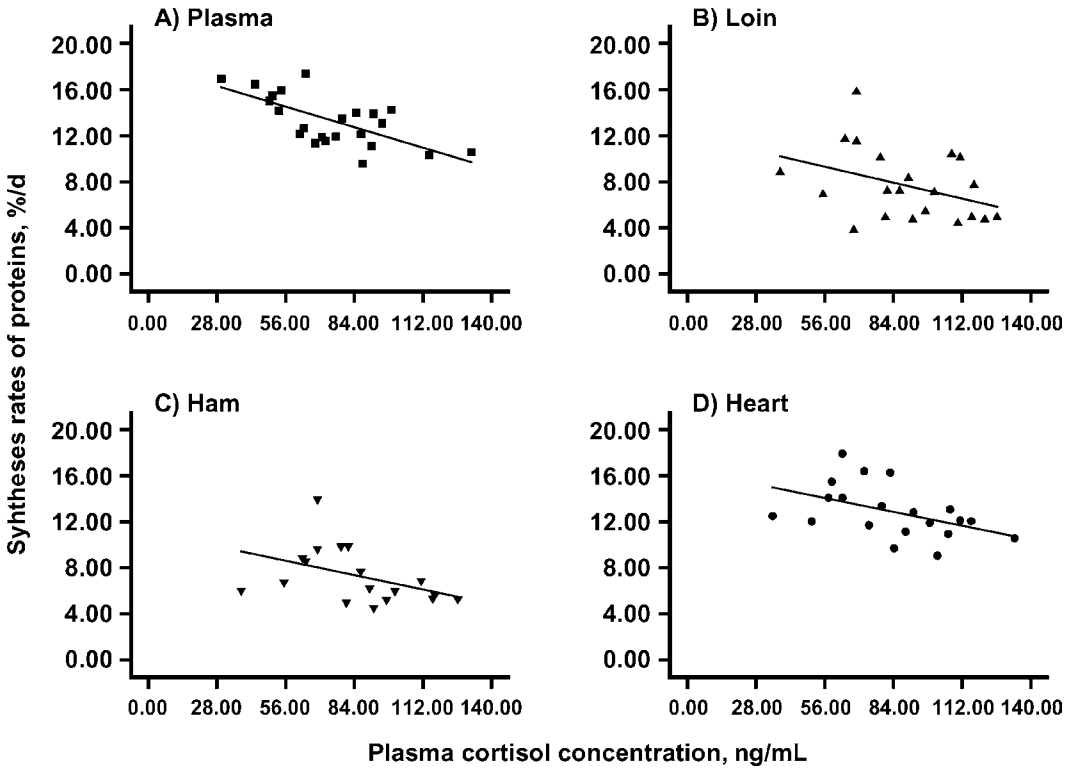

Figure 9. Quantitative relationships between fractional protein synthesis rates in various cortisol-sensitive tissues and plasma cortisol concentrations after an intraperitoneal injection of a flooding dose of Phe (1.50 mmol/kg of BW) in fed weaned pigs. A) Plasma: linear regression, y = (18.1 ± 1.1) – (0.06 ± 0.02)x, n = 24, r^2 = 0.46; $P < 0.05$ for the intercept and slope estimates. B) Loin: linear regression, y = (12.1 ± 2.6) – (0.06 ± 0.04)x, n = 21, r^2 = 0.14; $P < 0.05$ for the intercept estimate and $P = 0.09$ for the slope estimate. C) Ham: linear regression, y = (11.1 ± 2.1) – (0.06 ± 0.03)x, n = 18, r^2 = 0.18; $P < 0.05$ for the intercept estimate and $P = 0.07$ for the slope estimate. D) Heart: linear regression, y = (16.5 ± 1.7) – (0.05 ± 0.02)x, n = 20, r^2 = 0.22; $P < 0.05$ for the intercept and slope estimates. Fractional protein synthesis rates and plasma cortisol levels were measured at 15, 30, 45, 60, and 75 min after an intraperitoneal injection of a flooding dose of Phe (1.50 mmol/kg of BW) containing L-[ring-$^2H_5$]Phe at 40 mol% (0.60 mmol/kg of BW), as described by Bregendahl et al. (2004). Plasma cortisol levels were measured with ^{125}I-radioimmunoassay (Clinical Assays GammaCoat Cortisol ^{125}I-RIA Kit, Stillwater, MN) and were observations of X. Yang and M. Z. Fan at the University of Guelph, ON, Canada.

Bregendahl et al. (2004) examined the time course of tracer Phe enrichment in the plasma of weanling pigs after an i.p. injection of a flooding-dose Phe (Figure 7). The tracer Phe enrichments in the plasma, organ, and tissue free pools reached the plateau levels after 15 min of the injection (Bregendahl et al., 2004), as demonstrated in Figure 7. The tracer Phe injection time had no effects on protein synthesis except in the plasma, and the cardiac and skeletal muscles (Table 4). A surge in cortisol concentration, presumably due to the abrupt human and animal interactions and handling for the tracer injection, was observed in the i.p. injected weanling pigs (Figure 8). Pearson correlation analyses further suggested that the cortisol surge in responses to the injection procedure was likely responsible for the time-dependent reductions in protein synthesis rates in plasma, cardiac, and skeletal muscles (Figure 9). The negative effects of glucocorticoids on skeletal muscle protein synthesis rates are mediated through protein synthetic initiation factors (Liu et al., 2004). Therefore, 2 strategies can be proposed to minimize the negative impacts associated with the i.p. injection-based flooding dose approach for measuring protein synthetic activities in plasma and muscles: i) improve human and animal interactions during the days of animal management before the tracer injection to reduce the cortisol surge; and ii) complete the muscle sampling first and then conduct the blood sampling within 20 min of i.p. tracer injection. Nevertheless, the flooding dose technique in combination with i.p. injection is a powerful approach for studying protein synthetic activities for small nonruminants under both controlled research and field test conditions.

RNA and DNA Synthesis Rates

Major technical aspects of measuring in vivo RNA and DNA synthesis rates in organs and tissues include the selection of precursor pools for the tracer enrichment analysis and kinetic calculations, types of tracers,

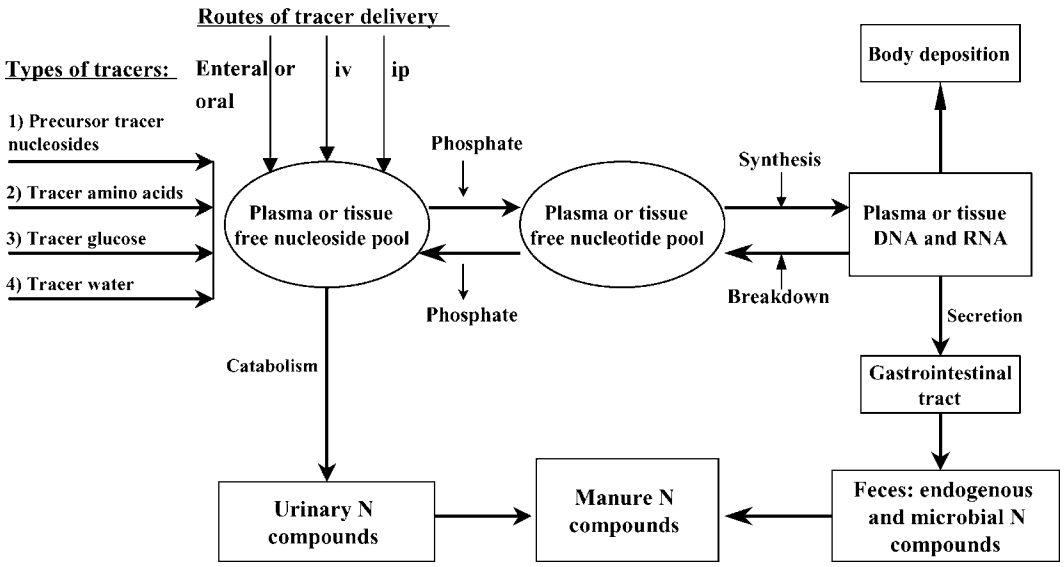

Figure 10. Schematic representation of the major methodological aspects affecting the measurement of fractional RNA and DNA synthesis rates of organs and tissues in vivo, and their impacts on determining the efficiency of N use in nonruminants.

and regimens of tracer delivery (Perez and Reeds, 1998; Neese et al., 2002), as illustrated in Figure 10.

Three major pathways are known to exist for the biosynthesis of nucleic acids RNA and DNA. These include i) the deoxyribonucleoside salvage pathway by using thymidine and uridine as substrates for DNA and RNA synthesis, respectively; ii) the de novo purine nucleoside synthesis pathway for RNA synthesis; and iii) the de novo purine deoxynucleoside synthesis pathway for DNA synthesis (Perez and Reeds, 1998; Neese et al., 2002; Zhang et al., 2004). Thus, 2 major precursor pools are available for probing RNA and DNA syntheses: the organ or tissue free thymidine and deoxyuridine pools, and the organ or tissue free purine and deoxypurine nucleoside or nucleotide pools (Perez and Reeds, 1998; Neese et al., 2002; Zhang et al., 2004).

As shown in Figure 10, 4 major types of tracers can be used for measuring RNA and DNA synthesis rates. Deoxyribonucleoside salvage pathway tracer nucleosides such as [^{3}H]thymidine and bromodeoxyuridine are used (Smith and Jarvis, 1978; Fan et al., 2001). These types of tracers are usually used in cell culture or with small animals. There are concerns associated with the use of these tracers, because they can only label a small fraction of de novo RNA and DNA synthetic activities (Neese et al., 2002; Zhang et al., 2004). The de novo purine nucleoside pathway can be labeled by using additional types of tracers for measuring RNA or DNA synthesis: i) using L-[^{15}N]Gly and L-[U-^{13}C]Gly for labeling the purine base (Perez and Reeds, 1998; Zhang et al., 2004); ii) using D-[U-$^{13}C_6$]glucose and $^{2}H_2O$ for labeling ribose or deoxyribose (Neese et al., 2002; Zhang et al., 2004). L-[^{15}N]Glycine and $^{2}H_2O$ are relatively low-cost tracers, whereas L-[U-^{13}C]Gly and D-[U-$^{13}C_6$]glucose are more expensive.

The deoxyribonucleoside salvage pathway tracer nucleosides such as [^{3}H]thymidine and bromodeoxyuridine are usually administered via a single i.p. or i.v. injection, followed by organ and tissue harvesting after the tracer injection (Fan et al., 2001). Oral feeding with $^{2}H_2O$ can be economically and conveniently applied after an i.p. priming dose of the tracer water (Neese et al., 2002). L-[U-^{13}C]Glycine, D-[U-$^{13}C_6$]glucose, and L-[^{15}N]Gly are delivered through a continuous infusion via a catheter for 6 to 8 h (Perez and Reeds, 1998; Zhang et al., 2004).

Cellular turnover rates, as measured by various techniques, are summarized in Table 5. Continuous infusion or oral feeding of L-[^{15}N]Gly is, in principle, a rapid, economic, and convenient approach for simultaneously measuring organ and tissue protein, RNA, and DNA synthesis rates under controlled research conditions. Oral feeding with $^{2}H_2O$ can be conveniently applied for measuring organ and tissue protein, RNA, and DNA synthesis rates under both controlled research and field test conditions.

CONCLUSIONS

Poor conversion efficiency of dietary CP, including protein, RNA, and DNA, is not only a classical productivity and profit issue but also a major sustainable development concern facing nonruminant production on the global scale. Metabolic losses of N compounds, including endogenous loss and catabolic loss, are the major reasons of N inefficiency, and are affected by postnatal developmental programming, diets, and management factors. Measurements of the synthesis rates of major N-containing polymers in the viscera, plasma, peripheral immune cells, and muscles through oral

Table 5. Cell life span in different organs and tissues measured via cellular DNA synthesis rates

Cell types and animal	Type of tracer used	Life span, d
Small intestinal epithelia, new born piglets[1]	[^{3}H]Thymidine	19
Proximal small intestinal epithelia, 16-d-old suckling pigs[2]	Bromodeoxyuridine	5
Distal small intestinal epithelia, 16-d-old suckling pigs[2]	Bromodeoxyuridine	10
Small intestinal epithelia, mature rats[3]	^2H_2O	3 to 5
Colonocytes, mature rats[3]	^2H_2O	2 to 5
Adipocytes, mature rats[3]	^2H_2O	60 to 100
Blood vessel endothelial cells, mature rats[3]	^2H_2O	20 to 50
Newly divided naïve CD4$^+$ T cells, mature rats[3]	^2H_2O	30 to 33

[1]Smith and Jarvis (1978).
[2]Fan et al. (2001).
[3]Neese et al. (2002).

feeding with ^2H_2O and/or i.p. injection of a flooding dose with a Phe tracer are emerging powerful tools for studying nonruminant nutrition, metabolism, and growth under research and field test conditions. These tools will be complementary to the classical mass-balance techniques in providing new knowledge and developing novel strategies. The application of stable isotope tracer methodology for measuring in vivo organ and tissue cellular protein, RNA, and DNA synthesis rates may dramatically improve our understanding in nonruminant nutrition, metabolism, and growth.

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