PURINE DERIVATIVES - MARKER FOR RUMEN MICROBIAL PROTEIN PRODUCTION

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ABSTRACT

Microbial cells are the major source of protein for ruminants. Knowledge of the microbial contribution to the nutrition is essential to develop feed supplementation strategies for improving ruminant production. The methods generally used for determining microbial protein production depend on the use of natural microbial markers or isotopes. The idea of using microbial purine compounds as a specific marker for rumen microbial biomass was suggested few decades back. Microorganisms have high concentration of purine containing compounds compared to plants and mammalian cells. Moreover, purines in dietary and endogenous materials are in general rapidly degraded by microbial enzymes in the rumen. They are therefore likely to be present in negligible concentration in digesta leaving the rumen. The microbial purines on the other hand remain intact in living microbial cells and pass via the abomasum to the small intestine. The purines present in the digesta entering the small intestine can therefore be expected to be almost totally of microbial origin. When the microbes enter the abomasum and the small intestine, they are degraded enzymatically to nucleotides and purine bases. These are then absorbed into the body of the animal. Although these purine compounds may be incorporated into tissues, the amount absorbed greatly exceeds tissue requirements and the majority is excreted via the kidneys. The amount of purine derivatives (PD) (allantoin, uric acid, xanthine and hypoxanthine) in the urine therefore tend to closely reflect and can be used to predict the flow of microbial purines into the intestines, thus quantifying the intestinal flow of microbial protein to the animal.

Keywords: Purine Derivatives, microbial protein, ruminants

INTRODUCTION

A major constraint to animal production in developing countries is poor nutrition due to inadequate nutrient supply. This results in low rates of growth and production as well as increased susceptibility to diseases and mortality. The small holder farmers in developing countries have limited resources available for feeding their livestock. Unlike

those in developed countries, they are unable to select their basal diet according to requirement for production. Therefore, the strategy for improving production has been to maximize the efficiency of utilization of available feed resources in the rumen by providing optimum conditions for microbial growth and then by supplementation to provide dietary nutrients to compliment and balance the products of digestion to meet the requirement. So, there is a need to optimize the plane of nutrition in ruminants for adequate production of microbial protein and supply of nutrients adequately for the maintenance of normal growth, production and reproduction. As the rumen microbes are rich in purines, the amount of purine derivatives (PD) in urine can be used as a tool for estimating microbial protein supply to the ruminants.

Purines

Purines are heterocyclic ring structures (nitrogenous bases) with varying functional groups. The purine bases, adenine and guanine are found in DNA and RNA. Rumen microorganisms have high concentrations of purine containing compounds (RNA and DNA) relative to their concentrations in plants and mammalian cells. Of the total nitrogen present in rumen bacteria, nucleic acids and proteins comprise 13 to 19 per cent and 75 to 85 per cent, respectively (Stangassinger *et al.,* 1995).

Purine derivatives

The term purine derivatives (PD) refer to the sum of allantoin, uric acid, xanthine and hypoxanthine. These are the end products of purine metabolism. All the four compounds are excreted in the urine of sheep, goats, llamas, red deer and camels; but xanthine and hypoxanthine are virtually absent from the urine of cattle, buffaloes and yaks (Chen and Gomes, 1992; Chen *et al*., 1990a; George *et al.,* 2011a). Purine derivatives in urine can be estimated by colorimetric and HPLC methods (George *et al.,* 2006a) and there exist good correlation (George *et al.,* 2006b) between these methods.

Degradation of dietary purines and the formation of microbial purines

In general, it appears that free nucleic acids and their derivatives from diet are rapidly degraded in the rumen (McAllan and Smith, 1973). Thus, the majority of purine nucleosides and free bases present in the small intestine of ruminants are microbial in origin (McAllan, 1980). A linear relationship exists between urinary excretion of purine derivatives and duodenal input of microbial nucleic acids (Fujihara *et al*., 1987; Chen *et al*., 1990a), and of microbial RNA (Antoniewicz *et al*., 1980; Balcells *et al*., 1991). Nucleic acid of diet and added nucleic acids in the rumen have reported to be rapidly degraded into free bases, nucleotide and nucleosides within the rumen (McAllan and Smith, 1973; Smith and McAllan, 1970). These substrates can be utilized as a source of carbon and nitrogen for microbial synthesis in the rumen (Jurtshuk *et al*., 1958) and may be incorporated as a precursor of nucleic acids by rumen microbes (Smith and Mathur, 1973). In the rumen, acetic acid, carbon dioxide and ammonia are the major end products of the degradation for purine bases (Jurtshuk *et al*., 1958).

Rumen microbes are rich in nucleic acids. Around 18 per cent of total nitrogen of rumen microbes is present in nucleic acids

of which 11 per cent is in purines (Topps and Elliot, 1965). The purine content of bacteria but not that of protozoa in the rumen can vary widely due to different species and types of bacteria, energy sources and the post feeding time (Arambel *et al*., 1982; Kanjanapruthipong, 1995). Microbial nucleic acids entering the small intestine (of which about 60-70% is RNA) are extensively degraded to mononucleotides (Armstrong and Hutton, 1975). Later these mononucleotides are acted upon by the phosphatase enzymes in the rumen. It is clear that a variety of nucleases and related enzymes occur in the small intestine of ruminants and these degrade nucleic acids to nucleosides. It was hypothesized that the rate of removal of sugar to release the free base was the rate-limiting step for the complete degradation of purine compounds in the intestines (McAllan, 1982). Pancreatic secretions of ruminants are particularly high in ribonuclease which ensures that microbial nucleic acids are extensively degraded in the small intestine (Davidson, 1972). These enzymes catalyze the hydrolysis of phosphodiester bonds and release poly or mononucleotides (McAllan, 1980). Two phosphodiesterases have been isolated from intestinal mucosa, which is essentially exonucleases, the first attacking polyribonuclease on one end of the nucleic acids and liberating the nucleoside-5-phosphate in a stepwise manner, and the second liberating nucleoside-3-phosphate from the other end. Intestinal 5'-nucleotidase and a non-specific alkaline phosphatase have also been found in ruminants. These nucleosides or their breakdown products, adenine, guanine and ribose are absorbed. Condon *et al*. (1970) found that adenine and guanine were completely absorbed from the small intestine, whereas nucleosides were less completely absorbed. Mammalian tissues also have purine nucleoside phosphorylase that would allow nucleosides, if they are absorbed as such, to be further degraded within the body. Net digestibility for nucleic acids in the small intestine of ruminants was 80 to 90 per cent for RNA and 75 to 85 per cent for DNA (McAllan, 1982). McAllan (1980) reported that true digestibility for nucleic acids infused into the duodenum of steers was 97 per cent, while Chen *et al*. (1990b) reported a digestibility of 91 per cent for an infused source of microbial purines. A value of 0.83 is generally used for calculations (Nolan, 1999). However, true digestibility is arguably more appropriate because purines flowing from the ileum are more likely to be derived from endogenous sources and should therefore be considered as part of non renal excretion of purines.

Enzymatic degradation of absorbed purines

The absorbed purines are immediately degraded by the enzyme xanthine oxidase, present in the intestinal mucosa. The mucosal cells of cattle and buffaloes are rich in xanthine oxidase, which increases the potential for oxidation of absorbed purines before they enter the bloodstream (Nolan, 1999). In ruminants, hypoxanthine and xanthine are converted to uric acid by xanthine oxidase, and uric acid is further converted to allantoin by the liver enzyme uricase (IAEA, 1999). Sheep mucosa in contrast has only trace amount of xanthine oxidase enzyme (Al-khalidi and Chaglassian, 1965), which results in a higher blood concentration of xanthine and hypoxanthine and their subsequent

excretion in urine by sheep relative to cattle and buffaloes (Chen *et al*., 1996). The presence of high activities of xanthine oxidase in cattle and buffalo plasma leads to the complete conversion of hypoxanthine and xanthine to uric acid.

Intracellular metabolism of absorbed nucleosides

The absorbed nucleosides are split by nucleoside phosphorylase to give free bases (Zollner, 1982). Guanine is degraded by guanase to xanthine, adenosine to form inosine and further catabolized to hypoxanthine (Berlin and Hawkins, 1968). Hypoxanthine and xanthine can be further oxidized or salvaged depending upon the existence of the xanthine oxidase in tissues particularly in the mucosal epithelium of the small intestine as well as in the blood. In sheep, hypoxanthine and xanthine are available to be reutilized or salvaged by other organs before reaching the liver (Chen *et al*., 1990a). But in cattle, the purine derivatives might be salvaged only in tissues of the small intestine, where xanthine oxidase can be detected (Al-khalidi and Chaglassian, 1965).

Excretion of purine derivatives

Estimation of rumen microbial protein production by quantifying the purine derivatives excreted in the urine is based on the assumption that, the absorbed purines are completely excreted via the kidney (Nolan, 1999). Both the works in sheep and cattle showed that approximately 15 per cent of the exogenous purines were not accounted for by urinary excretion (Chen *et al*., 1991). Direct infusion of allantoin into the blood of sheep also yielded incomplete recovery (Chen *et al*., 1991; Liang *et al*., 1994). Although the plasma purine derivatives can be excreted/secreted via non-renal routes i.e. the salivary (Chen *et al*., 1989), milk (Giesecke *et al*., 1994; Gonzalez-Ronquillo *et al*., 2003) and gut secretions (Berlin and Hawkins, 1968), the amount of excretions is negligible (Rosskopf and Giesecke, 1992) and does not appear to be related to urinary excretion (Gonda and Lindberg, 1997). The concentrations of purine derivatives in the plasma are also related with those in urine (McAllan, 1980).

Endogenous excretion

Purine nucleotides are broken down and re-synthesized from either *de novo* synthesis of purines or salvage of preformed purines. This cycle is a continuous process, a small proportion of the recycling purines are decomposed to hypoxanthine, xanthine, uric acid and allantoin, which are excreted in the urine. This fraction of purine derivatives, which originate from animal tissues, is called endogenous purines. Among the purine derivatives hypoxanthine can be reused for the synthesis of purine nucleotides, but when hypoxanthine is oxidized by xanthine oxidase to produce uric acid, the latter cannot be reused. Xanthine oxidase activity in the tissue is thus the key enzyme affecting the production of endogenous PD excretion (Chen *et al*., 1996). Estimates of the extent of endogenous release of purines have been made by conducting fasting trials (Osuji *et al*., 1996), by using the technique of maintaining animals by intra-gastric infusion of nutrients (Verbic *et al*., 1990; Funaba *et al*., 1997; Martin-Orue *et al*., 2000; Orellana-Boero *et al*., 2001; Pimpa *et al*., 2001), or by replacing

normal digesta entering the intestines with digesta devoid of purines. The endogenous PD excretion is higher in cattle, per unit of metabolic weight, than in buffaloes (Pimpa *et al*., 2003, Singh *et al*., 2007) and sheep, and is not inhibited as purine absorption increases. A consequence of these factors is that endogenous secretion of purines is always present in cattle and the relationship between purine absorption and urinary PD excretion is more linear (Verbic *et al*., 1990). A further consequence is that different prediction equations will be required for different species of livestock.

Relationship between feed intake and the excretion of purine derivatives and creatinine in urine

The urinary excretion of purine derivatives by different ruminants has been shown to be positively related to the digestible organic matter intake (Mayes, 1995; Chowdhury, 1998; George *et al*., 2006c). This was also proved in various studies conducted in Indonesian Ongole cattle (Soejono *et al*., 1999), Malaysian Kedah Kelantan cattle (Liang *et al*., 1999), Chinese Yellow cattle (Fang, 2000), Turkish Yerli Kara cattle (Cetinkaya *et al*., 1999), Chilean Friesian cattle (Orellana-Boero *et al.,* 2000), Brahman cattle (Jetana *et al*., 2003), Murrah Buffaloes (Dipu *et al*., 2006), Indian crossbred bulls (George *et al*., 2006c; Singh *et al*., 2007) and Barbari goats (George *et al*., 2011b). Excretion of purine derivatives per unit of digestible organic matter intake (DOMI) is 18.5 mmol/kg DOMI for *Bos taurus* (Giesecke *et al*., 1993) and 8.3 mmol/kg DOMI for buffaloes (Liang *et al*., 1994). An almost constant excretion of creatinine from different animal species with varying DOMI when expressed on metabolic weight, confirmed that it can be used as an internal marker, relating the ratio of purine derivatives to creatinine concentrations to the daily out put of purine derivatives (Chen *et al*., 1995a; IAEA, 2000). The ratio of purine derivatives:creatinine (mmol:mmol) concentrations in hourly urine samples (referred to as spot urine) when corrected for metabolic weight to allow comparison between animals is called PDC index and found to respond positively to changes in DOMI (Chen *et al*., 1995b; IAEA, 2000).

Mathematical models for the estimation of rumen microbial protein production using PD excretion

Prediction equations have been developed based on PD excretion rates in urine to estimate the supply of microbial protein in cattle, sheep, buffaloes and goats. The important parameters required for this are daily PD excretion through urine, endogenous PD excretion and the proportion of plasma PD excreted in the urine, or the recovery of exogenous PD from the urine. Chen *et al*., (1990b) exploited the intra-gastric nutrition technique to define the quantitative relationship between PD excretion and purine absorption in sheep. Animals were maintained on purine free nutrients and known amounts of purines (adenine and guanine) were infused into the abomasum. In their study they got the following curvilinear relationship between PD excretion, (Y, mmol/day) and purine absorption (X, mmol/day) where $W^{0.75}$ represents the metabolic body weight (kg) of the animal

$$Y = 0.84\ X + (0.150\ W^{0.75} e^{-0.25x})$$

Later this equation was modified by

Balcells *et al*. (1991) as

$Y = 0.87 X + (0.210 W^{0.75} e^{-0.14x})$

A similar study conducted in European cattle (Verbic *et al*., 1990) proved that the endogenous PD excretion in cattle was higher compared to sheep and they got the following linear relationship,

$Y = 0.85 X + (0.385 W^{0.75})$

Liang *et al*. (2002), established similar equations for tropical swamp buffaloes (*Bubalus bubalis*) and zebu cattle (*Bos indicus*). The relationship between daily urinary PD excretion and daily microbial purine supply was established as $Y = 0.12 X + (0.20 W^{0.75})$ for buffaloes and $Y = 0.85 X + (0.30 W^{0.75})$ for zebu cattle.

Prediction equations were later developed for various breeds of cattle based on the recovery of labelled PD (nuclear technique) and the measures of endogenous excretion (IAEA, 1999).

For Kedah-Kelantan (KK) cattle:

$Y = 0.68X + 0.275W^{0.75}$

For Zebu crosses

$Y = 0.84X + 0.236W^{0.75}$

For Ongole cattle:

$Y = 0.85 X + 0.132W^{0.75}$

For Bali cattle:

$Y = 0.86 X + 0.145 W^{0.75}$

Prediction equations were also developed for various species of Indian ruminants

For Crossbred cattle (Singh, 2007)

$Y = 0.83 X + 0.296 W^{0.75}$

For Murrah buffaloes (*Dipu et al*., 2006)

$Y = 0.74X + 0.117 W^{0.75}$

For Muzzafarnagari sheep (Dipu *et al*., 2008a)

$Y = X + (0.217 \text{ kg } W^{0.75} e^{-0.25x})/0.84.$

For Barbari goats

$Y = X + (0.250 \text{ kg } W^{0.75} e^{-0.25x})/0.76$ (George *et al*., 2011b)

Where Y = PD excretion (mmol/d), X = Purine absorption (mmol/d) and $W^{0.75}$ = Metabolic body weight (kg) of the animal.

Use of PD technique under field conditions

Analysis of spot urine samples for PD and creatinine and linking the information to feed intake, makes possible to study the nutritional adequacy in ruminant livestock under field conditions. Antoniewicz *et al*. (1981) already observed a linear correlation between the ratio of allantoin-N to creatinine-N and ME intake in sheep, suggesting the possibility of using the allantoin:creatinine ratio in spot urine as the parameter. Subsequent studies were carried out in steers, dairy cows and sheep to examine the diurnal variations in spot urine measurements (Chen *et al*., 1992; 1995a; Gonda and Lindberg, 1994, Singh *et al*., 2007., George *et al*., 2006c., George *et al*., 2011b). Various studies were carried out under the FAO/IAEA research programme (Makkar, 2004). Many studies used the direct (PD/Creatinine) concentration ratio in their presentation of results (Chen and Ørskov, 2004). However, data of the direct (PD}/{creatinine} ratio can only be compared within the same animal or among animals of the same body weight, since the daily creatinine excretion is a function of body weight.

PDC index

In the absence of total urine collection, PD:C ratio may provide an index to detect differences in PD excretion assuming that creatinine excretion depends only on the body mass and is constantly excreted through out the day. Each derivative and the total PD concentration were expressed as a ratio to creatinine concentration, which

in turn had to be divided by metabolic live weight to allow comparison between species. The ratio of PD:C (mmol:mmol) concentrations in hourly urine samples (referred to as spot urine) when corrected for metabolic weight to allow comparison between animals is called PDC index (Chen *et al*., 2003). Total PD:Creatinine ratio (mol/molkgw$^{0.75}$) was 118, 46, 37 and 33 for cattle, camels, buffaloes and sheep, respectively (Moscardini *et al.,* 1999). However, data of the direct {PD}/{creatinine} ratio can only be compared within the same animal or among animals of the same body weight, since the daily creatinine excretion is a function of body weight.

PDC index = [PD]/[Creatinine] X W$^{0.75}$

Where W is the body weight (kg), [PD] and [Creatinine] are PD and creatinine concentrations in urine, in mmol/L. Differences in PDC index should now be due to urinary PD output. Data of PDC index can be compared among animals of different body weights (but the same breed). By definition, the PDC index and PD excretion has a following linear relationship:

PD excretion = PDC index X C, Where, C is the daily creatinine excretion (mmol/kg W$^{0.75}$) for a specific breed of animals, and should have been previously measured using complete urine collection. From this equation, total daily excretion of PD can be calculated from the PDC index, yet without the need for total urine collection. However, the ratio of purine derivates/allantoin with creatinine in urinary spot samples was found to be not suitable for detecting small differences in microbial nitrogen supply in cross bred bulls (George *et al*., 2006c).

Purine nitrogen index

During microbial fermentation, part of the dietary nitrogen is converted to ammonia, a proportion of which is captured by rumen microorganisms for the synthesis of microbial protein. The remaining ammonia is absorbed from the rumen and is finally excreted as a source of nitrogen in the urine. Purine Nitrogen Index (PNI) refers to the ratio of purine derivative (PD) nitrogen to total nitrogen in urine (Chen *et al*., 1999). This has been proposed as an index of the utilization of nitrogen in the rumen (PD nitrogen) compared to the amount wasted in the urine. The purine nitrogen index (PNI) correlates with nitrogen balance and could be used as an indicator for assessing efficiency of dietary nitrogen utilization (Dipu *et al*., 2008). If a diet or a dietary regime has poor conversion efficiency, proportionally less dietary nitrogen is converted to microbial protein and more excreted in the urine resulting in a low PNI. Endogenous nitrogen output, nitrogen intake, protein degradability, digestibility of protein and inefficiency of absorbed amino acids can all affect the value of PNI (Chen *et al*., 1999). PNI could be used as one of the criteria to help formulate ruminant diets that are biologically more efficient and produce less nitrogen waste. A practical application would be to set an empirical threshold criterion for a specific group of ruminants and diets with PNI values lower than this threshold are graded as unsatisfactory with respect to nitrogen utilization (Chen *et al*., 1997). Even though PNI can be used as a tool for rapid feed evaluation in ruminants it has certain limitations. The index does not offer any explanation to the cause of poor efficiency and it is not sensitive for

detecting small differences (George *et al*., 2011c). However the latter limitation could be overcome by using it in a 'grouping' system.

Relationship between level of purine derivatives in plasma and urinary excretion of purine derivatives

The relationship between plasma PD level and urinary excretion of PD has been investigated by a number of workers (Chen *et al*., 1992; Giesecke *et al*., 1994; Fujihara *et al*., 2003; George, 2007; George *et al*., 2017). However, the results on whether plasma PD concentration was correlated with daily urinary PD output were inconsistent. It has been reported (Chen and Ørskov, 2004) that, the urinary excretion of PD is proportional to the plasma concentration of PD and glomerular filtration rate (GFR). If GFR is constant, then concentration of PD in plasma is linearly correlated with the influx of microbial purines from the intestines. However, if GFR and reabsorption of PD is variable, plasma level of PD will be related with neither the influx into the plasma nor the renal excretion. At increased levels of feed intake the influx will be obviously high. Therefore, if GFR is not changing with feed intake, the plasma PD can be used as an index of microbial protein supply. Therefore, if the plasma concentration is to be used to indicate the flow of microbial purines into the blood or the excretion of PD in urine, the variation in GFR needs to be taken into account. The studies conducted in cattle (Chen *et al*., 1995; Deshpande *et al*., 2013), buffaloe (Deshpande *et al*., 2013) and sheep (Kagiyama *et al*., 1996) revealed that the GFR in same animal may change with level of feed intake. However, in a study in crossbred bulls, GFR was found to be similar in animals fed at 100 and 80 per cent of voluntary dry matter intake (George, 2007).

CONCLUSION

The studies conducted so far indicate that purine derivatives excreted in the urine serves as an index for rumen microbial protein supply in ruminants. The use of spot urine samples to predict daily urinary PD excretion by calculating PDC index may be a simple and useful indicator of nutritional status of ruminants under field conditions. However, further research would be needed to define the partitioning factor between renal and non-renal disposal of plasma PD in ruminants, which may help us to understand better the source of variability between individuals and thus, more precision and sensitivity can be attached to the estimation of microbial protein supply based on the PD excretion.

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