

RESEARCH ON URINARY EXCRETION OF PURINE DERIVATIVES IN RUMINANTS: PAST, PRESENT AND FUTURE

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Abstract

RESEARCH ON URINARY EXCRETION OF PURINE DERIVATIVES IN RUMINANTS: PAST, PRESENT AND FUTURE

Research on urinary excretion of purine derivatives (PD), namely allantoin, uric acid, xanthine and hypoxanthine, in ruminants have been carried out with an objective to use the excretion of these purine metabolites as a parameter to estimate the intestinal flow of microbial protein. This paper reviews the published literature, from the first paper in 1931 to the current date, in a historical perspective. The current status of understanding in some key topics is discussed. The topics include: endogenous excretion, modelling the response of PD excretion to purine absorption, calculation of microbial N supply from PD excretion, use of spot urine measurement, possible use of plasma or milk PD as an alternative index, and applications in ruminant nutrition research. This review also covers the current understanding of PD excretion in different animal species, including sheep, cattle, goats, buffaloes, llamas, camels, yak and deer. Progress in analytical methods for the determination of purine derivatives is also discussed. Finally, areas of future research are highlighted. The paper stresses the need for more studies on metabolism of PD in the tissue, the kinetics of PD in the blood and physiological processes of renal excretion, so as to understand better the mechanism that accounts for the between-species and within-species variation in PD excretion. Development of simpler and more rapid methods for defining the endogenous excretion and purine input-output relationship is also an area for future work.

1. INTRODUCTION

Rumen microbes are rich in nucleic acids: around 18% of the total nitrogen (N) is present in nucleic acids or 11% in purines. Rumen microbes constitute the major source of protein supply to the ruminants. The purines from the rumen microbes are metabolized and excreted in the urine as their end products: hypoxanthine, xanthine, uric acid and allantoin. Research work has been carried out actively for the past 20-30 years into the urinary excretion of these purine metabolites, with an objective to use the excretion of these metabolites as a parameter to quantitatively estimate the supply of rumen microbial protein to the ruminant. The current paper attempts to provide an overview of the published work in a historical perspective, and to highlight the current status of understanding on this subject. A large number of references to published literature are cited in this review for the benefit of research students and future researchers. Areas of future research are also suggested. The general biochemistry of purines is however not covered in this review.

The term “purine derivatives (PD)” used in this review refers to the sum of allantoin, uric acid, xanthine and hypoxanthine. All four compounds are excreted in the urine of sheep, goat, llamas, red deer and camels; but xanthine and hypoxanthine are virtually absent from urine of cattle, buffaloes and yaks. In ruminants, hypoxanthine and xanthine are converted to uric acid by xanthine oxidase, and uric acid is further converted to allantoin by uricase. 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.

2. PAST AND PRESENT

The very first work reported in the literature in this topic dated 1931. Over the 70 years, most progress was made from 1980, as judged from the amount literature available. The history may be broadly divided into two phases, 1) observation phase: changes in PD excretion with dietary components were observed; 2) modelling phase: the quantitative response of PD excretion to purine absorption were modelled. 1985 may be subjectively used as the dividing point for the two phases: the observation phase before 1985 and the quantitative phase after 1985.

2.1. The observation phase

French researchers Torronine & Mouroit [1] noted in 1931 that allantoin excretion in the urine of sheep increased with level of protein intake. In their paper, they asked the question: “Does allantoin excreted in the urine by mammals originate from the partial degradation of protein?” Morris & Ray [2] in 1939 found that allantoin and uric acid in the urine decreased during a 7-day starvation in sheep, goat and cows, and further confirmed that these purine metabolites were associated with some dietary factor. Blaxter & Martin [3] infused casein into the rumen and abomasum of sheep and observed an elevated allantoin excretion only with the ruminal infusion. The result indicated that the unknown factor was associated with fermentation in the rumen. It was hypothesised that the increased allantoin excretion was due to a greater extent of microbial growth when additional protein was provided in the rumen. Elliott & Topps [4] further showed the response of allantoin excretion to dietary protein intake in cattle. Topps & Elliott [5] demonstrated that allantoin excretion in urine was correlated with the nucleic acid concentration in the rumen in sheep given diets of different levels of fermentable energy. Based on this observation, They suggested for the first time that allantoin in urine could serve a usual index of production of nucleic acids in the rumen. The term “purine derivative” was also used for the first time. Topps’s PhD student Razzque followed this line of research for the next few years [6]. In order to determine xanthine, hypoxanthine and uric acid, a method based on enzymatic conversion of xanthine and hypoxanthine to uric acid which was then determined spectrophotometrically was developed [7].

In 1970s to 1980s, more extensive observations were made to confirm that there was a linear relationship between allantoin excretion and level of feed (N or dry matter) intake [6,8-17], or the flow of nucleic acids in the duodenum [18-20].

Smith and McAllan in the 1970s carried out extensive studies on nucleic acid metabolism in the gut of ruminants. Their work was crucial for relating PD excretion to the production of microbial biomass in the rumen. Their research demonstrated that exogenous

source of nucleic acids (the sources used in their study were: yeast RNA, herring-sperm DNA, hay nucleic acids) were completely broken down in the rumen fluid; and so were the intermediate degradation products, nucleotides and nucleoside [21-23]. The indication was that nucleic acids in the feed would not contribute significantly to the nucleic acid content in the rumen, and the nucleic acids entering the duodenum of ruminants were essentially of microbial origin. This has provided the basis for using RNA as a marker of microbial biomass synthesis in the rumen. The same basis is shared by the use of PD excreted in the urine as an index of microbial biomass synthesis in the rumen.

In 1975, Rys et al. [24] were the first to suggest a conversion factor to calculate microbial N yield from allantoin: a conversion factor of 0.25 of nucleic acid N being excreted as allantoin-N, which was the result obtained from a study in rat [25]. The estimates of microbial N yield by this calculation however were considerably lower than those by other marker methods [14,24]. In retrospect, little confidence can be attached to the final result, since at that time little was known about the quantitative relationship between nucleic acid intake and allantoin excretion.

The work of Antoniewicz and co-workers in Poland must be acknowledged. They had carried out an active research programme into PD excretion in relation to microbial protein production in sheep in 1970s and 1980s. Some of their work spanned to the modelling phase.

2.1. The modelling phase

1980s - 1990s was the period of time when ruminant protein nutrition research was the most active. The new protein evaluation system was developed within this time – a system which is based on the concept of rumen un-degradable protein and microbial protein, instead of digestible crude protein. There was a greater need to quantify the supply of microbial protein to the host animal, since the data was required for the new system. This need has driven the research into developing simpler and more accurate method for measurement of microbial protein supply. The use of PD excretion in the urine has the potential for providing a non-invasive approach to quantify microbial protein supply. The scientific curiosity has been to model how allantoin and other metabolites are excreted in the urine in relation to the absorption of microbial purines.

Significant progress in modelling PD excretion using nuclear techniques was made under the Joint FAO/IAEA Division's Coordinated Research Project on "Development, standardization and validation of nuclear based technologies for measuring microbial protein supply in ruminant livestock for improving productivity" from 1996-2001 (reports can be found in IAEA-TECDOC-945 and 1093 [26,27] and the present publication). In this project, the research work on PD was extended to Zebu cattle, buffaloes and camels.

2.1.1. 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 in animal tissues. During this 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". It should be remembered that hypoxanthine can be reused for the synthesis of purine nucleotides, but

when hypoxanthine is oxidized by xanthine oxidase to produce uric acid, the latter can not be reused. Xanthine oxidase activity in the tissue is thus the key enzyme affecting the production of endogenous PD excretion.

The measurement of this endogenous contribution is an important parameter in modelling the PD excretion. In earlier work, the endogenous excretion was estimated by fasting [28,13]. The results were variable (measurement in sheep was 32-208 μmol allantoin/kg $W^{0.75}$ per day). The critique of using fasting excretion was that the prolonged starvation may alter the metabolic activities of the animal and thus the rate of nucleic acid degradation. The development of “intra-gastric nutrition technique” in the UK by Ørskov et al. [29] had provided the means to study endogenous excretion without affecting the nutrition of the animal. With this technique, the microbial fermentation in the rumen is eliminated but the normal nutrition of the animal is maintained by the nutrients supplied as VFA and casein continuously infused into the rumen and abomasum respectively. Antoniewicz & Pisulewski [30] adopted the technique in Poland to measure the endogenous excretion first. Subsequently, Sibanda et al [31], Fujihara et al. [32] in the UK, Giesecke et al [33] in Germany, and Lindberg & Jacobsson [34] in Sweden used this technique to study the endogenous excretion of PD in sheep. Despite that the measurements were made based on a small number of animals, the results were less variable (165-209 total PD $\mu\text{mol}/\text{kg } W^{0.75}$ per day in sheep). Fujihara et al. [32] for the first time proposed to measure total PD, i.e. allantoin, uric acid, xanthine and hypoxanthine, instead of allantoin only.

The work of Fujihara in the UK laboratory was furthered by Chen and co-workers. For the next a few years, they had conducted systematic measurements of the endogenous PD excretion in sheep and cattle using the intra-gastric nutrition technique. The endogenous PD excretion was 168, 166, 514 $\mu\text{mol}/\text{kg } W^{0.75}$ in sheep, pig and cattle respectively [35]. A marked difference in the endogenous excretion between sheep and cattle was observed. Chen et al. [35] suggested that this could be due to the difference in the xanthine oxidase state: the enzyme is absent in the blood of sheep but present in high activities in the blood and other tissues of cattle. The influence of protein nutrition on the endogenous excretion was also examined. Chen et al [36] demonstrated in sheep that the daily allantoin excretion fluctuated according to the previous nutrition status, or induced by an abrupt depletion of protein intake.

Balcells, Guada and co-workers in Spain had developed an alternative technique called “gastric emptying” to measure endogenous excretion in sheep [37]. In this technique, animals were fitted with a re-entry cannula at entry to duodenum, at which digesta leaving the abomasum is replaced with a synthetic fluid without nucleic acids and placed back to the duodenum. The endogenous PD excretion determined in sheep was similar to that observed in animal nourished by intra-gastric nutrition.

A review on the endogenous excretion in sheep and cattle is given by Stangassinger et al. [38].

2.1.2. Response of PD to purine uptake

Condon et al. [39], Smith et al [40] and Razzaque [41] studied the recovery of dietary purines in sheep using ^{14}C tracers. In their work, ^{14}C -purine was infused into the abomasum, or ^{14}C -purine labelled bacteria cells were injected into the rumen, recovery of the dosed ^{14}C activities in the urine was low and variable (0.15-0.41), but radioactivities were also detected in tissue and in gas. Condon & Hatfield [42] and Antoniewicz et al. [19] infused two levels of

RNA to the abomasum of sheep fed with mixed rations. The recovery, calculated based on the increment over the control, was also variable (0.22-0.79 as allantoin).

The revelation of the quantitative relationship between PD excretion and purine absorption was hindered by technical difficulty of getting the data of total intestinal purine uptake – due to the fact the “background” purine uptake from the basal diet (i.e. the control) was not quantified. The “background” level may not necessarily stay unchanged with the treatments. Moreover, as demonstrated by Chen et al. [43], the recovery of absorbed purine as urinary PD increased with the purine input in sheep, following a curvilinear pattern (see end of this section). Thus, without the data of total purine uptake (background plus infused), the results obtained were confounded. The variable recovery results observed by the different authors could all be correct at different positions of the X axis in the response curve of PD excretion vs. purine input (see later section).

In 1990, Chen and co-workers again exploited the intragastric nutrition technique to define the quantitative relationship between PD excretion and purine absorption in sheep and cattle. Animals were maintained with purine free nutrients and known amounts of purines in 6-8 steps infused into the abomasum. The range of purine input was from 0 to the amount equivalent to microbial purine uptake when the animals were to be fed with energy three times maintenance requirement. In sheep, a curvilinear relationship was observed [43]:

$$Y = 0.84 X + 0.150 W^{0.75} e^{-0.25X} \dots\dots\dots (1)$$

In cattle, a linear relationship was observed [44]:

$$Y = 0.85 X + 0.385 W^{0.75} \dots\dots\dots (2)$$

Again a difference between sheep and cattle was observed. It was suggested that the fate of the absorbed exogenous purines on passage through the intestine was important and determined the shape of the response curve [44]. In cattle, because of high activities of xanthine oxidase in the intestinal mucosa [45], exogenous purines could be completely converted to end products which are not usable by tissues. In sheep, the intestinal xanthine oxidase activity was low allowing exogenous purines to be available for salvage in the intestine and the liver. Tracer study had shown that exogenous purines were retained into tissue (discussed above). The studies of Chen et al. [43] and Balcells et al.[46], which showed that re-usable purines were present in the portal blood of sheep but not in cattle, provided further supporting evidence.

Both the work in sheep and cattle also showed that approximately 15% of the exogenous purines were not accounted for by urinary excretion. Direct infusion of allantoin into the blood of sheep also yielded incomplete recovery [47]. One possibility was disposal by non-renal route, e.g., saliva or direct secretion into the rumen. Chen et al. [48] then further demonstrated that indeed, allantoin and uric acid were presence in the saliva of sheep, and that PD entering the rumen would be completely broken down and would not get back to the urine (later confirmed by Sura et al. [49]. In a further study of the renal clearance of allantoin, Chen et al [47] reported that the tubular reabsorption of allantoin in sheep was about 1 mmol/d, already saturated even by endogenous production of allantoin, and allantoin entering the glomerular tubule was quantitatively excreted in the urine.

In a modelling approach, Chen et al. [43] proposed a general mechanistic model to depict the quantitative relationship between amount of purine absorbed (X) and the PD

excretion (Y). The model may apply to sheep, cattle and other species. A more detailed description is given here.

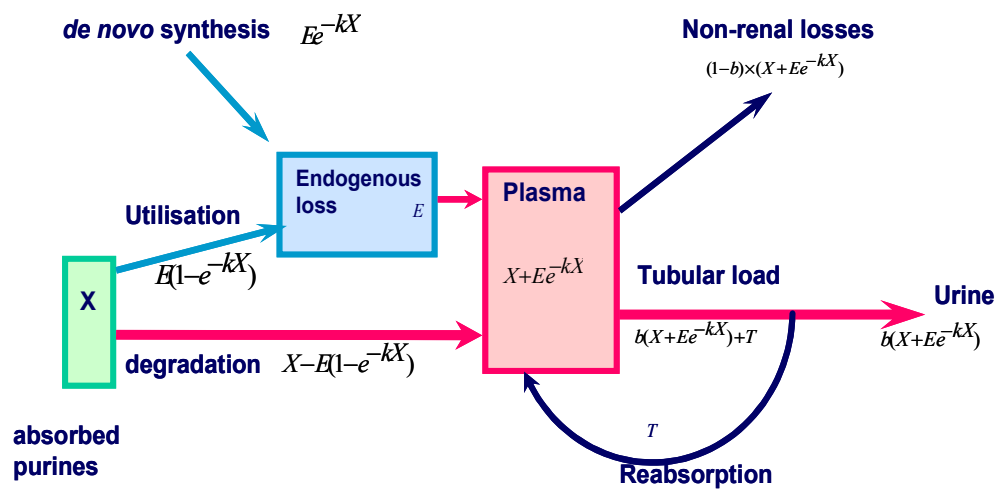


FIG. 1: A model which describes the quantitative relationship between purine absorption and the excretion of purine derivatives in the urine

The model is illustrated in Fig. 1. The endogenous production of PD is denoted as “E” in the diagram. The animal will need to replace the endogenous purine loss by either de novo synthesis (i.e. creating purines from amino acids) or/and salvaging preformed purines if available. When no exogenous purines is available, the loss has to be replaced all by de novo synthesis. However, when exogenous supply is increased, there is evidence in the literature to show that de novo synthesis is reduced and even turned off. For simplicity, the amount of de novo synthesis is described in this model as Ee^{-kX} , where X is the exogenous purine supply. Accordingly, the amount of exogenous purines utilised is $E(1 - e^{-kX})$, the rest is converted into PD and enter the plasma. The amount of PD entering the plasma is: $X + Ee^{-kX}$.

This model also assumes a constant partitioning between the renal excretion and non-renal route of disposal. The factor “b” represents the proportion of the plasma PD to be disposed of by renal excretion, thus urinary excretion of PD:

$$Y = bX + bEe^{-kX} \dots\dots\dots (3)$$

Y is curvilinearly related to X. When exogenous purine input is zero (i.e. X=0), bE becomes the measured endogenous excretion, a constant per kg $W^{0.75}$.

When the model is applied to cattle in which exogenous purines is not unavailable for utilization ($e^{-kX} = 1$), the equation becomes:

$$Y = bX + bE \dots\dots\dots (4)$$

Y is linearly related to X. Again, when X=0, bE becomes the measured endogenous excretion.

Equations 1 and 2 showed that experimental data fitted into this model. It is interesting to note that the value of ‘b’ is similar for cattle (0.85) and sheep (0.84). Subsequent studies in the UK laboratory and those from other laboratories also gave similar results (one should bear in mind the intercept may not be estimated accurately).

| | |
|------------------|--|
| Sheep[37]: | $Y = 0.87 X + 0.210 W^{0.75} e^{-0.14X}$ |
| Sheep[50]: | $Y = 0.81 X + 0.176 W^{0.75} e^{-0.25X}$ |
| Cattle[51]: | $Y = 0.87 X + 0.531 W^{0.75}$ |
| Cattle[52]: | $Y = 0.87 X + 0.785 W^{0.75}$ |
| Zebu cattle[53]: | $Y = 0.85 X + 0.147 W^{0.75}$ |

Equations (3) and (4) should also apply to other animal species. The xanthine oxidase activity in the intestinal mucosa may provide an indication as to whether a linear or curvilinear response would be expected.

The nonlinear model helps to explain why many studies in sheep previously reported in the literature had shown a variable recovery of the purine administered post-ruminally (but the total purine uptake was not measured). By definition, the recovery is calculated as $\frac{\Delta Y}{\Delta X}$,

i.e. the increment in PD excretion (ΔY) as proportion of the increment in purine uptake (ΔX). In fact the recovery is the first derivative of the equation $Y=f(X)$. Taking the example of Equation (1) for sheep, the recovery at any point of X is:

$$f'(X) = 0.84 - 0.25 \times 0.15 \times W^{0.75} \times e^{-0.25X}$$

The recovery can range from 0.24 (when X is close to zero) to 0.84 (when X is large, e.g. 20 mmol/d) for a 40 kg sheep; and from 0.14 to 0.84 for a 50 kg sheep. Clearly, the recovery value can be variable depending on X and body weight W. Therefore, in the sheep the recovery at isolated points without the data of total purine supply did not give any meaningful information about the true relationship between PD excretion and purine absorption.

Taking the example of Equation (2) for cattle, the recovery at any point of X is:
 $f'(x) = 0.85$

It can be seen that the recovery is a constant and not affected by X. This implies that one can simply use the regression approach, whereby known amounts of purines in several steps are supplied to the animal post-ruminally, to reveal the slope of response curve in cattle, even though the background level purine uptake in the control is unknown or incorrect (however, the intercept will be variable if the data of total purine uptake are inaccurate).

More data have become available for the purine input-output relationship in recent years, with the credit to the work of Balcells and co-workers from Spain. Balcells et al. [37] used duodenum infusion of purine bases to examine PD excretion in relation to purine absorption, by directly infusing purine bases into the duodenum. Quantitative data were generated in the subsequent applications of this technique [54-56].

2.1.3. Studies on other animal species

1) Goats

The first report of studies on allantoin in goat was made by Laurent and coworkers [13,57,58]. They noted that allantoin excretion in urine of goats was highly correlated with the intake of digestible organic matter and the authors suggested the use of allantoin excretion as an index of microbial protein synthesis in the rumen. Similar observation of the response

of allantoin excretion to feed intake was also made by Lindberg et al.[15]. Many studies on goats were carried out by Matsumoto in Japan and Lindberg in Sweden. Matsumoto and coworkers demonstrated that allantoin excretion was related with the amount of yeast provided to milk-fed goats and with the RNA contents in rumen digesta of goats [59-61]. Allantoin excretion was used as an indication of the microbial bacteria synthesis in their studies on the effect of defaunation [62-64]. Lindberg used preruminant goat kids to measure the endogenous PD excretion, and to examine the quantitative response of PD excretion to different levels of RNA supply [65,66]. Lindberg concluded in a review paper that urinary allantoin excretion could be used to indicate the microbial N supply status in goat [67]. More parameters which can be used to calculate microbial N supply from PD excretion were available by a recent study of Belenguer et al. [55]. Known quantities of purine bases were infused into the abomasum, and 0.74 of the infused purines were recovered as PD in the urine when interpreted using a linear regression model. The profile of xanthine oxidase activities in intestine, blood and tissue was also similar to that of sheep.

Allantoin, uric acid and xanthine plus hypoxanthine are present in goat urine, as in sheep urine. The available data suggest that goats are very similar to sheep in the endogenous PD excretion, and also in the pattern in which PD excretion responds to purine absorption. While Belenguer et al. [55] interpreted their data using a linear model, a curvilinear model may give a better fit to the data. Unfortunately, the results of this work were also confounded by the fact that there was no measurement of the “background” level of purine flow in the control. It was not possible to define a clear response curve of PD excretion to purine absorption from that dataset. More studies are needed in this area.

2) Buffaloes

Vercoe [10] first reported in 1976 that allantoin excretion was positively correlated with the digestible dry matter intake in buffaloes (*Bos bubalus*), but compared with the cattle, the excretion was much too low. Subsequent studies of Liang et al [68,70], Chen et al. [69], and Thanh et al [71] confirmed the same observation. The endogenous excretion, as indicated by measurement made during fasting, was also lower than cattle. The xanthine oxidase profile in intestine, blood and other tissues on the other hand was similar to cattle [69], thus it is expected that the pattern of PD excretion should be similar to cattle, i.e. the PD excretion is a linear function of purine uptake. Moreover, as expected, urinary PD only consists of allantoin and uric acid.

Published data showed that PD excretion per unit of digestible organic matter intake (DOMI) was about 3-4 times lower in buffaloes than in cattle (5 vs 18 mmol/kg DOMI). Since the urinary excretion of other nitrogenous compounds, creatinine and basal N, were also consistently lower as well when compared to cattle. Chen et al. [69] suggested that the possible difference was the partitioning of plasma PD between renal excretion and non-renal disposal and that a low glomerular filtration rate (GFR) in buffaloes may explain part of the difference.

In a recent study of Liang et al. [70], purine bases were infused into the duodenum of buffaloes and zebu cattle as a comparison. The relationships between urinary PD excretion (Y , mmol/d) and purine supply (X , mmol/d) were $Y = 0.12X + 0.20 W^{0.75}$ for buffaloes and $Y = 0.85X + 0.15 W^{0.75}$ for zebu cattle. There was a 4 times difference in the excretion rate. However, there was no difference in the absorption rate of purines from the small intestine between the two species of animals. Plasma partitioning, as measured by urinary recovery of

¹⁴C-uric acid tracer injected to the blood, appeared similar in buffaloes and cattle (0.75 and 0.60), although caution was made on the analytical procedure for measuring the recovery. Plasma partitioning thus did not seem to explain the difference in the urine excretion. Moreover, results from this work also indicated that GFR was not the main factor contributing to the difference in PD excretion rate between buffalo and cattle. The mechanism of buffaloes excreting less PD is yet to be understood.

One aspect is conclusive, the urinary excretion per unit of DOMI or intestinal flow of purines is low in magnitude, and the PD excretion values are more variable. Urinary PD excretion as an index of microbial N supply is less sensitive than with other species of ruminants, and its application should be made with caution.

3) Local breeds of cattle

Under the Joint FAO/IAEA Division's Coordinated Research Project (CRP) on "Development, standardization and validation of nuclear based technologies for measuring microbial protein supply in ruminant livestock for improving productivity", research on purine derivatives was extended to several local cattle species. These included: Malaysian Kedah-Kelantan cattle (*Bos indicus*), Sri Lankan Zebu cattle (*Bos indicus*), Turkish Yerli Kara cattle (*Bos indicus*) and cross-bred, Chinese yellow cattle (*Bos taurus*), Vietnamese yellow cattle (*Bos indicus*), and Bali cattle (*Bos sondaicus*) and Ongole cattle (*Bos indicus*) of Indonesia. The same experimental design and analytical procedures [27] were followed. The experiment protocol included measurement of endogenous excretion during fasting and measurement of the response of PD excretion at four different levels of feeding. A linear correlation between PD excretion and DOMI were observed in all animals. Based on fasting excretion and the slope of the regression, it appeared that these breeds of animals were similar to European cattle (*Bos taurus*). A summary of these studies was given by Makkar [72].

Pimpa et al. [53] examined more specifically the relationship between PD excretion and purine intake in Kedah-Kelantan cattle. Known quantities of purine bases were infused into the duodenum. The following relationship between PD excretion (Y mmol/d) and the intestinal flow of purines (X mmol/d) was observed: $Y=0.85 X+0.15 W^{0.75}$. The equation was similar to that of European cattle (see Equation 2).

From the work so far, it appears that *Bos indicus* resembles *Bos taurus*, in the excretion of PD. The indication of endogenous excretion is only from measurements during fasting, or by interpolating to zero intake using a regression approach. One should bear in mind that values by interpolation to zero feed intake may be variable due to errors of measurements in feeding trials (e.g., purine intake is not measured). The fasting measurements in *Bos indicus* cattle tended to be lower than *Bos taurus*. Osuji et al. [73] showed a fasting PD excretion of 0.17 mmol/kg $W^{0.75}$ for Zebu cattle in Africa.

4) Camels, llamas and yaks

Italian researchers Mura and co-workers were the first to examine the purine metabolites in urine and plasma of camels (*Camelus dromedaries*) in comparison to zebu cattle, in a qualitative approach. They noted that xanthine and hypoxanthine were present in urine and plasma at a higher concentration than uric acid [74], while allantoin was still the main component. Camels lacked xanthine oxidase in the liver and also had a lower level of

purine degradation enzymes than zebu cattle, and the authors suggested that this was in part the animal's N-economy strategy so that exogenous purines could be reused [75,76].

Quantitative aspects of PD excretion in camel were examined in a greater detail by Guerouali et al. [56] under the FAO/IAEA research programme. Urinary PD contained allantoin, xanthine plus hypoxanthine, and uric acid (in order of magnitude). Xanthine oxidase was detected in liver and intestine, but absent from the blood. Total PD excretion was linearly correlated with DOMI (the slope was 11 mmol PD/kg DOMI). When purine bases were infused into the duodenum, the recovery of the infused purines as PD in the urine averaged 0.52. This recovery factor was different from cattle and sheep. The fasting excretion was 0.240 mmol/W^{0.75} per day. Based on the fact that exogenous purines are available for utilization (or salvage) by the animal, it is expected that the response curve of PD to intestinal absorption of purines should be curvilinear as in the case of sheep. There were no sufficient data points in the work to define this equation.

Bakker et al. [77] measured the PD excretion in llamas (*Lama glama* and *L. guanicoe*). Urinary PD contained allantoin, uric acid and xanthine plus hypoxanthine (in order of magnitude), but during fasting the proportion of xanthine plus hypoxanthine were higher than uric acid. Fasting excretion averaged 0.177 mmol/W^{0.75} per day. The PD excretion was 12-18 mmol/kg DOMI, similar to sheep. It was also observed that llamas were unique in maintaining a high concentration of uric acid in the plasma by actively re-absorbing uric acid, but not allantoin, from the kidney tubules.

Long et al. [78] reported a preliminary study on yaks (*Bos grunniens*) in China. Urinary PD only contained allantoin and uric acid. Fasting excretion averaged 0.220 mmol/W^{0.75} per day. When the animals were fed with different levels of feed, PD excretion increased and correlated with the DOMI. The slope was 13 mmol/kg DOMI.

5) Wide animals

There are interesting studies in which urinary allantoin excretion is used as index of feed intake in free ranging elk (*Cervus elaphus*) [79-83]. Earlier studies in red deer (*Cervus elaphus*) by Razzaque et al in 1973 showed that the animal excreted proportionally more xanthine plus hypoxanthine than uric acid [84]. The PD composition was 59% allantoin, 40% xanthine plus hypoxanthine, and 1% uric acid.

2.1.4. Use of purine derivatives excretion to calculate microbial protein supply

The method to estimate the intestinal flow microbial N from PD excretion was described by Chen [85,86,27].

Step 1: the absorption of purines (X mmol/d) is estimated from the PD excretion in the urine (Y mmol/d) based on a previously established equation, for example, Equation (3) and (4) for sheep and cattle respectively.

For sheep: $Y = 0.84X + (0.150 W^{0.75} e^{-0.25X})$

The calculation of X based on the above non-linear equation can be performed by means of the Newton-Raphson iteration process, as shown below:

$$X_{(n+1)} = X_n + \frac{f(X_n)}{f'(X_n)}$$

where $f(X) = 0.84 X + 0.150 W^{0.75} e^{-0.25X} - Y$
 and the derivative of $f(X)$: $f'(X) = 0.84 - 0.038 W^{0.75} e^{-0.25X}$

Given an initial value of $X_1 = Y \div 0.84$ to feed into the above equation to calculate X_2 , and so on after 4 iterations, X_5 should have reached a constant value.

For European cattle $Y = 0.85X + (0.385 W^{0.75})$
 Thus, $X = (Y - 0.385 \times W^{0.75}) \div 0.85$

For Zebu cattle [53], $Y = 0.85X + (0.147 W^{0.75})$
 Thus, $X = (Y - 0.147 \times W^{0.75}) \div 0.85$

For other animals, more specific equation for the target animal should be used. In the authors' experience, when the equation developed for European cattle was used for cattle in tropical countries, lower values of microbial N estimate were obtained. This was probably because the endogenous excretion of those animals was lower than the value $(0.385 W^{0.75})$ used for European cattle. The equation recently acquired for Zebu cattle may be more suitable.

For lactating cows, the output of PD in the milk can be added to the urinary PD excretion so to improve the accuracy. Since the PD concentration in milk was rather constant (see discussion later), the milk output of PD can simply be calculated as milk yield times an average concentration previously measured.

Step 2: Microbial N yield is then calculated using:

$$\text{Microbial N (gN/d)} = \frac{X(\text{mmol/d}) \times 70}{0.116 \times 0.83 \times 1000} = 0.727 X \dots\dots\dots (5)$$

The factors used in Equation (5) are:

- (i) Digestibility of microbial purines is assumed to be 0.83. This is taken as the mean digestibility value for microbial nucleic acids based on observations reported in the literature.
- (ii) The N content of purines is 70 mg N/mmol.
- (iii) The ratio of purine-N: total-N in mixed rumen microbes was measured as 11.6:100 [85].

It should be stated that the ratio of purine-N: total-N may need to be redefined for different feeding regimes or for different animal species, in order to improve accuracy of the calculation.

There have been only a small number of studies in which the microbial N was estimated in sheep by both the PD method and a conventional method based on direct measurement of a microbial marker. The markers used were: 2,6-diaminopimelic acid (DAPA) [87], amino acid profile [87], RNA [88,90], and ¹⁵N [89]. The correlation relationship between estimates by PD method and those by another method had a slope close to 1 in all studies although the intercept varied on both side of zero (see Fig. 2).

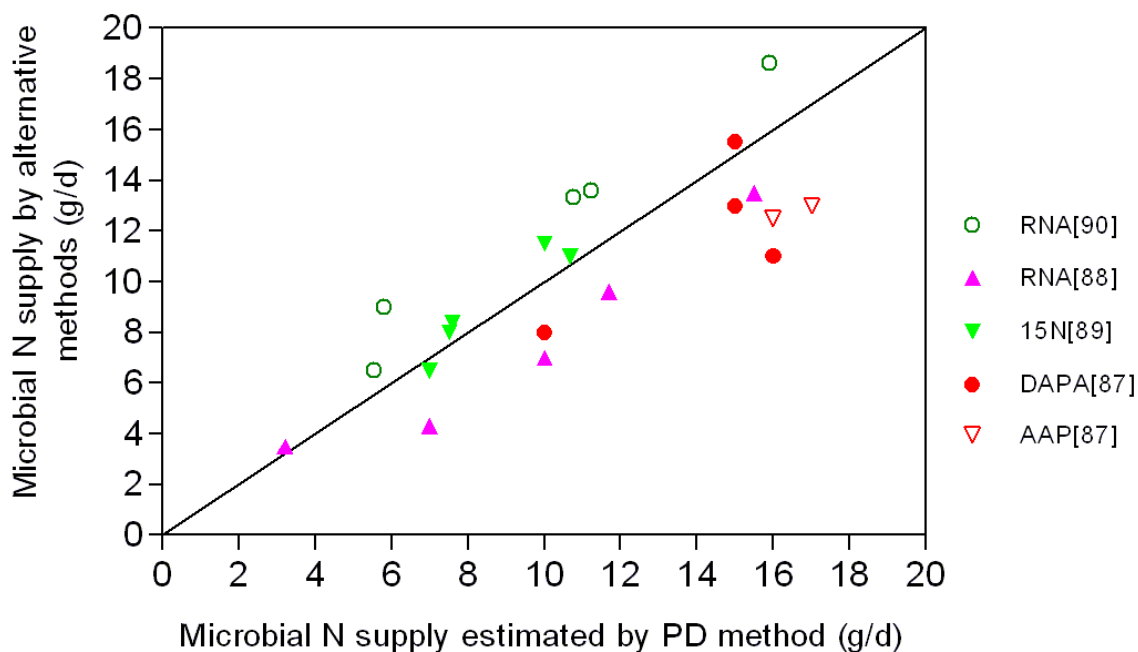


FIG. 2: Estimates of microbial N supply by PD method, plotted against the values of direct measurement using various markers: DAPA, amino acid profile, RNA, and ^{15}N .

Martin-Orue et al.[91] reported that microbial N estimated based on PD excretion in cattle closely matched, but were consistently lower than, the direct measurements based on intestinal flow of purine bases. So far, there have been few comparative studies conducted in cattle, but the values of microbial N synthesis estimated by the PD excretion for European cattle were within the range as expected from the fermentable energy intake. Obviously, a direct comparison would be useful. However, few of such comparative studies have been conducted due to the complexity of conducting direct measurements of intestinal flow of microbial markers and of the flow rate of digesta into the duodenum using cannulated animals. Moreover, there is also a problem as to “which method should be taken as the standard”. Indeed, all methods have limitations and there are inconsistencies in results depending on the choice of markers, as shown by Ling & Buttery [92]. A recent review on methods of measuring microbial protein was made by Tamminga & Chen [93].

Although, at the present stage the estimates of microbial N calculated based on PD excretion may not be taken as the absolute values, they should be valid for comparative purposes as in the studies to be presented in the next section. When used in sheep and cattle, reasonable confidence and reliability are attached to the results of estimation.

It seems an appropriate point here to discuss the choice of measurement of PD. All four compounds, allantoin, uric acid, xanthine plus hypoxanthine, are excreted in the urine of sheep, goat, llamas, red deer and camels; but only allantoin and uric acid in the urine of cattle, buffaloes and yaks. Since allantoin is the main components in all animals, the question is why not use allantoin only to estimate microbial protein supply. This may be valid for cattle, buffaloes and yaks, since the relative proportion of allantoin:uric acid was also rather constant (typically 85% allantoin:15% uric acid), but not valid for the other animals since the relative proportion of allantoin: uric acid: xanthine plus hypoxanthine was not constant,

depending on the level of exogenous purine absorption. Chen et al [43] showed in sheep that the composition of PD of exogenous origin differed from that of endogenous origin, thus the final composition depended on the relative size of these two sources. In cattle, however, the composition was the same for exogenous and endogenous PD, and thus the final composition was independent of the relative size of these sources. For more accurate estimation of microbial N supply, total PD should be used, even for cattle.

2.1.5. The use of PD as index of microbial protein supply in feeding studies

The use of urinary PD excretion as an indicator of rumen microbial protein synthesis has been extensively applied to ruminant nutrition research. The technique has become a useful tool to aid understanding how dietary factors affect microbial protein synthesis. Citations to some of these studies are given below:

1) Studies in sheep

- a) Effects of different sources and levels of energy/carbohydrates: [94-112].
- b) Effect of different N source and level: [87,113-128].
- c) Effect of rumen outflow: [86,129-134].
- d) Effect of fat or oil supplementation: [135-139].
- e) Effect of tannin: [140-144].
- f) Effect of monensin: [58,145].
- g) Effect of yeast addition: [146,147].
- h) Effect of defaunation: [148,149].
- i) Difference between sheep breeds in digestion: [150-152].

2) Studies in cattle

- a) Effects of level and source of energy/carbohydrates: [153-161].
- b) Effects of level and source of N: [162-174].
- c) Effect of feeding frequency: [175-176].
- d) Effect of rumen outflow rate: [177].
- e) Effect yeast addition: [178].
- f) Stages of rumen development in calves: [179].

3) Studies in goats

- a) Effects of level and source of energy: [180-181].
- b) Effects of level and source of N: [182-184].
- c) Effect of sulfate supplementation: [185-187].
- d) Effect of tannin: [140].
- e) Effect of monensin: [58].
- f) Effect of protozoa addition: [64].

It can be seen from the references cited above, that most studies were conducted in the last 10 years. It is expected that the technique will continue to be used in ruminant feeding research in the future, as a tool to improve utilization of feed sources. When it comes to interpretation of PD data, the researchers should bear in mind the variability of PD measurement. In the experience of the authors, the day-to-day, and between-animals,

variation of PD excretion could be up to 10%. Thus treatment effects should be viewed in respect of the size of variability in PD measurements. In order to minimize the day-to-day variability, measurements should be made with sufficient duration of continuous urine collection (at least 4-5 days).

2.1.6. Plasma, milk and spot urine measurements

1) Spot urine measurements

In order for the PD technique to be applied under farm conditions, it would be ideal if spot measurements such as spot urine, plasma or milk could be used instead of total urinary excretion. Antoniewicz et al. [188] in 1981 already observed a linear correlation between the ratio of allantoin-N to creatinine-N and feed ME intake in sheep - suggesting the possibility of using the allantoin:creatinine ratio in spot urine as the parameter. Subsequent studies were carried out by Chen et al. [189] in steers, Gonda & Lindberg [190] in dairy cows and Chen et al. [191] in sheep to examine the diurnal variations in spot urine measurement. More recent studies were carried out under the FAO/IAEA research programme mentioned in an earlier section (see summary by Makkar [72]). A review on spot urine measurements was given by Chen et al. [192] and more recently Chen et al. [193]. The conclusion from the studies so far can be summarized as follows: 1) PD to creatinine ratio correlates to feed intake and intestinal flow of microbial purines, and thus can be used as an indicator of microbial protein supply; 2) sufficient number of measurements should be made in order to reduce errors; 3) the variability of spot measurement would be greater than that based on total urine collection. Thus difference between treatments in microbial N supply must be large enough otherwise it may not be reflected by the spot measurements, as in the case of Iriki et al. [194] and Shingfield & Offer [195] where the authors did not find the spot measurements to have performed as expected.

Readers are brought to the attention of the expression of results. Many studies reported in the literature used the direct [PD]/[Creatinine] concentration ratio in their presentation of results. 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.

Chen et al.[193] proposed to use a term “PDC index”,

$$PDC\ index = \frac{[PD]}{[Creatinine]} \times W^{0.75} \dots\dots\dots (6)$$

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:

$$PD\ excretion\ (mmol/d) = PDC\ index \times C, \dots\dots\dots (7)$$

where, C is the daily creatinine excretion (mmol/kgW^{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. For practical applications, a banding system was suggested [191,193].

2) Plasma PD concentration

The possibility of using plasma concentration of allantoin (or total PD) as an alternative index of microbial protein supply has been investigated by a number of workers: Chen et al. [189-192,50]; Ehrentreich [196]; Giesecke et al. [197,198]; Iriki et al. [194], Kagiya et al. [199], and Matsumoto & Yonai [200]. The results on whether plasma PD concentration was correlated with the daily PD output were mixed. This should in fact be expected by the theoretical consideration of the kinetics of PD in the blood. Take allantoin as an example, allantoin enters plasma and exits by renal excretion or by non-renal disposal. At the steady state, when the influx is equal to outflow, plasma concentration reaches a constant level. Renal excretion is equal to, or proportional to, the plasma concentration multiplied by GFR. If GFR is constant, then plasma allantoin concentration is linearly correlated with (or proportional to) the allantoin influx or the urinary excretion. However, if GFR is variable, plasma allantoin concentration will be correlated with neither the influx into the plasma nor the renal excretion. Given the same influx, a higher GFR would give a lower plasma concentration at the steady state. GFR in the same animal may change with feed intake as shown by Chen et al. [191,192] and Kagiya et al. [199]. As concluded by Chen et al. [192], plasma PD is not an appropriate index of microbial protein supply unless changes in GFR are also taken into account.

3) PD output in milk

The notion of using milk allantoin concentration, or daily allantoin output in milk, as an indicator of microbial protein supply in dairy cows is an interesting one, since both the milk samples and milk yield data are available. Moreover, it would be useful for farmers if indication of microbial protein supply status can be integrated as part of the milk recording system. The research on this area was initially carried out in Germany by Giesecke and co-workers [196,201-203,198], and subsequently also by various people in other countries [172,192,204-210]. Conclusions from the research can be summarized as follows: 1) concentration of allantoin and uric acid in milk fell within a narrow range (lower than 1 mmol/L for total PD), 2) total output of PD in the milk only accounted for a small fraction of output in the urine (4-5 mmol/d in milk, accounting for 0.6-2.4% the urinary excretion [198]). There appeared to be a control on the concentration of PD in the milk in the mammary gland resulting in relatively constant concentrations in the milk, and the milk output could be practically calculated from milk yield. As an indicator of microbial N supply status of the animal, neither the output nor concentration of PD in the milk is a sensitive parameter [192,206-208].

2.1.7. Progress in analytical methods

The classical method used for the determination of allantoin in urine was developed by Young & Conway in 1942 [211]. The method is still being widely used today since it requires only a spectrometer. The disadvantage of this method is the procedure is rather lengthy and requires critical timing of operation. Thus only a small number of samples (20-30) can be analysed in a day. Accuracy of the analytical results depends heavily on whether the analysis is carried out routinely. Pentz [212] automated the procedure by adapting it to the AutoAnalyzer. Subsequently the automatic method was adopted and improved by Lindberg & Jansson [213] in Sweden and Chen et al. [214] in the UK, and became a useful aid in their studies on urinary PD in ruminant since large numbers of samples could be processed.

There are many reports on methods for determination of allantoin by HPLC based on reversed-phase chromatography. On a C18 reversed-phase column, allantoin is not retained

long enough for good separation from the polar solutes, thus the column length often needs to be extended by using two columns [215-222]. Yamamoto et al. [223] used ion-pairing agents to slow down the elution of allantoin. The advantage of the direct determination is its simplicity and the bonus of the possibility to measure uric acid, xanthine and hypoxanthine in the same run (if the concentration range for all compounds are right). The weakness is that when the concentration is low, the accuracy of allantoin measurement is affected since the allantoin peak is not well separated from the unidentified background noise when monitored at around 200 nm wavelength. Pre-column derivatization is an alternative, i.e. to change allantoin into a derivative which is detected at a unique wavelength [224-226]. In general, with the analysis of derivatized allantoin, the run time is short and analysis robust, but other purine derivatives are not included within the same run.

Methods for determination of allantoin up to 1995 were reviewed by Chen et al [227]. Since then, there has been development of newer methods. These are:

- 1) Methods based on GC-MS [228-231].
- 2) Methods based on capillary zone electrophoresis (CZE) [232-234]
- 3) Methods based on electrokinetic chromatography [235-236].
- 4) Method based on near infrared spectrometry [237].

The development of GC-MS methods will provide powerful tools for future research into the metabolism of purines and the kinetics of PD in plasma using stable isotopic tracers. On the other hand, from the other new methods and more to come in the future, the long-sought after techniques for routine PD analysis which are accurate, fast, robust and inexpensive may finally evolve.

Compared to allantoin, the measurements of uric acid, xanthine and hypoxanthine are much simpler. These three together could be determined as “total uric acid” after enzymatic conversion of xanthine and hypoxanthine to uric acid by xanthine oxidase [7,32]. A more recent account of the procedure is given in IAEA-TECDOC-945 [27].

3. FUTURE RESEARCH

In the personal view of the authors, there are four broad areas which require future research.

3.1. Modeling the response of PD excretion in relation to purine absorption

Despite major progress having been made in modeling the quantitative response of urinary PD excretion to purine absorption from the small intestine, some factors that affect PD excretion are still not completely understood. Although consistent purine input–output relationships have been observed for European cattle and sheep, there are variations among animal individuals of the same species/breed and much larger variations among different animal species.

For example, in sheep, consistent variation among animal individuals were observed in the urinary recovery of intravenously dosed PD [47,238]. The recovery of absorbed purines as urinary PD was 0.12 and 0.52 for buffaloes and camels [70,56], whereas that for sheep and cattle (both *Bos taurus* and *Bos indicus*) was both around 0.85 [43,44,70]. The reason for this

species difference is still not fully understood. What is the mechanism that makes buffaloes excrete less PD and other nitrogenous compounds than cattle? The understanding of this mechanism may help to explain variability within species.

In order to elucidate the mechanism that contributes to the species diversity, more research will be needed to study the metabolism in the tissue and kinetics of PD in the blood. It is known that the distribution of allantoin in plasma follows a two-compartment model. Some work in this aspect has been conducted by Kahn & Nolan [239]; Kahn, et al. [240] and Prasitkusol et al. [238]. Although it is known that there was a fairly constant proportion of plasma PD being disposed of by non-renal routes, but what are these routes?

More extensive studies are also required to examine the effect of renal physiology on PD excretion, with a focus on identifying the factors which affect the renal excretion of plasma PD. The effect of GFR and tubule exchange may be further studied. Prasitkusol [241] conducted extensive studies on GFR in sheep and cattle. Available data showed that within the same individual animal, the GFR may change with intake and the change in GFR did not seem to affect the total PD output in the urine, because when the GFR increased, plasma concentration decreased accordingly given a fixed plasma volume [50]. However, across different animal species, how much the variation in the PD excretion rate is due to variation in GFR needs to be further investigated.

The species difference in purine metabolism (including profile of xanthine oxidase) is an interesting subject on its own right. Differences may be viewed in the context of animal adaptation to their environments.

3.2. New research techniques

The development of newer methods to help defining the purine input-output relationship is also required. So far techniques based on post-ruminal infusion of purine bases have been used, but these techniques are complex and require surgical intervention of experimental animals. It will be ideal if simpler and more accurate techniques can be developed. The direction shall be to use radioactive or stable isotopic tracers to define the parameters for the already developed models. In sheep, goats, camels, llamas and other species in which exogenous purines can pass through the intestinal wall in a salvageable form, a curvilinear relationship could be expected (Equation 3). However, in cattle and buffaloes in which exogenous purines pass through the intestinal wall in a non-salvageable form, a linear relationship could be expected (Equation 4). The 'b' in these two equations refers to the proportion of plasma PD to be excreted in the urine. This parameter could be defined using tracer techniques, a preliminary procedure was provided by IAEA-TECDOC-945 [27]. Preliminary work showed this approach is promising. Chen et al.[228] used [1,3-¹⁵N₂] uric acid to measure the proportion of plasma uric acid to be excreted in the urine as uric acid and as allantoin in sheep. Prasitkusol et al. [238] used [4,5-¹⁴C] allantoin to measure the proportion of plasma allantoin to be excreted in the urine. The values for the proportion obtained in these studies were similar to the values observed from infusion experiments. Results of the two studies also implied that uric acid and allantoin may behave differently in the renal clearance and thus they may need to be measured separately. Further improvement, validation or new developments are required.

New techniques for determination of the endogenous contribution of PD in the urine also await development. Again, the use of ^{15}N - tracers may play a role. Some interesting study is made recently by Orellana Boero et al.[54] in which endogenous contribution was estimated based on the dilution of ^{15}N tracer infused.

It is envisaged in the future, with the aid of these new techniques, the quantitative relationship between PD excretion in the urine and the intestinal purine absorption can be identified rapidly for new breeds or species of animals, so that the application of using urinary PD to estimate microbial protein supply can be extended to more animal breeds and species.

3.3. Practical application

More work needs to be carried out to validate the results of the estimates of microbial N supply from PD excretion. Moreover, in order to achieve a more accurate estimation, parameters in Equation (5), for example, ratio of purine-N : total-N in rumen microbes, will need to be defined better under local conditions.

A system for the field application of spot urine measurement needs to be developed or refined. Although a “banding system” has been proposed by Chen et al. [193] based on the results of the FAO/IAEA coordinated research programme, the feasibility of the banding system in practice has yet to be determined. It is most likely that the parameters used in the system will have to be modified as more information becomes available.

The measurements of PD in urine of sheep, cattle and other species except buffaloes will continue to serve as indicator of microbial protein supply in ruminants and as a useful tool to aid understanding how various dietary factors affect rumen microbial protein production.

A new concept of “Purine Nitrogen Index (PNI)” was suggested by Chen et al. [242]. PNI is defined as the proportion of total-N present as PD-N in urine. It may reflect the efficiency rumen degradable N is used for microbial protein synthesis. This parameter, which can be determined using spot urine samples, can aid the formulation of N-efficient diets for ruminants (thus less N pollution to the environment). Further studies on PNI may be useful.

3.4. Analytical methods

Further development of analytical methods will be in two directions. One direction is to develop methods for field application. Simple, robust and inexpensive methods for determination of PD are required, so that the analysis work can be done in extension or veterinary laboratories. It would be even better if dipstick methods can be developed. Another direction is to develop methods for purine metabolism studies. The method should be accurate and able to detect concentration of micro gram per litre level. Methods based on GC-MS represent progress in this direction.

4. CONCLUSION

From the first work of 1931 to today, over the 70 years we have progressed a long way in our understanding of the purine derivative metabolism and renal excretion in ruminants.

Most active progress was made in the last 20 years. Our information of PD excretion has been improved from qualitative to quantitative. Using a modeling approach, the urinary PD excretion can be predicted in a quantitative manner. We can now estimate microbial N supply in sheep and cattle with reasonable confidence. Further research will help us to understand better the source of variability in PD excretion between animal species and between animal individuals of the same species, so that more confidence can be attached to the estimation of microbial protein supply based on the PD excretion.

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