CHAPTER 7

7. PREDICTION OF MICROBIAL YIELD FROM THE RUMEN USING URINARY EXCRETION OF PURINE DERIVATIVES

7.1 Introduction

In ruminants, the predominant source of potentially absorbable (exogenous) nucleic acids is the rumen microbial biomass which is also the major protein source for the ruminant host. Topps and Elliot (1965) foresaw a role for the measurement of urinary excretion of allantoin and uric acid as an indicator of rumen biomass and efficiency of protein utilisation in ruminants. These researchers noted the close correlation between the ruminal concentration of nucleic acids and the rate of urinary excretion of allantoin plus uric acid, but did not appear to use this information to predict rumen microbial outflow. Some years later, McAllan and Smith (1971) also suggested that nucleic acid concentrations in rumen digesta could be used to predict microbial-N concentration in the digesta, recognising from their earlier work (Smith and McAllan 1970) that non-microbial nucleic acids had a short half-life in the rumen and would therefore contribute minimally to the total nucleic acids in the digesta.

Rys and co-workers (1975) were among the first workers to draw attention to the possible role of urinary purines as an index of intake and rumen fermentation. These researchers assumed that 18% of the microbial-N leaving the rumen was in nucleic acids, and that 25% of the N in these nucleic acids appeared in urinary allantoin, and thus argued that microbial-N outflow rate from the rumen was given by urinary allantoin-N excretion rate divided by 0.045 (0.18 x 0.25). This factor is, however, dependent on many factors and is a potential source of error in any prediction.

Vercoe (1976) demonstrated that digestible dry matter (DM) intake and urinary allantoin excretion in cattle and buffaloes were closely correlated. A similar relationship also exists for sheep (Kahn 1991) (Figure 7.1). It is reasonable to assume that these relationships stem from the generally close correlation between digestible DM intake and microbial yield of protein and nucleic acids from the rumen, combined with a second association between entry of nucleic acids into the intestines and excretion of purine metabolites in the urine. Other workers have demonstrated either linear (Antoniewicz *et al.* 1980; Giesecke *et al.* 1984) or

curvilinear (Chen *et al.* 1990b; Balcells *et al.* 1991) responses in urinary excretion of allantoin or total purine derivatives to stepwise increases in rates of intra-duodenal infusion of nucleic acids in sheep.

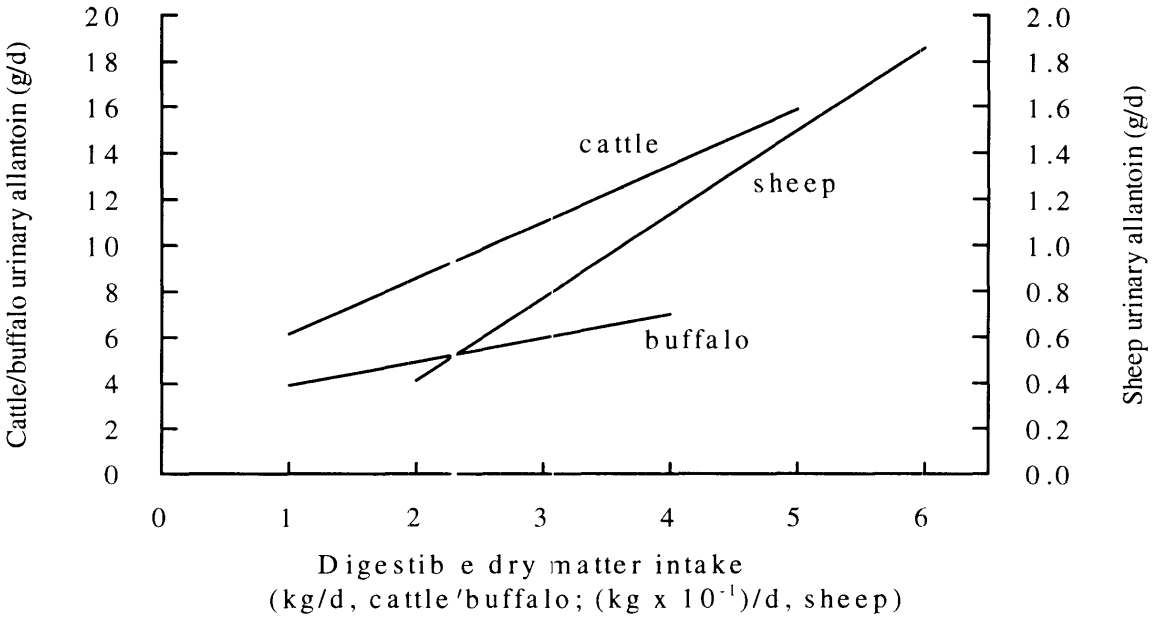

Figure 7.1. Relationships between urinary allantoin excretion (Y, g/d) and digestible dry matter intake (X, kg/d) of sheep, cattle and buffalo given forage based diets. The regression equations for each species are:

Sheep: $Y = -0.312 + 3.62X$ ($r^2 = 0.72$); Kahn (1991)

Cattle: $Y = 3.72 + 2.43X$ ($r^2 = 0.66$); Vercoe (1976)

Buffalo: $Y = 2.86 + 1.04X$ ($r^2 = 0.53$); Vercoe (1976)

In the remainder of this Chapter, the current knowledge of purine origin, synthesis and metabolism in ruminants as depicted in Figure 7.2 is reviewed.

7.2 Nucleic acids as precursors of purine derivatives excreted in urine

Nucleic acids contain two classes of nitrogenous base, purines and pyrimidines. Pyrimidine bases present in DNA (cytosine and thymine) and RNA (cytosine and uracil) are all derivatives of the parent compound pyrimidine, a compound with a heterocyclic conjugated single ring structure. Purine bases (adenine and guanine) are derivatives of the parent compound purine, a heterocyclic double ring structure formed by the fusion of a pyrimidine ring and an iminazole

ring (Davidson 1972). Both adenine and guanine can be condensed with a pentose (RNA) or deoxypentose (DNA) sugar to form a nucleoside. Nucleotides can be formed as a result of phosphoric esterification of nucleosides (Davidson 1972).

7.3 The origin and fate of nucleic acids in the rumen

Nucleic acids in the rumen can be associated with feed, microbial or endogenous sources. Values for various feeds show that the contribution of nucleic acid-N to total-N (on a DM basis) can vary from 1–4%, 8–11% and 15–25% in cereals and protein concentrates, hay and legumes respectively (Smith and McAllan 1970; McAllan 1982). Some forages have also been reported to contain small amounts of free purines, allantoin and uric acid (Ferguson and Terry 1954).

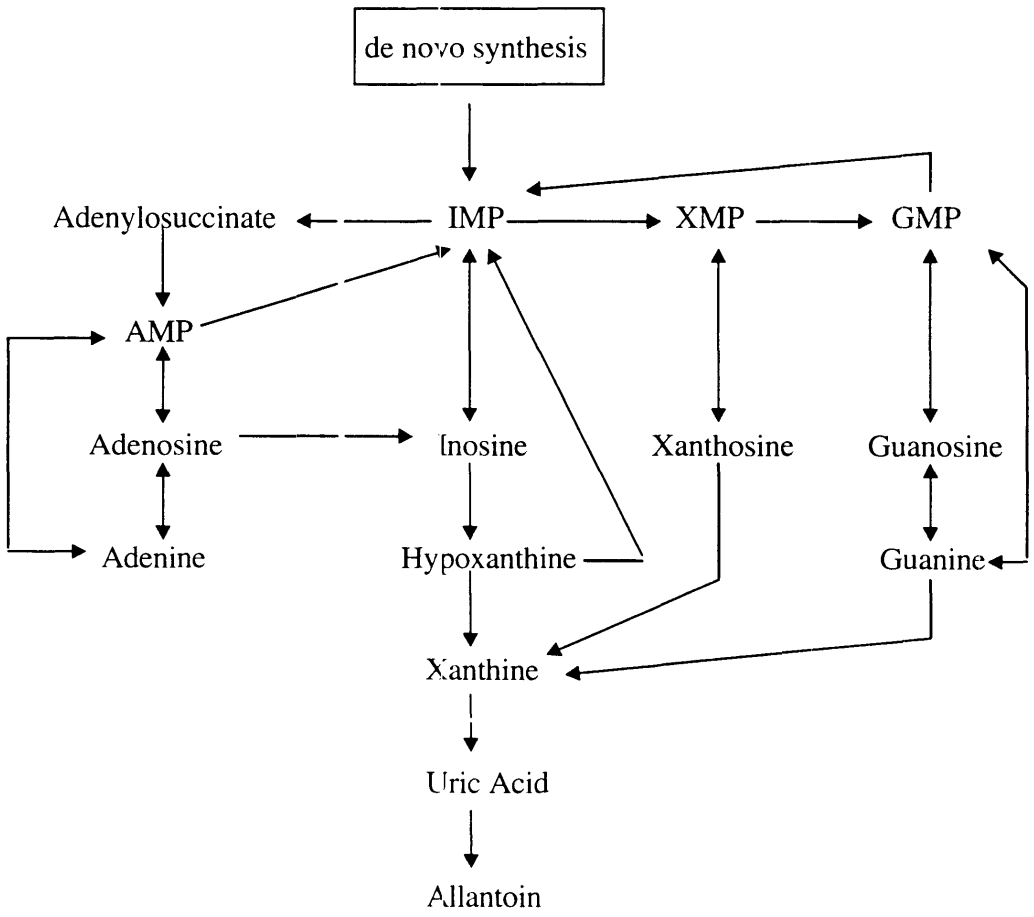

Figure 7.2. Pathways of purine metabolism in ruminant tissues (derived from Lehninger 1977; Stryer 1988).

Complete ruminal degradat on of feed nucleic acids, or a knowledge of the amount of dietary purines reaching the intestines, is essential to enable any relationship between urinary excretion of purine derivatives a id exogenous purine absorption to be used to estimate microbial flow to the intestines. S nith and McAllan (1970) found that the ratio of RNA:DNA in rumen fluid was similar to that ir rumen bacteria and was independent of the diet, indicating that negligible amounts of dietary nucleic acids are present in rumen fluid. These researchers also found that pure nucleic acids a dded to the rumen were rapidly degraded.

Later, McAllan and Smith (1973) conducted experiments in which purine bases, nucleosides and nucleotides were incubated, *in vitro*, with whole bovine rumen contents, and found that virtually no traces of these compounds were left in the incubating medium after 4 h. Purine bases were degraded to va ying extents, with xanthine being the most resistant. In contrast, Jurtshuk *et al.* (1958) reported that adenine was neither decarboxylated nor deaminated after *in vitro* incubation with a washed cell suspension of bovine rumen bacteria. In addition, Smith and co-workers (1978) have shown that non-microbial RNA may contribute up to 15% of the RNA entering the duodenum of young steers receiving equal amounts of hay and concentrate.

Chen *et al.* (1990c) found that allantoin incubated *in vitro* in rumen fluid was degraded rapidly enough to ensure no net movement out of the rumen. These same authors infused allantoin into the rumen and the abomasum of animals nourished by intragastric infusion of VFA and casein (Ørskov *et al.* 1979) and did not record any increased recovery of allantoin in urine. On balance it seems that non-microbial nucleic acid contributes an insignificant portion of the total nucleic acid outflow from the rumen.

7.3.1 Variations in the nucleic acid content of rumen micro-organisms

In the prediction of rumen microbial outflow, the purine content of mixed ruminal bacteria must be known. Prediction would be easier if a constant factor were universally applicable, but this is unlikely to be so. The nucleic acid content of bacteria varies in response to many factors but in general the amounts of DNA and RNA depend largely on growth rate (Maeng *et al.* 1976; McAllan 1982) with the RNA:protein content increasing with specific growth rate (Bergen *et al.* 1980). Variation in the ratio of RNA-N:total-N appears to be greater in isolated pure strains grown *in vitro*, than for mixed bacteria in the rumen (McAllan 1982). In the latter, the majority of RNA-N:total N ratios are in the range 6.5–8.5% with higher values occurring soon after feeding, and in the absence of protozoa (Smith and McAllan 1974). Variation in the concentration of RNA and the ratios RNA-N:total-N (Merry and McAllan

1983) and purine:total-N (Cecava *et al.* 1990a) also exist between bacteria that are associated with the fluid (FAB) and solid (SAB) phase of rumen digesta. Both the concentration of RNA and the ratios RNA-N:total-N and purine:total-N are greater in FAB. DNA-N:total-N ratios are somewhat lower than that for RNA at about 5.0–6.5% (Smith and McAllan 1974; McAllan 1982) but show greater variation between bacterial populations. However, within a population, the ratio DNA-N:total-N shows less variation throughout the day (McAllan 1982).

7.4 Intestinal digestion of nucleic acids

Nucleic acids in duodenal digesta are degraded by pancreatic ribonuclease and deoxyribonuclease with the former being particularly abundant in the ruminant (Armstrong and Hutton 1975; Smith 1979; McAllan 1982). The combined effects of these enzymes yields polyribonucleotides and oligodeoxyribonucleotides (Armstrong and Hutton 1975). These compounds are further degraded by phosphodiesterases associated with the intestinal mucosa to yield both 3 prime or 5 prime mononucleotides (Armstrong and Hutton 1975; McAllan 1982). Other enzymes that can remove the phosphate group to form nucleosides are also present in the small intestine (Armstrong and Hutton 1975; McAllan 1982). The existence of nucleoside-cleaving enzymes in ruminant intestinal mucosa is equivocal. However, McAllan (1982) reported that when adenosine and cytosine were infused into the small intestine of steers, appearance of their breakdown products (i.e. the respective bases) followed. Hence, it seems likely that absorption of both nucleosides and the corresponding base occur in the ruminant (Wilson and Wilson 1962). Absorption of lower metabolites such as uric acid and allantoin is unlikely (Sorenson 1960; Chen *et al.* 1990c). Once absorbed from the gut of ruminants, purine nucleosides and bases may be either degraded or incorporated into host nucleic acids (Ellis and Bleichner 1969; Smith *et al.* 1974; Razzaque *et al.* 1981).

Nucleic acids are highly digestible with 80–90% RNA and 75–85% DNA apparently disappearing in the small intestine (Smith and McAllan 1971). These estimates underestimate the true digestibility because of the addition of nucleic acid in sloughed cells or secretions. Storm and co-workers (1983) reported a true digestibility value of 86% for microbial nucleic acids following infusion of isolated rumen micro-organisms into the abomasum of sheep. Chen and co-workers (1990b) reported a value for the true digestibility of microbial purines of 91% following infusion of a microbial nucleic acid concentrate into the abomasum of lambs. In support of the high true digestibility of nucleic acids, Razzaque *et al.* (1981) reported that only 7% of the radioactivity that was associated with the nucleic acids of a bacterial culture

injected into the rumen of sheep appeared in the faeces after 48h. Other experiments (McAllan 1980) have revealed differences between the various bases and nucleosides in removal from the small intestine, with the free bases adenine and guanine being completely removed.

7.5 Synthesis of purines in the ruminant host

Biosynthesis of purine ribonucleotides in the ruminant host can occur via two distinctly different processes, often referred to as the *de novo* and the 'salvage' pathways (Lehninger 1977). The salvage pathway utilises preformed purines of both endogenous and exogenous origin. For purine anabolism *de novo*, the starting material is 5-phospho-ribosyl-1-pyrophosphate (PRPP) on which a purine ring is built using amino acids and other molecules. The activated (PRPP) molecule is also of importance in the salvage pathway and is a precursor of the pyrimidine nucleotides (Lehninger 1977).

Studies of the salvage pathway have shown that free purines and their corresponding nucleosides are reused in nucleotide and nucleic acid biosynthesis in vertebrates. When nucleotides are formed via the salvage pathway, PRPP, the starting material of the *de novo* pathway is combined with purine bases under the influence of *nucleotide pyrophosphorylases* to yield either adenosine monophosphate (AMP) or guanosine monophosphate (GMP) (Lehninger 1977). Another less important salvage pathway involves the combination of ribose-1-phosphate with purine bases to form the purine nucleoside. This reaction is catalysed by enzymes termed *nucleoside phosphorlyases* (Davidson 1972). Hypoxanthine can also be salvaged as can xanthine, though the latter only at a very low rate (Hitchings 1978). Subsequent products of purine catabolism are unable to be salvaged and thus proceed along the catabolic route towards uric acid and allantoin.

The energetic cost of the formation of purine mononucleotides via salvage is 2 ATP (Lehninger 1977). In contrast, the formation *de novo* of GMP and AMP requires a total of 8 and 7 high energy phosphate bonds respectively (Lehninger 1977). In humans up to 90% of the free purines formed may be salvaged and recycled, and are subsequently essential to purine economy (Lehninger 1977). It also appears that the salvage pathway is of quantitative importance in sheep (Smith *et al.* 1974; Razzaque *et al.* 1981) but not cattle (see section 7.6). This is hardly surprising given the considerable energetic advantage of the salvage pathway over *de novo* synthesis.

7.6 Degradation of purines in the ruminant host

Purine degradation has been studied extensively. Cellular DNA is strongly conserved and tends to have a low turnover rate. In contrast, some types of RNA (e.g. messenger RNA) are rapidly turned over. DNA and RNA are hydrolysed by nucleases, diesterases and other enzymes to oligonucleotides and then to mono-nucleotides and nucleosides. The latter may be reutilised in nucleic acid synthesis or further degraded. Glycosidic linkages between purine bases and sugar units are cleaved to yield free purine bases and these too may be salvaged or degraded further and excreted.

AMP and adenine can be degraded enzymatically eventually yielding hypoxanthine which in turn is oxidised by the flavoproteins *xanthine dehyrodrogenase* (EC 1.1.1.204) or *xanthine oxidase* (EC 1.2.3.2), to xanthine and then to uric acid (Nishino 1991). Guanine nucleosides are catabolised to their free bases which, after deamination, yield the intermediate xanthine (Lehninger 1977). Uric acid is further catabolised via *uricase* with the release of CO_2 and the formation of allantoin, which is the major end product of purine degradation in ruminants (Lehninger 1977).

The intestinal mucosal cells represent the first available site for degradation of absorbed purines. In cattle, intestinal tissue is rich in xanthine oxidase which increases the potential for the irrecoverable degradation of absorbed purines and reduces the potential for purine synthesis from exogenous purine bases (Roussos 1963). In contrast, the intestinal mucosa of sheep has only trace amounts of xanthine oxidase activity (Al-Khalidi and Chaglassian 1965) which limits degradation of absorbed purines and increases the potential for their utilisation via the salvage pathway.

Once in the blood, purine products are subject to degradation by xanthine oxidase and uricase. Chen and co-workers (1990a) showed xanthine oxidase to be absent from sheep plasma but present at a considerable concentration in cattle plasma. These authors also found uricase to be absent from bovine blood but present at very low levels in the blood of sheep. The liver of sheep contains high levels of xanthine oxidase and uricase (Chen *et al.* 1990b) and is the major site for purine catabolism to allantoin.

7.7 Relationship between purine absorption and excretion in urine

It is clear that purine absorption from the gut and urinary excretion of purine derivatives are closely related. However, if such relationships are to be used for predictive purposes, any variability occurring under different conditions must also be known or predictable.

Various workers (Antoniewicz *et al.* 1980; Giesecke *et al.* 1984; Chen *et al.* 1990b; Balcells *et al.* 1991) have argued that the relationship between urinary excretion and absorption of purines is either linear or curvi-linear. Chen and co-workers (1990b) hypothesised that non-linearity occurs because purines are synthesised *de novo* when absorption of precursors from the gut is low, and these are added to urinary purines of exogenous origin. They advanced a model to describe their data obtained from sheep nourished intragastrically and infused into the gut with different amounts of a microbial (single cell) preparation.

In this model, the increase in the urinary excretion of purine derivatives (Y, mmol/d) with increasing level of absorbed exogenous purines (X, mmol/d) was described by the following equation:

$$Y = bX + cW^{0.75}\exp^{(-kx)}$$

$W^{0.75}$ represents metabolic body weight (kg), and these authors argued that the co-efficient (b = 0.84) obtained when this model was fitted to their data, implied that 16% of the absorbed plasma purine derivatives were lost via routes other than urinary excretion. The rate constant (k = –0.25) described the replacement of endogenous *de novo* synthesis of purines by the salvage of preformed purines, and the coefficient (c = 2.06 mol/d) represented excretion of endogenous purine derivatives when absorption was zero. Chen and co-workers (1990b) argued that the negative exponent (k = –0.25) allowed for the likelihood that the contribution of *de novo* synthesis of purines to the total urinary purine excretion is reduced by feedback occurring as the availability of absorbed (exogenous) preformed purines increases, i.e. the contribution of purines synthesised *de novo* decreases as absorption increases. They further argued that the contribution of *de novo* synthesis becomes insignificant when absorption of purines reaches 8.3 mmol/d; the value expected when a 40 kg sheep is fed a maintenance energy ration. This hypothesis seems reasonable for mammals in general, as the *de novo* formation of adenylate and guanylate is known to be controlled by three regulatory enzymes that are allosterically inhibited by AMP and GMP concentrations (Lehninger 1977). Thus *de novo* synthesis can be expected to cease once levels of AMP and GMP are adequate. The results of Condon *et al.* (1970) also support this hypothesis: they found that sheep receiving a normal supply of exogenous purines from the rumen microbes did not incorporate intestinally infused labelled glycine (a precursor for *de novo* synthesis) into their tissue nucleic acids. If the hypothesis is true, a corollary is that the complication of determining the endogenous

contribution to the excretion of urinary purine derivatives will largely be removed when animals are on diets that supply more than their maintenance energy requirements.

Whilst this model and the associated assumptions seem generally plausible, it should be recognised that the hypothesis concerning feedback control of *de novo* synthesis has not been experimentally verified. Why mammals in general, or ruminants in particular, need to synthesise any purines *de novo* remains questionable. However it has been suggested (Henderson and Paterson 1973) that most animal cells satisfy their purine requirements by both *de novo* and salvage synthesis. In animals on sub-maintenance diets, the salvage pathways will use purine catabolites from tissues as a major source of intermediates for resynthesis of purines, and no *de novo* synthesis would be required if this process were reasonably efficient. Thus, the level of metabolic activity of various tissues may affect the relative levels of purine excretion and catabolism and the net release of purine derivatives into the salvageable pools, and would therefore modify the model. Discussion relating to this point is considered at a later stage in this Chapter (see 7.7.2).

When the contribution of endogenous purines to total excretion was ignored, Chen and co-workers (1990b) calculated that the recovery of infused purines in urine as total purines after 24 h ranged from about 93–78% when animals were infused intragastrically with 5 to 25 mmol microbial purines/d. When corrected for the calculated true digestibility of the microbial nucleic acid concentrate (91.3%), the recovery increased to 102–85%. However, when the quantity of absorbed purines exceeded 8.3 mmol/d, urinary recovery remained constant at *c.* 85%. This confirmed to Chen and co-workers (1990b) that the contribution of endogenous purines to total excretion declined with increasing absorption of purines.

A greater urinary recovery of absorbed purines (93%) was observed by Balcells *et al.* (1991) when sheep were conventionally fed whilst receiving a duodenal infusion of varying levels of yeast RNA. Kahn (1991) reported that when sheep were infused intravenously with adenine, the net molar recovery (as urinary allantoin plus uric acid) was on average 73% and did not differ as infusion levels rose from 0–5.3 mmol/d for animals on a basal diet that provided for about 5 mmol/d of exogenous purines, i.e. total 5–10.3 mmol purines/d. Inclusion of the contributions of hypoxanthine and xanthine would make the recovery estimate of Kahn (1991) similar to that of Chen and co-workers (1990b). However, these recoveries are not easily reconciled with an average net recovery of absorbed purines of 98% in two lambs abomasally infused with a microbial mixture (Fujihara *et al.* 1987), and thus more work is needed to elucidate the non-renal routes of excretion of purine derivatives.

The urinary recoveries of an absorbed (unlabelled) purine load reported by various workers (Fujihara *et al.* 1987; Chen *et al.* 1990b; Balcells *et al.* 1991; Kahn 1991) have been substantially higher than those reported for labelled purines (Smith *et al.* 1974; Razzaque *et al.* 1981; Kahn 1991). When mixed rumen bacterial cultures labelled with [8-^{14}C] adenine were administered to the rumen of sheep, recovery of the total absorbed radioactivity in urine after 24 h was 21% (Razzaque *et al.* 1981) and 5% (Smith *et al.* 1974). In the study by Kahn (1991) in which recoveries of an intravenous load of adenine averaged 73%, urinary recovery of ^{14}C from intravenously administered ^{14}C-adenine over the same 24 h period amounted on average to only 9%. The difference in recovery between labelled and unlabelled adenine derivatives in these studies implies that absorbed labelled purine molecules or their products enter a large tissue pool with a low turnover rate and thus have a low probability of being excreted within 24 h of their arrival in blood.

Various tissues may contain part of this pool. For example, Murray (1971) reported a pool size for adenylate nucleotides of 1.3 μmol/ml of red blood cells (RBC) in humans, whilst Marger and co-workers (1967) reported values (μmol/ml RBC) for purine nucleotides in rabbits of 1.5–1.9 for adenine, 0.25–0.35 for guanine and 0.04–0.06 for hypoxanthine. Muscle tissues contain a large fraction of the purine pool even though nucleic acids represent only 0.1–0.2% of their mass.

7.7.1 The contribution of endogenous purines to urinary purine excretion

As discussed previously, urinary purine derivatives are derived from degradation of purines that have been synthesised from endogenous (via *de novo* or salvage pathways in host tissues) and exogenous (microbial) sources. One method of studying the relative importance of the net endogenous contribution is to eliminate the flow of purines to the intestine. However, this may affect the magnitude of the endogenous contribution to urinary purine derivatives (Chen *et al.* 1990b).

In a simple experiment, Rys and co-workers (1975) observed that allantoin fell markedly in sheep over successive days of fasting to a constant low value. Likewise, Antoniewicz and Pisulewski (1982) found that the excretion of allantoin declined rapidly as the hay diet of wethers was replaced by gastric infusion of purine-free nutrients over a period of 10 days. Meanwhile the excretion of creatinine which remained stable and the reduction of the allantoin-N:creatinine-N ratio was therefore solely a function of reduced allantoin excretion.

With lambs intragastrically nourished by purine-free nutrients, a urinary excretion value of 150μmol/kgW$^{0.75}$/d for endogenous purine derivatives (allantoin, uric acid, xanthine and hypoxanthine) was reported (Chen *et al.* 1990b). This value is in reasonable agreement with the values of 165 (Fujihara *et al.* 1987) 168 (Chen *et al.* 1990a) and 177 (Lindberg and Jacobsson 1990) obtained in lambs and sheep also nourished by intragastric infusion but is somewhat lower than the 208 obtained in sheep receiving a duodenal infusion of varying levels of yeast RNA (Balcells *et al.* 1991)

7.7.2 Dietary effects on urinary excretion of purine derivatives of endogenous origin

Turnover of intracellular nucleic acids, particularly RNA, and lysis of cells, will presumably increase the pool of endogenous purines from which a fraction of urinary purines originate. Hence it seems reasonable to suggest that those factors that dictate the extent of cellular turnover may also influence the endogenous contribution to urinary purine derivatives. However, a review of the relevant literature suggests that the effect of varying the intake of energy and protein on the urinary excretion of purines in gastric fed sheep (dysfunctional rumen) is equivocal.

Changing the quantity and quality of nutrients available for absorption in ruminants is difficult because of ruminal microbial fermentation. Thus, the bottle or intragastric fed ruminant has been used to ascertain the relationship between excretion of purine derivatives in urine and energy and nitrogen intake. When the milk diet of the bottle fed kid was progressively substituted with a nitrogen-free liquid formulation, Linberg (1989) observed that the excretion of endogenous purine derivatives in urine was only marginally affected. However, a closer examination of the data showed that endogenous urinary purine derivatives decreased by *c.* 20% when 80% of the goat's milk was replaced with a nitrogen-free liquid formulation. Further indication that increasing N intake stimulates urinary purines of endogenous origin is evinced from the data of Lindberg and Jacobsson (1990). These authors intragastrically fed sheep varying levels of volatile fatty acids and protein and concluded that neither the level of protein or energy influenced urinary excretion of endogenous purine derivatives. When the data presented by Lindberg and Jacobsson (1990) was examined it seemed apparent that increasing protein intake beyond 600 mg N/kgW$^{0.75}$ stimulated the excretion of endogenous purine derivatives in urine for all energy levels (Figure 7.3). For

protein intakes less than 600 mg N/kgW$^{0.75}$, the effect of energy and protein on endogenous purine excretion was inconsistent.

More recently, Chen and co-workers (1992b) have shown that the endogenous excretion of allantoin may be more closely correlated to cumulative N retention (the sum of daily N retention) rather than intake of protein. Hence it appears that endogenous urinary purines increase with the mass of proteinaceous tissue which presumably, in ruminants, is an indirect measure of the pool of nucleic acids. In contrast to those results previously discussed, Fujihara *et al.* (1987) observed a 'remarkable stability' of endogenous excretion of urinary purine derivatives in steers nourished by intragastric infusion when subjected to large changes in casein nitrogen supply.

Whilst large changes in energy and protein intake may influence the excretion in urine of endogenous purine derivatives such changes are unlikely to be experienced in most situations. Of more concern is the apparent relationship between endogenous excretion and protein mass. If this relationship is confirmed, then protein mass, which at times is only poorly predicted from live weight, needs to be considered in the stratification of animals to experimental treatments.

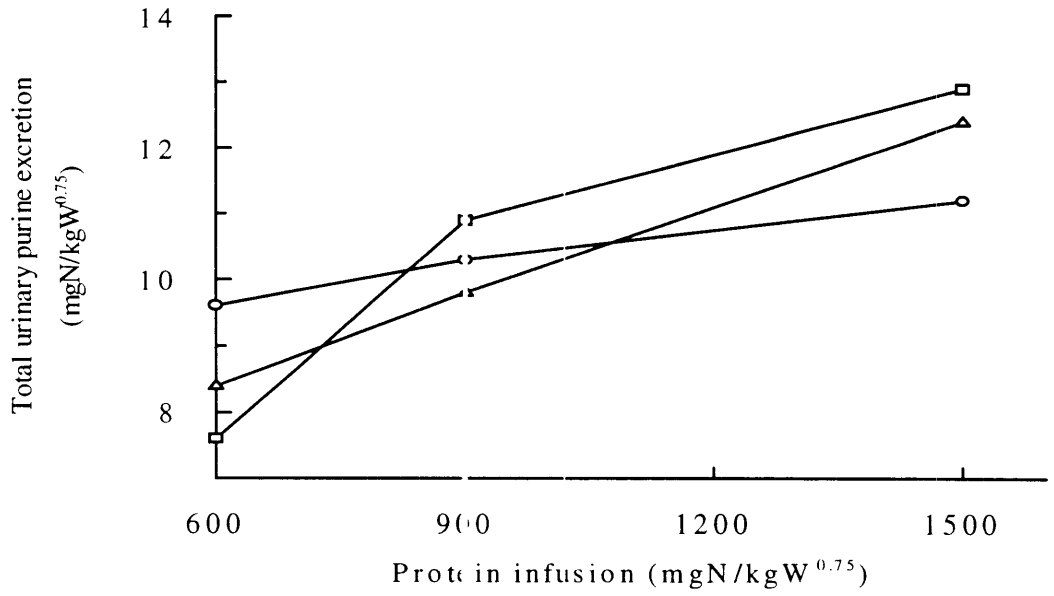

Figure 7.3. Excretion of purine derivatives in the urine of sheep intragastrically nourished with varying levels of protein (600, 900 or 1500mg N/kgW$^{0.75}$) when gross energy intake was 340 (circle), 450 (triangle) and 630 (square) kJ/kgW$^{0.75}$). Prepared from the data of Lindberg and Jacobsson (1990). Standard errors not given in the original paper.

7.7.3 Changes in the proportions of purine derivatives in urine

A further problem associated with prediction of purine absorption from urinary excretion of purines is that the proportions of allantoin, uric acid, xanthine and hypoxanthine may vary with the level of exogenous purine absorption. Various workers (Chen *et al.* 1990b; Balcells *et al.* 1991) have found that increasing the load of exogenous purines results in an increase in the proportion of urinary purine derivatives excreted as allantoin. Chen and co-workers (1990b) observed that allantoin accounted for an increasing proportion of total purine excretion in urine until total purine excretion was *c.* 12 mmol/d at which stage, allantoin represented *c.* 0.8 of total purines in urine. These authors suggested that an explanation for this may relate to the sites of derivative formation in the body.

In sheep, all extra-hepatic cells contain only trace amounts of xanthine oxidase whereas the blood contains no xanthine oxidase and only traces of uricase (Al-Khaldi and Chaglassian 1965). Endogenous purine products can be degraded in the tissue to hypoxanthine, plus small amounts of xanthine and uric acid. Further catabolism of these derivatives to allantoin requires passage through the liver where high levels of xanthine oxidase and uricase exist (Chen *et al.* 1990a). The circulatory system is such that these endogenous derivatives may be filtered from the blood before being carried to the liver. In contrast, exogenous purine derivatives formed from absorbed purine products are carried to the liver in the portal blood. The major site of degradation of these derivatives is likely to be the liver where they will readily form allantoin. If there are different sites of catabolism for endogenous and exogenous purine products, it is predictable that a greater proportion of urinary purine derivatives will appear as allantoin as the exogenous purine load increases.

The possible complication of changes in the proportions of urinary purine derivatives with increasing excretion of total purine derivatives is obviated by the close linear relationship between allantoin and total purine excretion (Figure 7.4). Data collected in the period 1992–1995 from *c.* 70 sheep that were used in a range of trials conducted at the University of New England with low quality roughages and nitrogenous supplements indicate that for every additional mole of total urinary purines, allantoin excretion increases by 0.894 mol.

7.7.4 Non-renal excretion of purine metabolites

To use the excretion of urinary purine derivatives as a predictor of the outflow of microbial protein from the rumen, it is necessary to know the proportion of absorbed purines excreted via non-renal routes and test whether this is a constant. As there is apparently no allantoinase in

ruminant tissues (Lehninger 1977) the other potential route of loss of purine metabolites is into the gut. However, glyoxylic acid, the degradation product of allantoin, is present in the urine of both sheep and cattle (Van Der Horst 1960) suggesting that in these species catabolism of purines beyond allantoin is possible. Chen and co-workers (1990b) concluded that *c.* 16% of purine excretion occurred via routes other than through the urine in intragastrically fed sheep and this was therefore either excreted into the gut, or incorporated into tissues. However, Balcells and co-workers (1991) observed that non-renal excretion of purines was of lesser importance and accounted for only 7% of purine absorption.

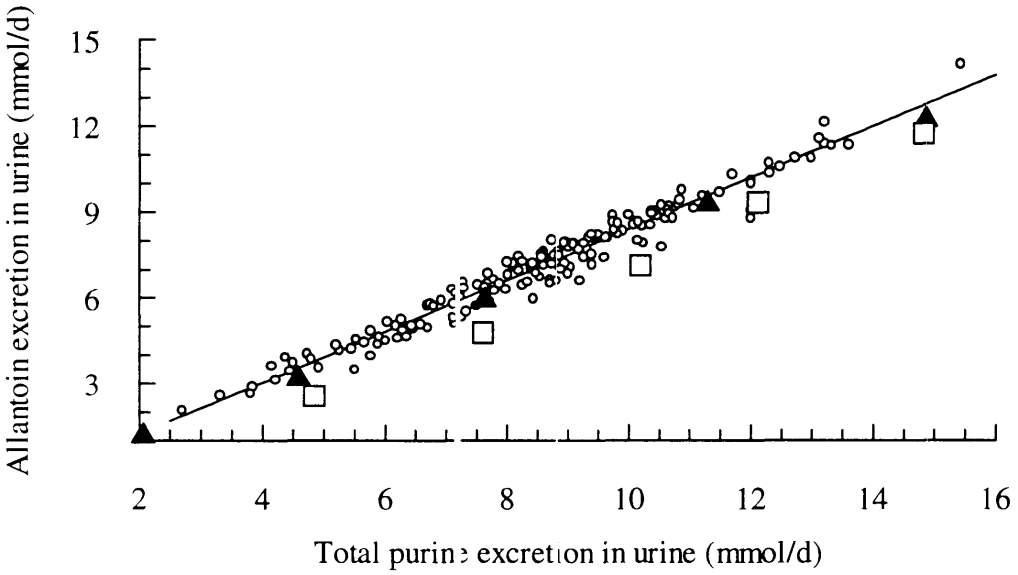

Figure 7.4. Urinary allantoin increases linearly with total purine derivative excretion in urine collected from sheep from a range of experiments (see text). The relationship between allantoin excretion (Y, mmol/d) and total purine excretion (X, mmol/d) was derived from 167 observations and is described by the equation Y = –0.54 (s.d. = 0.121) + 0.89 (s.d. = 0.014) X, r^2 = 0.96. The data of Chen *et al.* 1990b (filled triangle) and Balcells *et al.* 1991 (unfilled square) are superimposed for comparative purposes.

Incomplete urinary recovery of intravenously infused allantoin has also been reported in sheep (Chen *et al.* 1991). In these studies, allantoin was infused into the jugular vein and urinary recovery of infused allantoin ranged from 62–105%. Chen and co-workers (1991) suggested that the allantoin not recovered in urine may have been excreted via saliva or entered the lower gut. The between-sheep variation in urinary allantoin recovery reported by Chen

and co-workers (1991) suggests that the quantitative importance of non-renal excretion of allantoin varies between animals.

7.7.4.1 Salivary loss of purine derivatives

There is evidence that in sheep (Chen *et al.* 1990c), allantoin and uric acid occur in saliva at concentrations greater than in plasma, indicating that allantoin and uric acid can be actively taken up by the salivary glands. Assuming a salivary flow of 10 *l*/d, Chen and co-workers (1990c) estimated that the amount of purine derivatives secreted in the saliva would account for about 10% of that excreted in the urine. However, in contrast, Surra and co-workers (1993) reported salivary concentrations of allantoin and uric acid in sheep that were only 3–4% of that reported by Chen and co-workers 1990c. The salivary concentrations of allantoin and uric acid observed by Surra and co-workers (1993) were considerably lower than that expected in plasma and when multiplied by estimated saliva flow rate (7 *l*/d) accounted for only 1% of the daily purine excretion in urine.

Purine products and derivatives that are recycled to the rumen via the saliva are likely to be rapidly degraded (Belasco 1954; McAllan and Smith 1973; McAllan 1982; Chen *et al.* 1990c) and thus the likelihood of their reappearance in urine will be very low. Allantoin and uric acid that enters the gut is rapidly degraded to glyoxylate and urea and the latter is completely converted to ammonia and carbon dioxide by bacterial urease (Belasco 1954; McAllan 1982). Infusion of allantoin into the rumen or abomasum of normally fed sheep, or into sheep and steers nourished by intragastric nutrition, did not change the levels of urinary excretion of purine derivatives (Chen *et al.* 1990c).

Thus confusion exists concerning the quantitative importance of non-renal excretion of purine derivatives in sheep. This confusion adds uncertainty to the estimates of microbial yield based on the urinary excretion of purine derivatives. In addition, the equations that are used to predict microbial yield which are based on the relationship between purine absorption and urinary excretion may be inappropriate if the fraction of purine excretion via non-renal routes (e.g. saliva) varies. The quantitative importance of salivary allantoin as a mechanism for allantoin recycling to the rumen was examined as part of the studies reported in this thesis (see Chapters 8, 9).

7.7.4.2 Loss of purine carbon via expired gases

If negligible quantities of purine derivatives are lost via saliva, then it is suggested that excretion of purine derivatives to the small intestine would be the most likely means to account

for the incomplete urinary recovery of absorbed purines. Secretion of purine derivatives to the lower gut would expose these compounds to enzymatic attack from enteric micro-organisms.

Razzaque and co-workers (1981) gave a ^{14}C-labelled bacterial nucleic acid source to lambs via a rumen tube and reported that c. 8% of the total amount of radioactivity absorbed appeared in exhaled gases in the following 24h. These authors suggested that the ^{14}C-labelled gases were not derived from the tissue catabolism of uric acid to allantoin but most likely arose from "degradative reactions within the gut". However, because the ruminal degradation of the bacterial nucleic acid preparation was not estimated, it is possible that the ^{14}C-labelled CO_2 arose from ruminal rather than intestinal degradation.

There is evidence for transport of purine derivatives into the intestinal lumen of hamsters (Berlin and Hawkins 1968) and humans (Sorenson 1960) and for degradation of purines by enteric micro-organisms in mice (Zvilna *et al.* 1975 in Razzaque *et al.* 1981) and humans (Sorenson 1960). Berlin and Hawkins (1968) studied the transport of purines in hamster intestine *in vitro* and showed that xanthine and uric acid were secreted into, but not absorbed from, the intestinal lumen When humans were given injections of labelled uric acid into the blood no uric acid was found in faeces (Sorenson 1960). However, when bacteriostasis was achieved in the gut, radioactive uric acid was excreted in faeces (Sorenson 1960). Zvilna and co-workers (1975 in Razzaque *et al.* 1981) observed that, following an oral dose of labelled adenosine, much less $^{14}CO_2$ was expired in mice whose intestinal microflora had been eliminated. These results suggest that, in some mammals, uric acid and xanthine are secreted into the gut and are then degraded to CO_2 and NH_4^+ by enteric micro-organisms. Hence, it seems reasonable to presume that other purine derivatives, particularly allantoin, may experience the same fate in ruminants.

The quantitative importance of transfer of allantoin to the lower gut and its subsequent degradation to yield amongst other metabolites CO_2 was investigated as part of the studies reported in this thesis (see Chapters 8 and 9).

7.8 Conclusions

The conditions that are essential for satisfactory prediction of microbial yield from urinary excretion of purine derivatives include the following:

(a) The ratio of renal:non-renal excretion of purine derivatives should be substantially constant and not influenced by the level of purine absorption.

(b) There should be no interaction between the level of protein and energy intake and the excretion of endogenous purine metabolites.

In addition to these conditions, it is necessary to use several factors in order to predict microbial yield from urinary excretion of purine metabolites. These factors include:

(i) The percentage of purine-N in the total-N of rumen micro-organisms. Variations which occur between fluid- and solid-associated bacteria, and in response to various factors such as time after feeding and specific growth rate will make predictions uncertain.

(ii) The true digestibility of microbial purines in the small intestine. This is normally greater than 85%.

(iii) The relationship(s) between the amount of absorbed purines and renal excretion of total purine derivatives (or allantoin). Available evidence suggests that 84–93% of the theoretical recovery is found in urine; the remainder probably enters the gut. At below-maintenance levels of feeding, derivatives from *de novo* synthesis of purines may cause exogenous purine absorption to be over-estimated, and this needs to be accounted for. However, net accretion or loss of tissue purines may also alter the relationship. Allantoin alone may be a useful urinary index because of the close linear relationship between total urinary purines and urinary allantoin.

Prediction should also be possible on the basis of relationships between exogenous purine absorption and net flux of allantoin (or total purines) through the blood pool (estimated by tracer dilution techniques). Prediction on the basis of blood concentrations of purine derivatives may also be a possibility as flux rates and concentrations of metabolites are often directly correlated.

Some of the studies that are later described in this thesis were established to provide information on the metabolism of allantoin. In these experiments ^{14}C-allantoin was administered intravenously to sheep and estimates of allantoin production, urinary and salivary excretion and transfer to carbon dioxide were made.

CHAPTER 8

8. ALLANTOIN METABOLISM IN SHEEP

8.1 Introduction

Urinary excretion of purine derivatives is proportional to the outflow of microbial cells from the rumen and is the basis of a non-invasive technique for estimating microbial yield from the rumen. Allantoin is the predominant purine derivative in the urine of sheep with lesser amounts of uric acid, hypoxanthine and xanthine. Numerous workers (Fujihara *et al.* 1987; Chen *et al.* 1990b; Balcells *et al.* 1991) have examined the relationship between inputs of either nucleic acids or RNA into the digestive tract of sheep and the excretion of purine derivatives in urine. Their research has shown that a close relationship exists but that urinary recovery of exogenous purines is incomplete (Chen *et al.* 1990b; Balcells *et al.* 1991).

Incomplete urinary recoveries have also been reported following infusion of allantoin into the jugular vein of sheep (Chen *et al.* 1991). Saliva has been suggested as one route of non-renal excretion of purines (Chen *et al.* 1990c) but this has recently been disputed (Surra *et al.* 1993).

Although urinary excretion of purine derivatives is closely correlated to the level of exogenously supplied purines, the nature of the relationship may alter if the losses of purine derivatives into the gut are changed by diet or for other reasons. The measurement of allantoin flux through the plasma pool should avoid the problems of non-renal losses and provide an even better indicator of microbial yield from the rumen. To date, there are no reports of estimates of allantoin flux rates through the blood of sheep. The aim of the present series of experiments was to estimate the flux rate of allantoin through the plasma pool and further investigate the metabolism of allantoin.

8.2 Materials and Methods

8.2.1 Animals and conditions

Two rumen-cannulated Merino cross Border Leicester wethers, 2 year old, weighing 39.2 and 43.1 kg live weight were used for the experimental work. These animals were fed hourly (automatic overhead feeder) equal portions of a ration of 650 g/d oaten chaff (*Avena sativa* L.;

89.3% DM and 1.2% N) plus 250 g/d lucerne chaff (*Medicago sativa* L.; 87.2% DM and 2.8% N) on an as fed basis. Animals were maintained on this feeding schedule for 12 days prior to the start of the experiment. Throughout the experiment, the animals were kept in metabolism crates with continuous lighting and unrestricted access to water. Indwelling jugular catheters were placed in each sheep 24 h before the single injection of tracer.

8.2.2 Experimental procedure

A solution containing 28 μCi of [4,5-^{14}C] allantoin (Amersham International plc; 38μCi/mg) and 15 mg of allantoin carrier in 10 ml of medical grade physiological saline was injected into the jugular catheter of each sheep. The catheter was immediately flushed with saline (10 ml) to ensure quantitative transfer of all radioactive allantoin.

To quantify the conversion of allantoin-C to HCO_3^--C it was necessary to measure the flux rates of HCO_3^- in blood and rumen fluid. Therefore, 2 days after the allantoin injection, 42 μCi of ^{14}C-HCO_3^- and 5 mg of Na_2CO_3 carrier in 10 ml of medical grade saline, made slightly alkaline with sodium hydroxide, was injected into the jugular catheter of each sheep. The catheter was immediately flushed with saline (10 ml) to ensure quantitative transfer of all radioactive material. One day later, 44 μCi of ^{14}C-HCO_3^- and 6.5 mg of Na_2CO_3 carrier in 50 ml of water, made slightly alkaline with sodium hydroxide, was injected through a stainless steel probe into the rumen of each sheep. The probe was immediately flushed with water (50 ml) to ensure quantitative transfer of all radioactive material to the rumen.

8.2.3 Sample collection and storage

Samples of blood, rumen fluid and saliva (the latter only for the allantoin experiment) were taken immediately before the injection of each isotope to determine the background levels of ^{14}C-allantoin and ^{14}C-HCO_3^-. Blood and rumen fluid (sampled as closely in time as possible) were sampled a further 13 times (5, 15, 30, 60, 90, 120, 180, 240, 360, 540, 720, 960, 1440 min) over the next 24 h for the study of the dynamics of allantoin metabolism and 10 times (15, 30, 45, 60, 90, 120, 180, 240, 300, 400 min) over the next 7 h when ^{14}C-HCO_3^- injections were made. Samples of saliva were taken a further 4 times (120, 240, 540, 1440 min) over the next 24 h following administration of radioactive allantoin using a similar method to that employed by Chen *et al.* (1990c). In this method, samples of saliva were obtained by swabbing the mouths of the experimental sheep with small pre-weighed sponges and these were squeezed to secure the saliva.

Blood samples (12 ml) were withdrawn from the jugular catheter and evenly divided. One fraction (6 ml) was used for the determination of the specific radioactivity of blood bicarbonate by the method of Leng and Leonard (1965). The remainder (6 ml) was placed into a heparinized tube, cooled on ice (30 min), centrifuged for 10 min at 3000 *g* and the plasma was then separated. Rumen fluid (10 ml) was withdrawn through nylon gauze and a portion (4 ml) was used to determine the specific radioactivity of rumen bicarbonate (Leng and Leonard 1965).

Urine was collected during 12 h periods for the first day and then daily for the next 3d after injection of allantoin tracer. A sufficient quantity of thymol was added to the urine collectors so that the final concentration in urine was *c.* 2.2mM. Immediately prior to the end of each sampling period, the metabolism crates were washed with distilled water to ensure transfer of all urinary residues into the collecting vessel. Urine was subsampled (20 ml) at the end of each collection period. Faeces were collected daily and subsampled (10%). Plasma, saliva, urine and faecal samples were frozen immediately after collection or separation and then stored at -20°C until later analysis.

8.2.4 Tracer purity

The radiochemical purity of the [4 5-^{14}C] allantoin as determined by Amersham International plc was 96% using thin layer chromatography (TLC) and 96.3% using high performance liquid chromatography (HPLC). To ensure integrity of the labelled allantoin upon delivery, crystalline [4,5-^{14}C] allantoin was dissolved in 4 ml of saline, subsampled and further diluted. The diluted material was then injected (20 µl) into a pre-equilibrated HPLC system. Analysis of the resulting chromatograms showed the existence of a single peak which had the same elution time and absorbance spectrum as unlabelled allantoin. The effluent from these chromatograms was fractionated and the associated radioactivity determined on a scintillation spectrophotometer (LSC: Packard Instrument Company). In this way, the radiochemical purity was assessed to be 96.6% which was in close agreement with the product specification.

8.2.5 Analytical procedures

Allantoin concentration was determined in diluted urine (50 fold) using HPLC with a modified version of the method of Balcells *et al.* (1992b). The primary modification was the use of two C_{18} reversed-phase Nova-Pak® (Waters, Millipore Corporation) columns (3.9 mm x 300 mm) connected in series. The effluent was monitored at a higher wavelength (214 nm) and the flow rate was reduced to 0.6 ml/min. The precision of allantoin determinations was checked by

repeated injections (n = 3) of four different urine samples. The suitability of this technique was further assessed by calculating the recovery of allantoin added at four levels to two different urine samples.

8.2.6 Separation and quantification of allantoin in plasma

Allantoin concentration in plasma was determined using the same column assembly as used for determinations in urine. A dual-pump delivery system (Waters Associates, U.S.A) with automatic injector (WISP 710 b) was used for analyses. Optimal separation of allantoin from other polar compounds present in plasma was achieved with filtered (0.45 µm, HVLP Millipore Corporation) solutions of 0.1% orthophosphoric acid (A) and 0.1% orthophosphoric acid with 40% acetonitrile (B). The flow rate was 0.6 ml/min and was made up of 100% (A) from 0–10 min, then reducing to 0% (A) by 20 min using a linear gradient and held at 0% (A) until 35 min to ensure cleaning of the column. Initial conditions were then re-established by increasing (A) from 0–100% over the period 35-38 min using a linear gradient and then 100% (A) was maintained until 53 min.

Plasma (400 µl) was placed into a regenerated cellulose filter assembly (Ultrafree mc™ 10,000 NMWL exclusion, Millipore Corporation) and centrifuged at 4° C at 3000 *g* for 15 min followed by a further 60 min at 12000 *g*. Allopurinol (1 mM) was added to the filtrate to act as an internal standard and 20 µl of the resulting solution was injected. The suitability of the plasma technique was assessed by calculating the recovery of allantoin added at two levels to two different plasma samples.

8.2.7 Specific radioactivity of allantoin

To determine the radioactivity in urinary allantoin, 0.2 ml of urine was acidified with glacial acetic acid to ensure the removal of any $^{14}C\text{-}HCO_3^-$. The acidified urine solution was then mixed with 0.8 ml water and 10 ml of a toluene/Triton-X solution (9:4 v/v and containing 0.4% 1,4-bis-[2-(5-Phenyloxazoly)]-Benzene (PPO) and 0.02% 2,5-Diphenyloxazole (POPOP)) and counted on a scintillation spectrophotometer (Packard Instrument Company Inc). The external standard method was used to correct for quenching

Radioactivity associated with plasma was determined from a subsample of plasma filtrate. A weighed amount of filtrate was acidified (glacial acetic acid) to ensure the release of any $^{14}C\text{-}HCO_3^-$ and then dried in a vacuum desiccator. The dried material was then dissolved in 1 ml of water and mixed with the toluene/Triton-X solution as for determination of radioactivity in urine. This procedure was also used for saliva samples. To ensure specificity

of the label for allantoin, several urine and plasma samples were fractionated via HPLC and then the radioactivity in each fraction was determined.

8.2.8 Calculation of allantoin kinetics

The specific radioactivity (SR) versus time data from the three tracer experiments were fitted by either a single or double exponential model by an iterative procedure based on the simplex algorithm that minimised the residual sum of squares (Ezfit: Noggle 1992). Estimates of pool size, space of distribution, proportion of carbon in the HCO_3^- pool derived from allantoin-C (transfer quotient, TQ) and net and total flux were calculated from these parameters (Shipley and Clark 1972) assuming steady state conditions applied.

8.3 Results

Determination of allantoin concentration in four urine samples by HPLC was found to be highly precise with the coefficient of variation (c.v.) being on average 2%. Recovery of known amounts of allantoin added to urine and plasma samples was 95% (c.v. = 3.0%) and 101% (c.v. = 1.3%) respectively.

Throughout the experiment both sheep consumed the entire ration of 900 g/d (as fed). Following intravenous injection of ^{14}C-allantoin there was a rapid appearance of radioactive allantoin in urine. After 12 h, both sheep had excreted in urine *c.* 80% of the ^{14}C-allantoin injected. Urinary excretion of ^{14}C-allantoin was monitored for the next 3.5 days when the cumulative recovery was *c.* 94% of that injected (Figure 8.1). Fractionation of urine samples

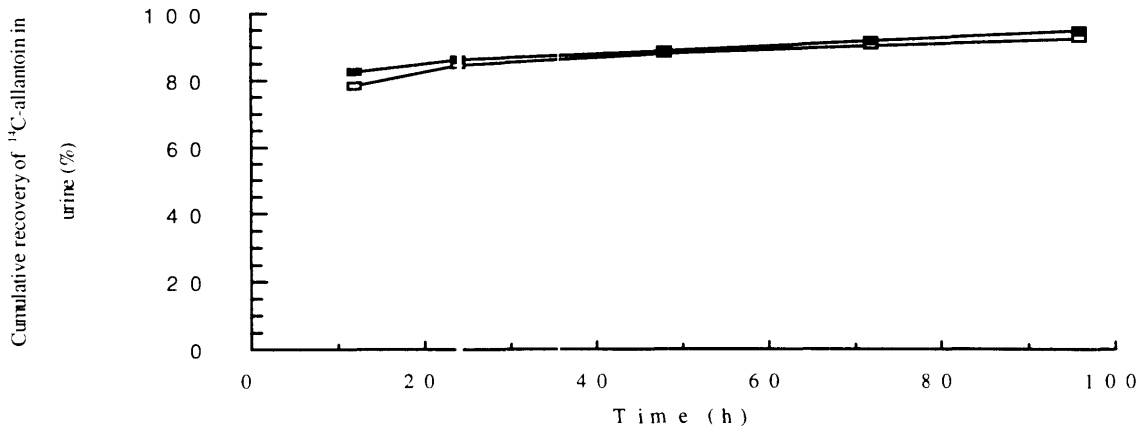

Figure 8.1. Cumulative recovery of ^{14}C-allantoin in urine of sheep 1 (unfilled square) and sheep 2 (filled square) following an intravenous injection of ^{14}C-allantoin. Values adjusted for dose purity of 0.966.

via HPLC verified that the radioactivity in urine was specific to allantoin.

Following intravenous injection of ^{14}C-allantoin, the SR of allantoin in plasma declined in a manner that was well described by an equation of the form:

$$SR_t = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} \quad (1)$$

Where SR_t is the specific radioactivity of allantoin at time t, A_1 and A_2 (μCi/gC) and k_1 and k_2 (min^{-1}) are the intercept values and rate constants for the primary and terminal components of the curve respectively. The resulting parameter estimates for each sheep (Equations 2, 3) provided a good fit to the data (Figure 8.2). The terminal component of the SR versus time curve for both sheep became apparent after 2–3 h.

Sheep 1 $$SRt = 503 e^{-0.0237t} + 317 e^{-0.0050t} \quad (2)$$

Sheep 2 $$SRt = 354 e^{-0.0210t} + 278 e^{-0.0052t} \quad (3)$$

Allantoin concentration, pool size, space of distribution, flux rates, transfer quotients and urinary excretion are shown in Table 1. The appearance of ^{14}C-HCO_3^- in the blood and rumen was extremely low and the transfer quotient for HCO_3^--C in both blood and rumen fluid was insignificant. Quantification of the transfer of allantoin-C to HCO_3^--C in blood and rumen fluid was achieved by multiplying the respective TQ by the total flux of carbon through the blood or rumen fluid HCO_3^--C pool (determined by $H^{14}CO_3^-$ injection) (Table 8.1). The mass of allantoin-C that was transferred to HCO_3^- in blood and urine was:

Sheep 1: 10 mg C/d allantoin-C to blood HCO_3^--C
2 mg C/d allantoin -C to rumen fluid HCO_3^--C

Sheep 2: 35 mg C/d allantoin-C to blood HCO_3^--C
9 mg C/d allantoin-C to rumen fluid HCO_3^--C

Table 8.1. Kinetic data from a s ngle injection of ^{14}C-allantoin into the jugular vein of two sheep.

Parameter	Sheep 1	Sheep 2
Allantoin in plasma[A] (mg C/l)		
mean	5.4	5.7
s.d.	0.23	0.35
n	14	14
Pool size (mg C)	33.3	42.6
Space of distribution (l)	6.1	7.4
Total flux (mg C/d)	790	860
Net flux (mg C/d)	460	550
Transfer quotient x 10^5		
Fraction of carbon derived from allantoin		
in blood HCO_3^-	9.01	3.26
in rumen HCO_3^-	6.18	4.32
Urinary excretion (mg C/d)	382	318
HCO_3^- total flux (g C/d)		
blood	296	385
rumen fluid	46	137

Allantoin data are corrected for dose purity of 0.966.

[A] Mean, s.d. are from 14 blood samples taken over 24 h (see Materials and Methods).

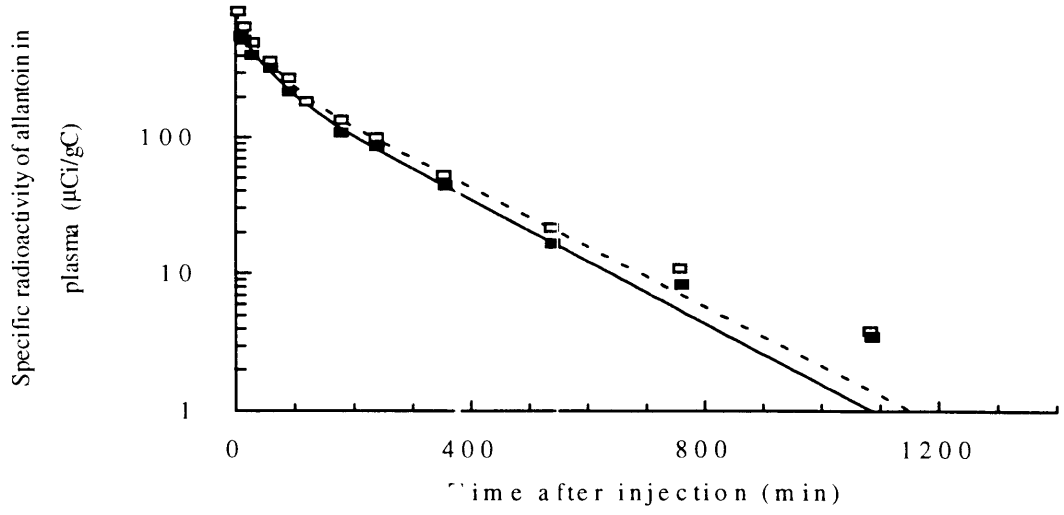

Figure 8.2. Decline in the specific radioactivity of plasma allantoin following intravenous injection of 28 μCi ^{14}C-allantoin. Equations 2 (dashed line) and 3 (solid line) have been fitted to the data for sheep 1 (unfilled square) and sheep 2 (filled square) respectively.

8.4 Discussion

The appearance of *c.* 94% of the ^{14}C-allantoin in urine over the 4 days following its intravenous injection, confirms that renal excretion is the major route of loss of allantoin in sheep (Chen *et al.* 1990b; Balcells *et al.* 1991). The routes of loss of ^{14}C-allantoin are similar in the rat: when [4,5-^{14}C] allantoin was given as a single injection into the dorsal tail vein of 3 rats, 84% (s.e. = 2.0) of the ^{14}C-allantoin injected was recovered in urine after 24 h (Kahn unpublished). Unlike hypoxanthine and xanthine, allantoin cannot be salvaged (i.e. reused in the synthesis of purines) and as there is apparently no allantoinase in ruminant tissues (Lehninger 1977) is considered to be the end-product of purine oxidation in ruminants (Stryer 1988). Consequently, the labelled carbon atoms of ^{14}C-allantoin stay associated with allantoin and disappear from the blood plasma at a rate determined by the sum of the excretory pathways.

To ensure that ^{14}C-allantoin was not transferred from blood and sequestered into other pools or metabolites, two rats (Kahn unpublished) were killed 24 h after injection with [4,5-^{14}C] allantoin. Approximately 1% of the injected radioactivity was found to be associated with organs and muscle. This is confirmation that, at least in the rat, allantoin in blood is not sequestered into pools that are associated with major organs or muscle. It has also been confirmed that sheep given an intra-jugular injection of [4,5-^{14}C] allantoin excreted less than 1% of the injected radioactivity in faeces in the 24 h following isotope injection (Kahn unpublished).

The existence of a second exponential component in the curves of allantoin specific radioactivity versus time became apparent 2–3 h after injection with ^{14}C-allantoin. This suggests that either allantoin-C was incorporated into other metabolites and then recycled back to allantoin or that allantoin existed in two kinetically distinct compartments (eg. blood and extracellular fluid). The second suggestion is most likely as current knowledge of the biochemistry of purine degradation (Stryer 1988) eliminates the possibility that allantoin-C can be recycled via other metabolites.

The double exponential model provided a good fit to the SR versus time data. However, visually there is a suggestion of a possible third exponential component becoming apparent 9 h after injection with ^{14}C-allantoin (Figure 2). A third component could not be fitted mathematically with confidence and did not significantly change the kinetic data. This component was presumably a result of ^{14}C-allantoin re-entering the primary pool from another, much larger compartment (eg. body water).

The net flux of allantoin represents the allantoin-C entering the allantoin-C pool for the first time during the experiment. The difference between net and total flux would generally be taken to represent allantoin-C recycled to the sampled allantoin pool within the time course of the experiment. However, given that allantoin-C is unlikely to recycle via metabolic pathways (Stryer 1988), the "recycling" in this experiment is likely to represent the cycling between two or more kinetically distinct compartments such as blood water and interstitial water.

Plasma concentrations of allantoin averaged 113 and 120 μM for sheep 1 and 2 respectively. These values are within the range reported for sheep by Chen *et al.* (1991) but are higher than those reported by Balcells *et al.* (1992a) and Chen *et al.* (1990b). If allantoin is distributed in non-plasma fluids at the same concentration as in plasma it would have been distributed in 6.1 and 7.4 l for sheep 1 and 2 respectively. This represented 16–17% of live weight for these sheep which indicates that the volume of distribution for allantoin was less than that occupied by extracellular fluid (*c.* 20%, Eckert *et al.* 1989).

It is clear that purine absorption from the gut and urinary excretion of allantoin in sheep are closely related (Chen *et al.* 1990b; Balcells *et al.* 1991); urinary recovery of absorbed purines was 84% (Chen *et al.* 1990b) and 93% (Balcells *et al.* 1991), implying that a proportion of absorbed purines are lost via non-renal routes. These losses may represent excretion via non-renal routes (i.e. transferred into the gut) or as a result of retention of absorbed purines in the tissues. However, urinary recovery of allantoin in sheep is also incomplete, ranging from 62 to 105% (mean 72% S.E.M = 7%) following an intra-jugular infusion of allantoin (Chen *et al.* 1991). As tissue utilisation of allantoin is unlikely, Chen and co-workers (1991) suggested that the allantoin not recovered in urine may have been lost via saliva, gastro-intestinal secretions or by diffusion of allantoin across the gut wall.

It has been suggested that the equations that are used to predict microbial yield which are based on the relationship between purine absorption and urinary excretion may be inappropriate if the fraction of excretion via non-renal routes (e.g. saliva) changes (Chen *et al.* 1990c). Chen and co-workers (1990c) suggested salivary purine loss may be 0.1 that excreted in the urine but did not determine if the fraction of purine loss via saliva was affected by factors such as diet. Close examination of their data revealed that the salivary concentration of allantoin and uric acid differed between the two experimental sheep by a factor of 2.3. If saliva flow did not change accordingly, the fraction of purine loss via saliva would have a large between-sheep variance. Allantoin entering the rumen via saliva will be completely degraded to ammonia and carbon dioxide (Belasco 1954), which will increase the SR of rumen fluid

HCO_3^- and eventually blood HCO_3^- and will not contribute to the urinary excretion of allantoin.

In the studies reported here, only negligible amounts of radioactivity were found in saliva samples following the intra-jugular injection of labelled allantoin and it was calculated that about 1% of the radioactivity injected into the sheep was transferred to the rumen via saliva. In support of this, the contribution of allantoin-C to blood and rumen fluid HCO_3^- (Table 1) was small and was about 5% of the net flux of allantoin through the blood pool. This also indicates that very little allantoin was secreted in saliva and that degradation of allantoin to carbon dioxide is quantitatively unimportant in sheep. The smaller transfer of allantoin-C to rumen fluid (*cf.* blood HCO_3^-; Table 8.1) is consistent with the findings of Surra *et al.* (1993) who concluded that recycling of purine derivatives to the rumen in saliva is negligible in sheep.

In the studies reported here the incomplete urinary recovery of an intra-jugular load of allantoin reported by Chen and co workers (1991) was further investigated. Assuming that urinary excretion is the only route of loss of plasma allantoin, the ratio of daily urinary allantoin excretion:allantoin net flux in plasma (over 24 h) will be unity. For this assumption to be valid, any variation in daily allantoin excretion must be paralleled by variation in allantoin net flux. For the two sheep used in this experiment, urinary excretion of allantoin accounted for only 83% (sheep 1) and 58% (sheep 2) of calculated allantoin net flux in plasma. The departure from unity may be partially explained by end point errors in urine collection contributing to variation in urinary allantoin excretion that was not matched by changes in net flux. Alternatively, the net flux during the period of measurement (effectively only the few hours after tracer injection) may not represent the true long-term mean flux rate. Nevertheless, the departure from unity observed in this study, provides further support for the suggestion that allantoin may be lost from the plasma via routes other than urinary excretion. However, the data presented here strongly indicate that, in the sheep, allantoin does not enter saliva in significant amounts and the degradation of allantoin to carbon dioxide is quantitatively unimportant.

CHAPTER 9

9. SENSITIVITY OF ALLANTOIN FLUX TO CHANGES IN ALLANTOIN SUPPLY

9.1 Introduction

The hypothesis that the microbial protein present in digesta leaving the rumen might be determined by reference to the nucleic acids present in the same digesta was proposed by McAllan and Smith (1971). This concept was based on the observation that, in ruminants, non-microbial nucleic acids have a short half-life in the rumen and therefore contribute minimally to the total nucleic acids in the digesta (Smith and McAllan 1970). Thus, the nucleic acids present in digesta leaving the rumen are predominately microbial in origin. Thus, if the ratio of nucleic acid-N:total-N, or more particularly, RNA-N:total-N of mixed rumen bacteria is known, the flow of microbial protein from the rumen may be quantified by analysis of the flow of nucleic acids in rumen digesta outflow.

More recently, several studies (Chen *et al.* 1990b; Balcells *et al.* 1991) have extended the hypothesis of McAllan and Smith (1971), and confirmed that purine absorption from the gut and urinary excretion of purine derivatives (predominately allantoin) are closely related. Consequently, urinary excretion of purine derivatives has provided a means for estimating the outflow of microbial nitrogen from the rumen without the need for surgical intervention in the animal. The latter being required by other existing methods.

Unfortunately, the recovery of purine derivatives in urine in response to infusions of nucleic material (Chen *et al.* 1990b; Balcells *et al.* 1991) and allantoin (Chen *et al.* 1991) has been variable and consistently less than unity. Saliva has been suggested as one route of non-renal loss (Chen *et al.* 1990c) but this has been disputed by Surra *et al.* (1993). In the studies reported in Chapter 8, only negligible amounts of radioactivity were found in saliva samples following the intra-jugular injection of labelled allantoin and it was calculated that about 1% of the radioactivity injected as allantoin into the sheep was transferred to the rumen via saliva. In the same experiment the transfer of allantoin-C to blood and rumen HCO_3^- amounted to about 5% of plasma allantoin net flux. Thus the conclusion made in Chapter 8 was that very little allantoin was secreted in saliva and that degradation of allantoin to carbon

dioxide is quantitatively unimportant in sheep. However, the latter may explain the fraction of the ^{14}C-allantoin dose that was not recovered in the urine of sheep (Chapter 8).

In the studies reported in this Chapter, the quantitative importance of non-renal loss of allantoin in sheep was further investigated. Accordingly, a continuous intra-jugular infusion of ^{14}C-allantoin was established in 2 sheep and the background (microbial origin) flux of allantoin was supplemented by an intra-jugular infusion of unlabelled allantoin (tracee). The difference between the background net flux of allantoin in plasma and the net flux after infusion of tracee was used as a measure of the recovery of unlabelled allantoin. Similar estimates derived from urine were used to indicate the fraction of infused unlabelled allantoin that was lost via the urine.

9.2 Materials and Methods

9.2.1 Animals and conditions

Two rumen-cannulated Merino cross Border Leicester ewes, 1 year old, weighing 30.4 and 31.2 kg live weight were used. These animals were fed hourly (automatic overhead feeder) equal portions of a ration of 510 g/d oaten chaff (*Avena sativa*; 89.3% DM and 1.2% N) plus 190 g/d lucerne chaff (*Medicago sativa*; 87.2% DM and 2.8% N) on an as fed basis. Animals were maintained on this feeding schedule for 14 days prior to the start of the experiment. Throughout the experiment, the animals were kept in metabolism crates with continuous lighting and unrestricted access to water. Indwelling jugular catheters and foley bladder catheters (5 ml, Paediatric Bard Limited, West Sussex, U.K) were placed in each sheep 24 and 18 h before continuous infusion of tracer began.

9.2.2 Experimental procedure

An intra-jugular infusion of isotopically labelled allantoin was maintained for 17 h. Details of the infusion protocol are as follows

1. A solution containing [4,5-^{14}C] allantoin (Amersham International plc; 38μCi/mg) and 15 mg of allantoin carrier in 180 ml of medical grade physiological saline was infused (0.101 ml/min) into the jugular catheter of each sheep for 17 h.
2. Medical grade physiological saline was infused (0.200 ml/min) into the jugular catheter of each sheep from 0–10 h.

3. A solution of unlabelled allantoir (21.2 mM, tracee) dissolved in medical grade physiological saline was infused (0.194 ml/min) into the jugular catheter of each sheep for the period from 10–17 h; this replaced the saline.

9.2.3 Sample collection and storage

Samples of urine, blood and rumen fluid were taken immediately before the start of the infusion period to determine the background levels of ^{14}C-allantoin and ^{14}C-HCO_3^-. Blood (12 ml) and urine (drained by the bladder catheter between sampling times) were sampled 2 hourly for 6 h, then hourly for 2 h and then half-hourly for a further 2 h. At this point (10 h after the start of infusion), unlabelled allantoin was included in the infusate, and blood and urine were then sampled hourly for the next 7 h. Rumen fluid (10 ml) was sampled at the time interval for blood and urine for the period from 8.5–17 h.

Blood samples (12 ml) were withdrawn from the jugular catheter and evenly divided (only blood sampled in the period from 8.5–17 h was divided). One fraction (6 ml) was used for the determination of the specific radioactivity of blood bicarbonate by the method of Leng and Leonard (1965). The remainder (6 or 12 ml) was placed into a heparinized tube, cooled on ice (30 min), centrifuged for 10 min at 3000 *g* and the plasma was then separated. Rumen fluid (10 ml) was withdrawn through nylon gauze and a portion (4 ml) was used to determine the specific radioactivity of rumen bicarbonate (Leng and Leonard 1965).

Bladder catheters were removed 11 h after the end of the intra-jugular infusion and urine was then collected for the next 18 h (composite sample) and then daily for the following 2 days. Urine was preserved with thymol (final concentration of thymol in urine *c.* 2.2mM) and subsampled (20 ml) at the end of each collection period. Faeces were collected daily and subsampled (10%). Plasma, urine and faecal samples were frozen immediately after collection or separation and then stored at –20° C until later analysis.

9.2.4 Tracer purity

The radiochemical purity of the [4 5-^{14}C] allantoin as determined by Amersham International plc was 96% using thin layer chromatography (TLC) and 96.3% using high performance liquid chromatography (HPLC). The purity of the radio-label was checked upon delivery by the method described in Chapter 8 and was found to be 96.1% which was in close agreement with the product specification and the 96.6% purity reported in Chapter 8.

9.2.5 Analytical procedures

Allantoin concentration and specific radioactivity in urine and plasma were determined according to the procedures described in Chapter 8.

9.2.6 Calculation of allantoin kinetics

The data were split into two distinct periods. The first period was taken from the start of the ^{14}C-allantoin infusion to the commencement of the infusion of unlabelled allantoin (0–10 h). The second period was during the infusion of unlabelled allantoin (10–17 h). The midpoint in time between successive urine collections was taken as the time indicative of the SR of that urine sample.

The specific radioactivity (SR) versus time data was fitted by an iterative procedure based on the Gauss-Newton algorithm that minimised the residual sum of squares (GraFit: Leatherbarrow 1992). Estimates of plateau SR (μCi/gC) were obtained from the curve fitting procedure and the net flux of allantoin was calculated by dividing the infusion rate of ^{14}C-allantoin (μCi/min) by the plateau SR.

9.3 Results

There were no feed refusals for either sheep at the end of the experiment (17 h after the start of infusions). However, sheep1 accumulated refusals from 11–12 h but had consumed these 2 h later. Recovery of ^{14}C-allantoin in urine (corrected for dose purity) collected over the 4 days following commencement of the ^{14}C-allantoin infusion accounted for 102 (sheep 1) and 92% (sheep 2) of that infused.

After the start of the ^{14}C-allantoin infusion (period 1), the specific radioactivity (SR) of allantoin in plasma and urine increased in a manner that was best described by a first order rate equation of the form:

$$SR_t = B(1-e^{-kt}) \qquad (1)$$

Where SR_t is the specific radioactivity of allantoin at time t, B (μCi/gC) and k (min^{-1}) are the plateau SR and rate constants respectively. The resulting parameter estimates for each sheep (Table 9.1) provided a good fit to the data (Figures 9.1, 9.2).

When unlabelled allantoin was infused (period 2), the SR of allantoin in plasma and urine declined exponentially until a lower plateau was reached some 2–3 h later. The decline in SR was best described by a single exponential plus offset algorithm.

$$SR_t = A_0 e^{-kt} + B \qquad (2)$$

Where A_0 (μCi/gC) is the intercept value and k and B are as previously described. The parameter estimates for each sheep (Table 9.1) provided a good fit to the data (Figures 9.1, 9.2).

The concentration of allantoin in plasma remained fairly constant during period 1. Intra-jugular infusion of unlabelled allantoin (0.196 mgC/min) increased the plasma concentration of allantoin in period 2 by 2 mgC/l (Table 9.2).

Table 9.1. Parameter estimates for allantoin SR versus time curves.

Parameter	sheep 1		sheep 2	
	estimate	s.e.	estimate	s.e.
Period 1				
first order rate				
plasma				
B	112	2.6	143	2.6
k	0.015	0.0027	0.015	0.0020
urine				
B	155	2.8	168	2.4
k	0.014	0.0011	0.014	0.0009
Period 2				
single exponential plus offset				
plasma				
B	80	1.8	97	1.9
k	0.025	0.0082	0.020	0.0058
urine				
B	87	1.2	89	2.8
k	0.014	0.0005	0.011	0.0008

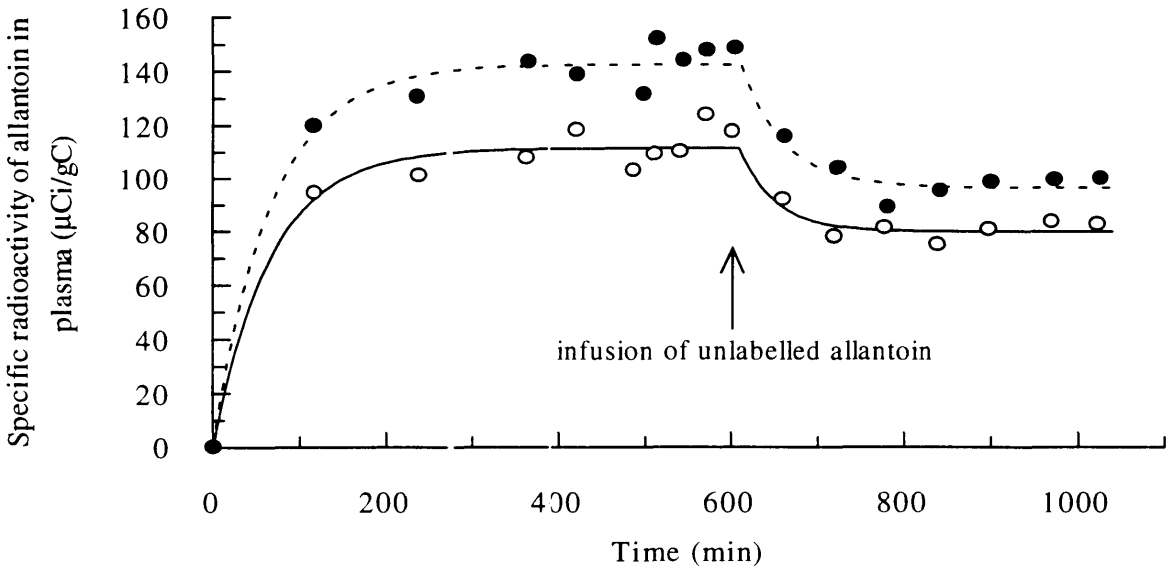

Figure 9.1. Specific radioactivity of allantoin in blood plasma over the experimental period. Data for sheep 1 (unfilled circle) fitted by dashed line for sheep 2 (filled circle) by a solid line.

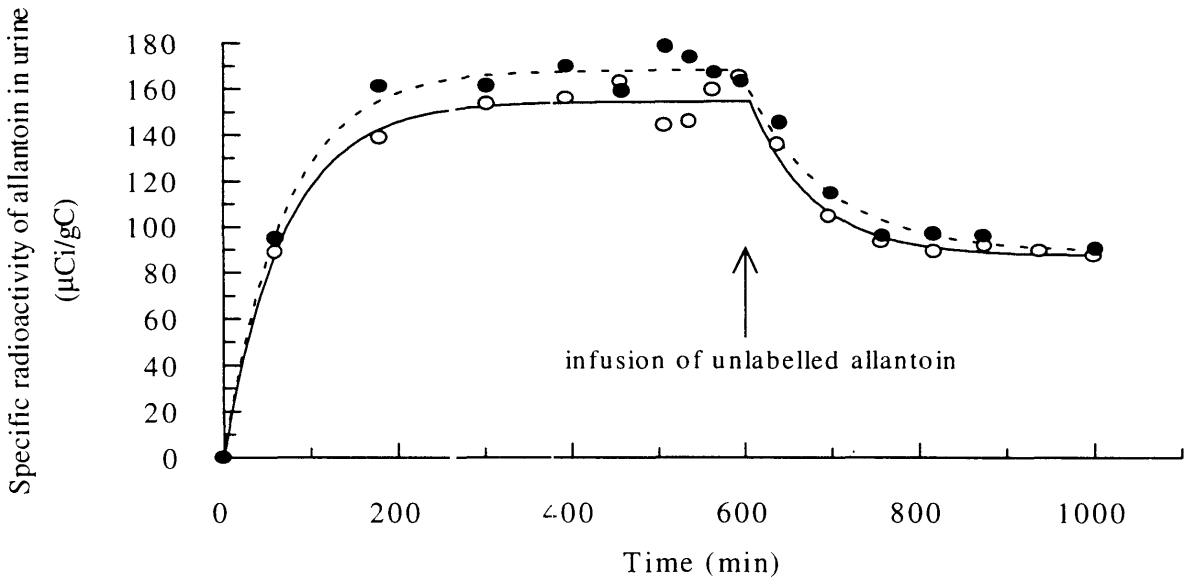

Figure 9.2. Specific radioactivity of allantoin in urine over the experimental period. Data for sheep 1 (unfilled circle) fitted by dashed line for sheep 2 (filled circle) by a solid line.

Table 9.2. The concentration of allantoin in blood plasma for period 1 and for the final 2 h of period 2.

Parameter	sheep 1			sheep 2		
	mean	s.d.	c.v. (%)	mean	s.d.	c.v. (%)
allantoin in plasma (mg C/l)						
Period 1 (0–10 h)	3.1	0.23	7.4	3.7	0.18	5.0
Period 2 (15–17 h)[A]	5.1	0.06	1.2	5.7	0.27	4.7

[A] Calculations only include data from 15–17 h of period 2 in order to ensure that the allantoin in blood plasma was equilibrated with the infusate.

The estimates of net flux of allantoin were similar for both sheep during period 1 but differed depending on whether they were derived from blood or urine (Table 9.3). Net flux of allantoin increased in period 2 and the estimates made from samples of plasma or urine became similar. The recovery of infused unlabelled allantoin (tracee) was calculated as the difference between the estimates of allantoin net flux (mgC/min) made in periods 1 and 2 expressed as a fraction of the tracee infusion rate. Based on estimates of net flux in plasma and urine, the recovery of tracee averaged 0.55 and 0.83 respectively. Transfer of allantoin-C to either the blood or rumen fluid HCO_3^- pool was insignificant (Table 9.3) and similar in magnitude to that reported in the previous experiment (see Chapter 8).

9.4 Discussion

Upon termination of the intra-jugular ^{14}C-allantoin infusion, urinary excretion of ^{14}C-allantoin had accounted for 90 and 78% of the ^{14}C-allantoin infused into sheep 1 and 2 respectively. Excretion of ^{14}C-allantoin in urine continued over the next 66h and at that time had accounted for 102 and 92% of that infused. Prolonged excretion of ^{14}C-allantoin in urine was also observed in the studies reported in Chapter 8. In those studies, *c.* 85% of the ^{14}C-allantoin given in a single intra-jugular injection was excreted in urine after 24 h. Excretion of ^{14}C-allantoin in urine continued over the next 4 days and at that time had accounted for *c.* 94% of that injected.

In sheep, the pool of allantoin in plasma (33–43 mg C) is relatively small in relation to the total flux of allantoin (see Chapter 8). The pool turns over approximately every hour (see Chapter 8), and a rapid passage of allantoin from blood to urine would be expected given the limited capacity for tubular reabsorption of allantoin in sheep (Chen *et al.* 1991). However,

the data from this study and that reported in Chapter 8 indicate that, in sheep, excretion of ^{14}C-allantoin in urine continues for a considerable period following its intra-jugular administration.

One possible explanation for the prolonged excretion of ^{14}C-allantoin in urine is that allantoin in plasma is able to exchange with another, much larger pool, with has a slow turnover rate. Labelled allantoin entering such a pool would have a low probability of reappearing from that pool in any finite time period. Body water would be the prime candidate for such a pool. This notion is supported by the studies reported in Chapter 8 which indicated that the SR versus time data for blood allantoin appeared to contain a third component.

Table 9.3. Kinetic data derived from blood plasma and urine of 2 sheep following intra-jugular continuous infusion of ^{14}C-allantoin (0–17 h) and unlabelled allantoin (10–17 h).

Parameter	sheep 1	sheep 2
PLASMA		
Net flux (mg C/min)		
0–10 h	0.257	0.237
10–17 h (tracee infusion)	0.357	0.350
Allantoin infusion (mg C/min)	0.195	0.196
Recovery (fraction of tracee infusion rate)	0.51	0.58
Net flux (mgC/kg intake)	528	487
Transfer quotient x 10^5		
Fraction of carbon derived from allantoin		
in blood HCO_3^-	4.86	5.73
in rumen HCO_3^-	3.17	4.69
URINE		
Net flux (mg C/min)		
0–10 h	0.185	0.201
10–17 h (tracee infusion)	0.328	0.380
Allantoin infusion (mg C/min)	0.195	0.196
Recovery (fraction of tracee infusion rate)	0.73	0.92
Net flux (mg C/kg intake)	381	413

Plasma concentrations of allantoin averaged 66 and 77 μM for the period prior to infusion of unlabelled allantoin. These values are similar to those reported for sheep (Chen *et al.* 1990b; Balcells *et al.* 1992a) but are lower than those reported in Chapter 8. However, because the blood concentration of allantoin is dependent on both the level of feed intake and on the protein status of the body (Chen *et al.* 1992b), comparisons between experiments may not be useful.

The plateau SR of allantoin estimated from urine, was greater than that estimated from plasma (Table 9.1) during period 1. Consequently during period 1, allantoin net flux (Table 9.3) estimated from urine was less than that estimated from blood plasma. The greater SR of allantoin in urine relative to blood plasma may have resulted from a greater proportion of tracer allantoin being filtered by the kidneys relative to tracee. Alternatively, tubular reabsorption, albeit limited (Chen *et al.* 1991), favoured tracee molecules. The evidence for either of these possibilities is scant and is dismissed from further discussion. An alternative explanation for the difference in plateau SR between blood plasma and urine is that, in this study, urine provided an integrated sample and thus absorbed any short term perturbations in allantoin concentration that may be evidenced in plasma. As a consequence of this, estimates of flux rates obtained from urine are likely to be more representative of the true allantoin flux rates.

Assuming that the sheep were in steady state with regards to allantoin metabolism, the difference between allantoin net flux in periods 1 and 2 represented the rate at which unlabelled allantoin was infused into the animal. The recovery of infused tracee estimated from blood plasma and urine was 51 and 73% for sheep 1 and 58 and 92% for sheep 2. The lower recovery of infused tracee estimated from plasma, may potentially be accounted for by cumulative errors in estimates of allantoin net flux associated with short-term perturbations in the concentration of allantoin in plasma. The lower recovery of tracee in sheep 1 was probably a direct result of a short term inappetance during period 2. Although this animal consumed its entire ration by the end of the experimental period, it is apparent that the period of inappetance interrupted some aspect of allantoin metabolism. This interruption may have resulted from a reduction in the rumen outflow of microbial nucleic acids or from a temporary change in the digestion and absorption of microbial nucleic acids.

The transfer of allantoin-C to HCO_3^--C in blood and rumen fluid was negligible and was of a similar magnitude to that reported in Chapter 8. This provides further confirmation that degradation of allantoin to carbon dioxide is quantitatively unimportant in the sheep.