

Excretion of Purine Derivatives by Holstein Cows Abomasally Infused with Incremental Amounts of Purines¹

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ABSTRACT

Five multiparous, ruminally cannulated Holstein cows (two lactating and three dry) weighing ($\bar{X} \pm$ SD) 667 ± 35 kg were used to study the effect of abomasal purine infusion on the excretion of purine derivatives. Cows were fed corn silage four times daily at 90% of ad libitum intake ($\bar{X} = 9.16$ kg of dry matter/d). Purines were infused into the abomasum as brewer's yeast suspensions in five incremental amounts (0 to 380 mmol/d) during five experimental periods according to a 5×5 Latin square design. Periods were 7 d; purine infusions were conducted during the last 4 d, and urine was collected during the last 3 d of each period. Ruminal purine outflow in all cows was measured during an experimental period immediately preceding and immediately following the five infusion periods and in each cow during the 0-mmol/d infusion period of the experiment. The relationship between total (milk plus urine) daily excretion of purine derivatives (allantoin plus uric acid) and total (abomasal infusion plus ruminal outflow) daily purine flow was quantified by linear regression analysis and was described by the relationship: $Y = 0.856X + 103$ ($r^2 = 0.93$). The slope (0.856) indicated that 86% of purines that reached the omasum were excreted as purine derivatives. In the two lactating cows, urinary purine derivatives accounted for 98.4% of the total purine derivatives that were excreted. Ruminal flow of microbial CP can be estimated from the CP:purine ratio of ruminal microorganisms and the excretion of purine derivatives.

(**Key words:** purine derivatives, purines, microbial protein synthesis, urinary excretion)

Abbreviation key: LDG = liquid digesta, MCP = microbial CP, PD = purine derivatives, SDG = solid digesta, WRC = whole ruminal contents.

INTRODUCTION

Standard in vivo methods to estimate the supply of ruminal microbial protein use exogenous (e.g., ¹⁵N ammonium salts) or endogenous (e.g., purines) microbial markers (2). The quotient of the marker to CP ratio in digesta divided by the marker to CP ratio in ruminal microbes describes the fraction of CP of microbial origin in digesta. Multiplying this fraction by the flow of CP in digesta yields an estimate of the ruminal flow of microbial CP (MCP). These procedures require the use of cannulas in the abomasum or small intestine, which is not desirable for the health of the animal, and the estimation of digesta flow, which is laborious and imprecise.

Topps and Elliott (14) reported a correlation between urinary excretion of allantoin and the concentration of purines in the rumen of sheep, suggesting that allantoin excretion is an index of the ruminal flow of microbial protein. Quantitative relationships between urinary excretion of purine derivatives (PD) and purine flow rates have been observed for sheep (1, 4) and cattle (16). Giesecke et al. (8) reported linear relationships between allantoin excretion in urine ($r^2 = 0.71$) and secretion in milk ($r^2 = 0.64$) and NE_L intake in Holstein cows, which is consistent with the concept that ruminal microbial protein synthesis is a linear function of NE_L intake (9). Giesecke et al. (8) further reported that allantoin excretion via the mammary gland accounted for a mean of 1.6% of total (renal plus mammary) excretion.

Data relating intestinal purine flow and PD excretion in cattle (16) were obtained from only two 300-kg Friesian steers that were nourished by total intragastric infusion (maximum exogenous purine

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supply = 171 mmol/d). Extrapolation of these data to lactating Holstein cows, which have substantially greater purine flows, seemed tenuous. Therefore, we determined the relationship between intestinal purine flow and urinary excretion of PD using five cows that were maintained in a normal nutritional state. The BW and purine flows more nearly approximated the flows in lactating Holstein cows. This trial required the development of a rapid analytical procedure to determine the purine content of microbial cells.

MATERIALS AND METHODS

Cows and Feeding

Five multiparous Holstein cows (two lactating and three dry) weighing ($\bar{X} \pm \text{SD}$) 667 ± 35 kg and were each fitted with a 10-cm ruminal cannula (Bar Diamond Inc., Parma, ID). Cows were fed a diet consisting of corn silage (37.5% DM) plus dicalcium phosphate (190 g/d). The silage contained (DM basis) 37.4% NDF and 6.7% CP and was fed in four equal portions daily at 0400, 1000, 1600, and 2200 h; the daily supplement of dicalcium phosphate was top-dressed on the 1000-h feeding. Cows were fed at 90% of ad libitum intake ($\bar{X} = 9.16$ kg of DM/d) to avoid orts. Daily silage samples were stored at -20°C ; weekly composites were analyzed for DM (60°C for 48 h), ground through a 1-mm screen, and analyzed for N (Carlo Erba NA 1500 N analyzer; Carlo Erba Instruments, Milan, Italy), NDF, and ADF (12). Cows had free access to water and trace-mineralized salt blocks throughout the trial. Body weight was recorded every 14 d. Lactating cows were milked twice daily at 0730 and 1830 h and were dried off immediately following the completion of period 5.

Experimental Treatments

Five equally spaced infusions of purines in the form of brewer's yeast (Labudde Feed Co., Grafton, WI) were administered during five experimental periods according to a Latin square design. Two additional periods were used, one immediately preceding the infusions and one following the final infusion period to allow for the determination of purine outflow from the rumen. All experimental periods were 7 d in duration. Suspensions of brewer's yeast containing 13% (wt/vol) DM were mixed in 15-L pails using an electric drill fitted with a mixing paddle, and the mixtures were stored at 5°C until infusion. During infusions, suspensions were mixed continuously on magnetic stir plates and were delivered to the cows

using a multichannel peristaltic pump (Pump III; Technicon Instruments, Tarrytown, NY). Suspensions were infused via vinyl tubing (1.2 m length, 3.18 mm i.d.) that passed through a small opening in the ruminal cannula plug, through the omasum, and into the abomasum. The tubing was secured in the abomasum with a Plastisol® flange (6 mm thickness, 10 cm diameter; Auburn Plastics, Chicago, IL) cemented 20 cm from the distal end of the tube. Placement of the tubing within the abomasum was confirmed immediately before and after each infusion. Infusions (3.2 to 20.1 L/d of solution; 62 to 380 mmol/d of purines) were initiated at 1800 h on d 3 of the experimental periods and were maintained for 96 h. Two cows had to be removed from the experiment prematurely (following completion of periods 5 and 6) as they neared parturition. Thus, only 32 of the 35 intended combinations of cow and period were completed.

Marker Dosing and Ruminal Sampling

Solid markers (90 g of Cr-NDF prepared from corn silage) and liquid markers (50 g of Co-EDTA dissolved in 500 ml of deionized water) were prepared according to the methods of Udén et al. (15) and were pulse-dosed into the rumen at 0700 h on d 4 of periods 1 and 7. Whole ruminal contents (**WRC**) were sampled at 0, 2, 4, 7, 11, 14, 17, and 24 h postdosing. Duplicate samples of WRC were dried (60°C for 48 h) and ground through a 1-mm screen. Samples were analyzed for DM (100°C for 24 h), ashed at 500°C for 16 h, and solubilized in 10 ml of concentrated HCl and a solution of 0.6% (wt/wt) LiOH to a final weight of 100 g. Concentrations of Co and Cr were analyzed by direct current emission spectroscopy (5). One liter of WRC also was obtained and preserved with formalin (25 ml of formalin/L of WRC) to determine concentrations of purines in digesta at 0700, 2100, and 2400 h, representing 3, 5, and 2 h postfeeding, respectively. Samples were squeezed through two layers of cheesecloth, the filtrates were centrifuged ($15,000 \times g$ at 4°C for 20 min), and the pellets were designated as liquid digesta (**LDG**). Particles that were retained by the cheesecloth were washed twice with a total of 1 L of McDougall's buffer, and the residue was designated as solid digesta (**SDG**). Both SDG and LDG were dried (60°C for 48 h) and ground with a coffee grinder (LDG) or through a 1-mm screen (SDG) and analyzed for purine content by HPLC.

Also during periods 1 and 7, total ruminal evacuations were conducted to obtain bacterial pellets and pool size estimates of SDG and LDG. The rumen of

each cow was emptied at 0800 h on d 2, the weight and volume of WRC were recorded, and duplicate 1-L samples were preserved with formalin as described previously. From each sample, 100 g were used to determine DM (60°C for 48 h), and the remainder was used for isolation of a bacterial pellet. The DM pool of LDG (in grams) was computed as the product of the volume of WRC and the mean quantity of DM in LDG contained in WRC. The DM pool of SDG (in grams) was then determined as total DM in WRC minus the DM in LDG. To obtain bacterial pellets, the remaining WRC were squeezed through two layers of cheesecloth and then were washed three times with a total of 3 L of McDougall's buffer. The ruminal fluid plus buffer wash was centrifuged at $550 \times g$ at 4°C for 10 min. The supernatant was centrifuged at $15,000 \times g$ at 4°C for 20 min, and the resultant bacterial pellets were dried at 60°C for 48 h. Bacterial samples were ground in a coffee grinder and analyzed for N (Carlo Erba NA 1500 N analyzer) and purines by HPLC. Procedures for marker dosing, ruminal sampling, and evacuation also were performed on the cow that did not receive brewer's yeast during the Latin square portion of the experiment (periods 2 through 6). Thus, three observations per cow were obtained for ruminal outflow of purines from microbial growth.

Urine and Milk Collection and Analysis

Total urine collections were made using indwelling Foley catheters (24 French, 75-ml balloons; C. R. Bard, Murray Hill, NJ), which were inserted at 1800 h on d 4 of each experimental period (24 h following initiation of brewer's yeast infusions). Cows received 25 ml/d of penicillin (300,000 U/ml; Butler Co., Columbus, OH) intramuscularly during the 3 d that the catheters were in place. Daily urine output was measured for 3 d. Fresh containers with 500 ml of 1.5N H₂SO₄ were attached to each cow at 0700 and 1800 h of each day; the final urinary pH was <3. After the volume of excreted urine was recorded, acidified urine was mixed, and duplicate 20-ml samples were taken, diluted to 100 ml with tap water, and stored at -20°C. After milk volume was recorded, milk was mixed, and duplicate 90-ml samples were preserved with 10 ml of 50% (wt/vol) TCA and stored at -20°C. Milk and urine samples were thawed, and milk was centrifuged ($15,000 \times g$ for 15 min) prior to analysis for uric acid and allantoin (7) and creatinine [urine only; (10)]. Total PD were calculated as the sum of allantoin plus uric acid.

Purine Flow and Excretion of PD

Passage rates of ruminal SDG and LDG were estimated by the fractional disappearance rates of Cr-NDF and Co-EDTA from the rumen. For each element, the fractional disappearance rate was determined by fitting the single exponential equation

$$X(t) = X(0) \times e^{-k_p t}$$

where $X(0)$ and $X(t)$ = concentrations of Cr or Co in the DM of the WRC at time 0 and sampling time t (in hours), respectively, and k_p = fitted passage rate (per hour). Parameter estimates were obtained by nonlinear regression using Marquardt's compromise implemented with the nonlinear regression procedure of SAS (13). The ruminal outflow of purines (millimoles per day) was calculated as ruminal outflow of purines (millimoles per day) = (grams of SP) \times (k_S) \times (24 h/d) \times (millimoles of purines per gram of SDG) + (grams of LP) \times (k_L) \times (24 h/d) \times (millimoles of purines per gram of LDG), where k_S = fractional disappearance rate of Cr-NDF, and k_L = fractional disappearance rate of Co-EDTA.

The mean value of the ruminal outflow of purines was computed from the three observations made for each cow. Total purine flow was the sum of the ruminal outflow of purines plus infused purines. Mean daily excretion of PD was computed, and the relationships between the excretion of total PD, urinary PD, urinary allantoin, and the ratios of urinary PD to creatinine and allantoin to creatinine versus mean total daily purine flows were evaluated. The experimental design (cow \times period means) required 20 observations for analysis, but only 19 observations were made because data were missing during the last infusion period for 1 cow because of calving.

Purine Analyses

Samples (100 mg of brewer's yeast or bacteria; 200 mg of SDG or LDG) were hydrolyzed in 17- \times 100-mm polypropylene tubes in 2 ml of 70% perchloric acid for 1 h at 100°C to liberate the purine bases. Aliquots of 250 μ l containing 1 mg (yeast or bacteria) or 0.1 mg (SDG or LDG) of allopurinol were added to samples as an internal standard, followed by 5 ml of borate buffer (33.3 g of sodium borate decahydrate plus 5.33 g of NaOH/L) and 2 ml of 12.4N KOH to adjust the pH to 10 and to precipitate the perchloric acid as potassium perchlorate. Samples were centrifuged (20 min at $30,000 \times g$), and 1 ml of supernatant was added to 4 ml of borate buffer. Samples were filtered (0.2- μ m membrane) and stored at 5°C prior to injection into an HPLC system (Gilson Medical

Electronics, Madison, WI) that consisted of dual pumps (model 302), a manometric module (model 802B), a dynamic mixer (model 811), and a variable wavelength detector (model 116; set to 254 nm). A 3.9- × 300-mm C-18 reverse-phase column (NovaPak®; Waters Corp., Millford, MA) maintained at 30°C with a column heater (CH-30; FiaTron Laboratory Systems Inc., Oconomowoc, WI) was used for the separations. Solvent A was a mixture (vol/vol) of 98.8% 10 mM potassium phosphate and 1.2% triethylamine adjusted to pH 6.5, and solvent B was a mixture (vol/vol) of 60% acetonitrile and 40% water. The two solvents were filtered through 0.45- μ m membranes and pumped in constant proportions of 0.95 of solvent A and 0.05 of solvent B at 1.0 ml/min. Purines were quantified by peak integration (Gilson HPLC system controller software 714; Gilson Medical Electronics). Peak purity was assessed by comparing the UV scans (220 to 350 nm) of eluant peaks corresponding to adenine and guanine in samples to scans of eluant peaks from the injection of respective pure compounds. All compounds were eluted within 6 min.

Hydrolytic efficiency was assessed from the ratios of chromatographic response from hydrolyzing incremental amounts (0 to 30 μ mol) of the nucleosides adenosine and guanosine to equimolar amounts of adenine and guanine, respectively. Sample matrix effects were assessed by comparing chromatographic response of adenosine and guanosine that had been processed in the presence of 100 mg of brewer's yeast or ruminal bacteria (four samples each, chosen at random) to the response of adenine and guanine, respectively.

Statistical Analyses

Infusion experiment. Relationships between the excretion of PD and purine flow were assessed using the general linear models procedure of SAS (13). First, data were fitted to a model that included the fixed effects of cow and period as well as the continuous effect of total purine flow (ANOVA model, appropriate to the Latin square design). This model accounted for all systematic variation and allowed for the assessment of the random (biological) variation in the relationship between the excretion of PD and purine flow in digesta. Second, a simple linear regression model containing only the effect of total purine flow was evaluated. This model was appropriate to the generalized predictive purposes of the experiment. Third, a quadratic term was added to the model to test for curvilinearity.

HPLC analyses. Responses to incremental amounts of purine base or nucleoside were quantified using simple linear regression. For both adenine and guanine, one estimate of slope and intercept was generated for each sample type (purine base, nucleoside, and four samples each of bacteria or yeast), resulting in 20 parameter estimates per data file. Parameter estimates were then forced to share a common value within and among sample types (purine base, nucleoside, bacterium, or yeast), and the effects of these constraints on fit were tested using the extra sum of squares principle (6). When a constraint had no effect on the fit of the model (i.e., when the F had $P > 0.05$), the parameter estimates did not differ, and the shared values are reported. This process was continued until the data files could be

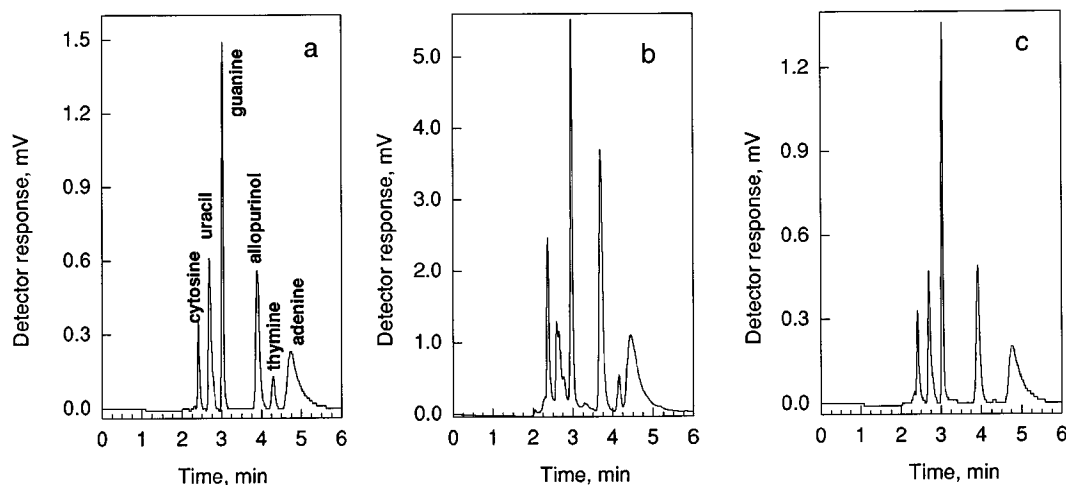


Figure 1. Chromatograms of standard mixture of nucleic acid bases (a), bacterial hydrolysate (b), and yeast hydrolysate (c).

described with the minimal number of parameters. When slopes differed, adenine or guanine recoveries were assumed to be affected by sample type.

RESULTS AND DISCUSSION

HPLC Analyses

Separation of a standard solution of nucleic acid bases as well as sample hydrolysates is presented in Figure 1. Large peaks corresponding to the retention times of each base are present in both bacterial (Figure 1b) and yeast (Figure 1c) hydrolysates, but only a small peak corresponds to thymine in yeast hydrolysate. To confirm the identity of adenine and guanine, HPLC eluants corresponding to these peaks were collected from three samples each of bacteria and yeast and two of standards, and the UV spectra were compared (Figure 2). Spectra obtained from the sample hydrolysates closely paralleled those obtained

from the corresponding standard compounds, confirming the peak identity based on retention time.

Hydrolysis and chromatography of adenosine resulted in parameter estimates that were similar to those obtained for adenine (Figure 3a), indicating complete hydrolysis of adenosine to adenine. However, when adenosine was hydrolyzed in the presence of bacteria (Figure 3b) or yeast (Figure 3c), the slope that was obtained from regression of the peak area on nucleoside concentration (4.02) was only 89.1% of that obtained for standard solutions (4.51), indicating significant effects of sample matrix on adenine recovery. Hydrolysis of guanosine to guanine was only 91.1% complete (Figure 4a). Moreover, hydrolysis and chromatography of guanosine in the presence of bacteria (Figure 4b) or yeast (Figure 4c) resulted in only 84.5% recovery as guanine. These data underscored the need to determine the completeness of purine recovery during hydrolysis and HPLC analysis. Recoveries did not differ within or among

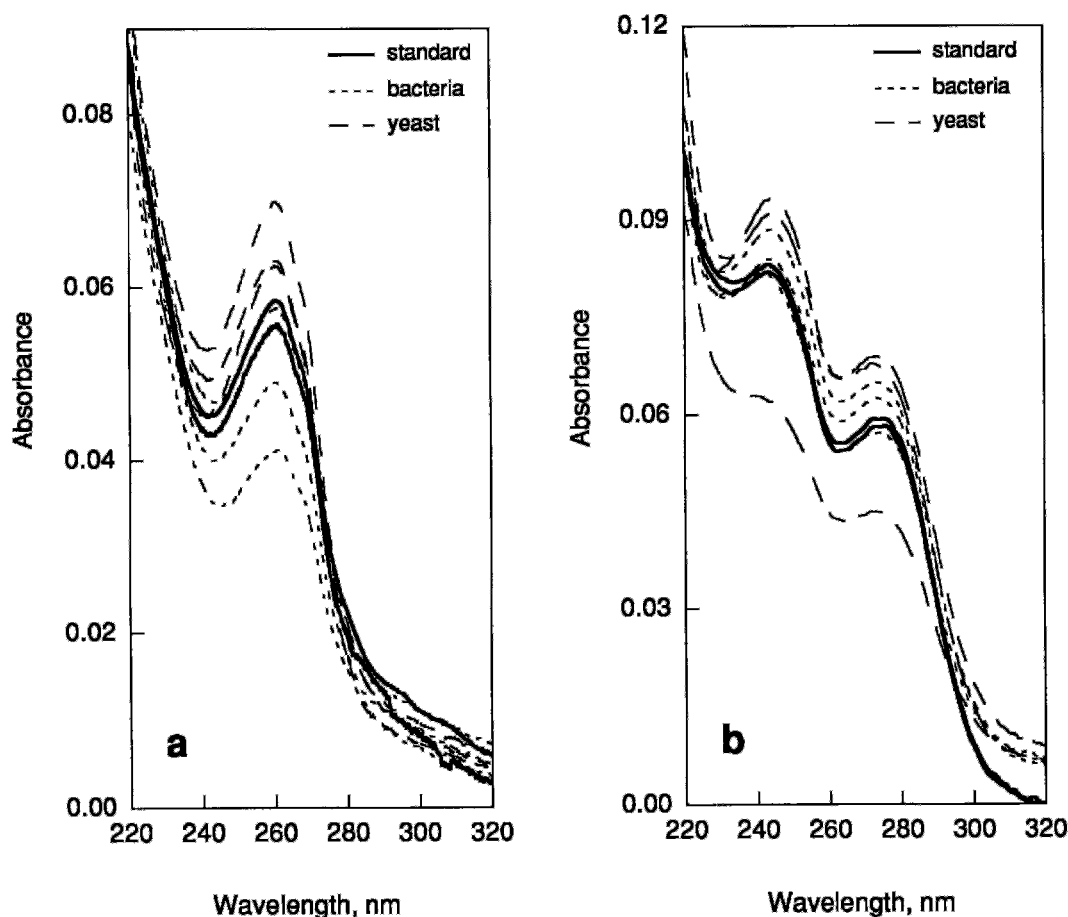


Figure 2. Ultraviolet spectra of HPLC eluants corresponding to adenine (a) and guanine (b) peaks in standard solutions (—), bacterial hydrolysates (---), and yeast hydrolysates (— — —).

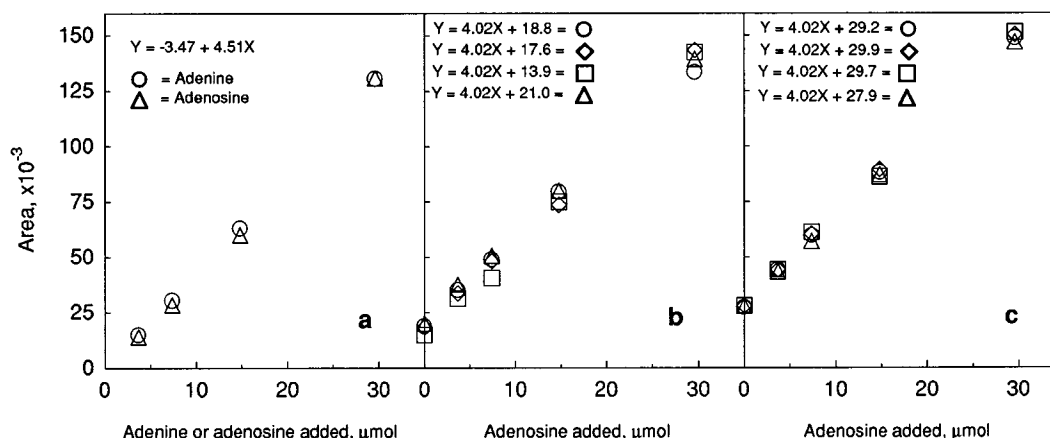


Figure 3. Chromatographic response to adenine in standard solutions (a), bacterial hydrolysates (b), and yeast hydrolysates (c). The regression equations shown on the graphs were generated using parameter sharing. Symbols in panels b and c correspond to replicate samples.

bacterial or yeast samples (Figures 3 and 4); thus, the same recovery factors for adenine and guanine were applied for both bacteria and yeast samples.

Excretion of PD

In the absence of infusions, the mean ($\pm\text{SD}$) flow of intestinal purines was equal to 91.2 ± 26.0 mmol/d or 9.65 ± 2.18 mmol/kg of DMI, which were the purine outflows arising from ruminal microbial growth. The relationship between total excretion of PD and purine flow was described by the ANOVA model with an r^2 of 0.97, indicating that random (biological) variation accounted for only 3% of the total variation. This result is in agreement with results of others (1, 4, 16)

who observed precise ($r^2 \geq 0.96$) relationships between the excretion of PD and purine flow in the intestines. The effects of cow and period were significant ($P < 0.05$) only for urinary excretion of creatinine. However, expression of the excretion of creatinine on the basis of BW (micromoles per kilogram of BW per day) removed the effect of cow ($P = 0.76$), indicating that creatinine was excreted at a constant rate relative to BW (10). This experiment was conducted to evaluate the prediction of purine flow in digesta from the excretion of PD, and, for this purpose, the effects of period and cow were immaterial. Therefore, only the parameter estimates obtained from the simple linear regression model are presented (Table 1).

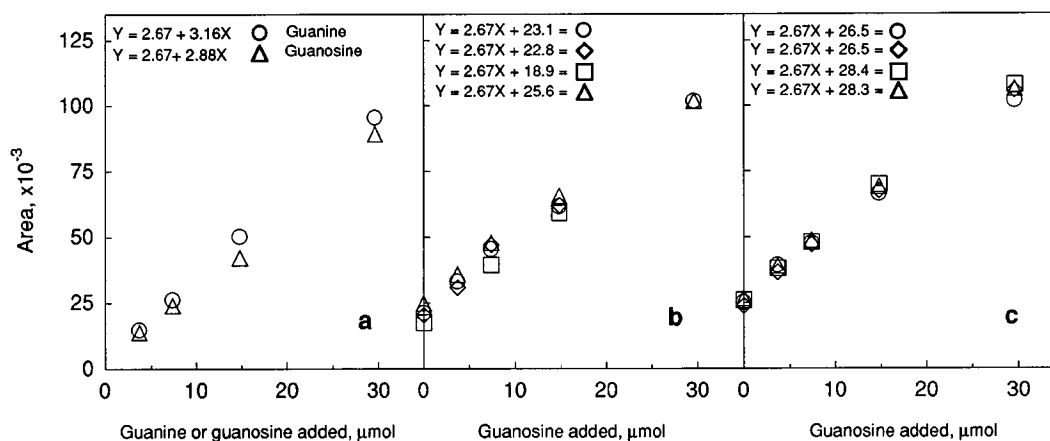


Figure 4. Chromatographic response to guanine in standard solutions (a), bacterial hydrolysates (b), and yeast hydrolysates (c). The regression equations shown on the graphs were generated using parameter sharing. Symbols in panels b and c correspond to replicate samples.

TABLE 1. Parameter estimates obtained from simple linear regression of urinary metabolite excretion on total daily flow of purines in the intestine (millimoles per day).¹

Dependent variable	Model r^2	Intercept			Slope		
		Estimate	SE	$P > t^2$	Estimate	SE	$P > t^2$
Total excretion of PD, mmol/d	0.93	103	19	<0.001	0.856	0.057	<0.001
Urinary excretion of PD, mmol/d	0.92	101	20	<0.001	0.860	0.060	<0.001
Urinary allantoin excretion, mmol/d	0.94	93.5	16.2	<0.001	0.772	0.048	<0.001
Urinary creatinine excretion, mmol/d	0.25	118	9	<0.001	0.066	0.028	0.03
Urinary allantoin:creatinine, mol/mol	0.88	1.03	0.13	<0.001	0.00438	0.00039	<0.001
Allantoin excretion, % of total PD	0.01	91.1	1.1	<0.001	-0.0015	0.00034	0.67

¹PD = Purine derivatives (allantoin plus uric acid).

²Probability that parameter estimate is different from 0.

In previous reports (1, 4, 16), a curvilinear relationship between the excretion of PD and purine flow in the intestines was observed at low purine flows. However, in our study, the smallest purine flow (150 $\mu\text{mol/kg}$ of BW per d) was greater than the maximum flow ($\sim 125 \mu\text{mol/kg}$ of BW per d) below which curvilinearity was observed. Therefore, it is not surprising that addition of a quadratic term was not significant ($P > 0.80$) for any of the responses tested. The fitted slopes obtained in previous experiments indicated 77 to 84% recoveries of intestinal purines as urinary PD. The 95% confidence interval (0.735 to 0.977) of the slope (0.856) that was obtained in this study encompassed those estimates, indicating a similar recovery of purines as PD. The goal of the present experiment was to predict purine flow and, hence, ruminal MCP flow from the excretion of PD. Computation of the 95% fiducial limits (6) of the predicted purine flow corresponding to the mean observed excretion of PD yielded an error rate of 5.0% (318 ± 16 mmol/d of purines), suggesting sufficient precision for quantitative prediction.

Excretion of both uric acid and allantoin increased ($P < 0.001$; Table 1) in response to purine flow. The proportion of PD excreted as allantoin was not affected ($P = 0.67$; Table 1) by purine flow, averaging 90.6%. Verbic et al. (16) reported that allantoin accounted for 85.5% of urinary PD excreted by steers and that this proportion did not change in response to purine flow. In two reports with sheep, excretion of the nonallantoin PD (hypoxanthine, xanthine, and uric acid) either did not (1) or did (4) alter in response to purine flow; the proportion of PD excreted as allantoin increased in response to purine flow in both of those trials.

Alterations in the excretion of total PD (allantoin plus uric acid) in response to purine flow were largely reflections of allantoin excretion (Figure 5). Also, the

ratio of urinary allantoin to urinary creatinine responded linearly to purine flow (Table 1; Figure 5). Chen et al. (3) found the ratio of urinary allantoin to urinary creatinine to be stable over time after feeding (CV among sampling times = 2.6%) in steers fed once daily, suggesting that this ratio is an index of PD excretion and, hence, MCP supply. Allantoin concentrations in plasma and milk were each correlated

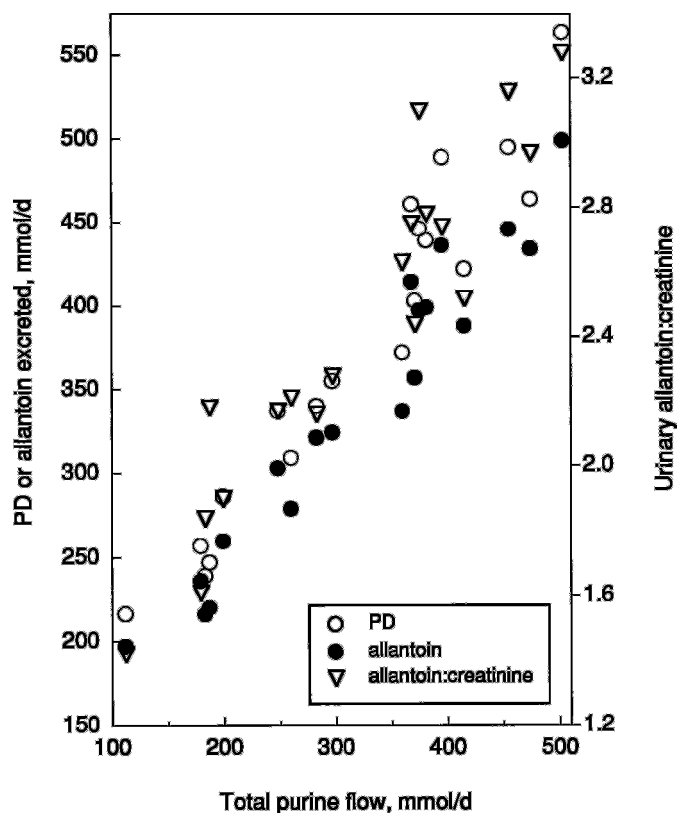


Figure 5. Relationship of intestinal purine flow to excretion of total purine derivatives (PD) and allantoin and to the ratio of urinary allantoin to creatinine.

with urinary excretion of allantoin (8), suggesting that milk allantoin also may be a useful index of MCP flow. Only two of the five cows in the trial were lactating; milk yield ($n = 8$) averaged 6.1 L/d. Under these circumstances, the relationship between secretion of allantoin in milk and excretion in urine ($r^2 = 0.18$) suggested that the secretion of allantoin in milk might not be responsive to purine flow in the intestines, which is consistent with the more extensive observations of others (R. J. Dewhurst, 1995, personal communication).

Only limited data are available for direct comparison of the excretion of PD to standard methods of estimating MCP flow. Pérez et al. (11) estimated MCP flow using PD excretion and ^{15}N as a microbial marker in ewes fed incremental amounts of barley. Flow of MCP increased linearly as estimated by both methods in response to barley supplementation, except at the highest barley concentration when the excretion of PD and, hence, predicted MCP flow, decreased. Pérez et al. (11) suggested that the use of the excretion of PD might be unreliable in diets containing a high proportion of concentrates, but no explanation for this effect was offered.

Under the conditions employed in this experiment, MCP flow may be estimated from the excretion of PD using the following equation: MCP (grams per day) = (grams of MCP/millimole of purines) \times [(millimoles of PD excreted per day - 103)/0.856]. However, further comparisons of this method using the excretion of PD to standard approaches of estimating MCP flow are needed to confirm this relationship.

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