

## Determination of Intestinal Disappearance of Lysine and Methionine Derived from Ruminally Protected Lysine and Methionine in Holstein Heifers

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**ABSTRACT :** In order to estimate the availability of ruminally-protected Lys (RPLys) and Met (RPMet), quantitative changes of free Lys and Met in the total abomasal outflow and feces were determined in three Holstein heifers with a T-shaped duodenal cannula. RPLys was prepared by coating Lys with fat and RPMet with a pH-sensitive polymer. RPLys and RPMet containing 30 g of each amino acid were supplied together to heifers, and total abomasal outflow was collected hourly from the T-shaped duodenal cannula for a 48 h period. Collected abomasal outflows were fractionated into liquid and solid phases to measure free Lys and Met concentration. At 2 wk after total abomasal outflow sampling, heifers were supplied RPLys and RPMet together again, and total feces were collected every 12 h for a 3 d period to estimate excreted RPLys and RPMet in feces. The amounts of Lys and Met recovered from the liquid fractions of abomasal outflow were estimated to be  $23.9\pm 8.3\%$  and  $68.6\pm 3.6\%$  ( $p=0.008$ ) of RPLys and RPMet ingested, respectively. Final intestinal disappearances of Lys and Met were estimated to be  $49.5\pm 2.6\%$  and  $78.2\pm 6.5\%$  ( $p=0.015$ ) of ingested RPLys and RPMet, respectively. (*Asian-Aust. J. Anim. Sci.* 2003. Vol 16, No. 4 : 549-554)

**Key Words :** Ruminally Protected Lysine, Ruminally Protected Met, Heifer, Intestinal Disappearance

### INTRODUCTION

Recent advances in amino acid nutrition for lactating dairy cattle have interested the feed industry in the production and usage of ruminally-protected amino acids (RPAA). RPAA are designed as crystal forms of amino acids to escape degradation by rumen microorganisms and to deliver eligible amino acids for effective absorption in the small intestine, and are used for improvement of animal production. Several technological approaches have focused specifically on the development of ruminally-protected Lys (RPLys) and Met (RPMet) since both of these amino acids are liable to be limiting in dairy cattle (NRC, 2001). It is well accepted that feeding of RPLys and RPMet is effective in increasing milk yield in dairy cattle. However, it is laborious and time-consuming to establish the effect of dietary amino acids on the performance of milking cattle. In order to develop a rapid method of evaluation for availability of RPAA, therefore, several procedures such as an *in vitro* method (Papas et al., 1984a), *in situ* methods (Overton et al., 1996; Berthiaume et al., 2000), and indirect methods measuring variations in jugular blood amino acid concentration have been developed (Papas et al., 1984b;

Wright et al., 1988; Bertrand et al., 1998; Blum et al., 1999). However, the results obtained using these methods yielded indirect or only partial findings, and not generally required indexes of the availability of RPAA such as 1) stability against biological and physical degradation by rumen microorganisms and rumination, 2) speed of passage through the rumen, and 3) absorption in the intestine.

Additionally, ruminal protection technique has been employed mainly for RPMet, but rarely for RPLys, because of the high water solubility of Lys. Therefore, development of RPLys is still a great challenge, and at the same time an appropriate technique to evaluate the availability of RPLys must be established. Measurement of the recovery rate of amino acid ingested from feces is a basic traditional method for estimation of the availability of nutrients. In the case of ruminants, physical degradation by rumination and digestion by rumen microorganisms should be taken into account. Yamazaki et al. (1987) established a procedure to continuously collect the total digesta flowing out from the abomasum in sheep. Therefore, it is expected that intestinal availability of RPAA can be estimated *in vivo* on the basis of disappearance of free amino acids in the intestine by measuring recovery of all amino acids in the abomasal digesta collected from a cannula in the duodenum and in the excreted feces.

This study was therefore conducted to estimate the availability of RPLys and RPMet in Holstein heifers with a T-shaped duodenal cannula by measuring recovery of free amino acids in duodenal digesta and feces.

### MATERIALS AND METHODS

RPLys used in this study was prepared by coating L-

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Received June 10, 2002; Accepted September 26, 2002

LysHCl with a fat, dehydrogenated tallow (Ajinomoto Co, Japan). RPMet consisted of DL-Met cores encapsulated with a pH-sensitive polymer (Ajinomoto Co, Japan). Their characteristics, evaluated by an *in vitro* method, are shown in Table 1. The stability of RPLys and RPMet in a buffer with the same pH as rumen fluid was evaluated by measuring either Lys or Met released from 1.0 g RPLys and RPMet added to 200 ml NaHPO<sub>4</sub> buffer at pH 7.0 after incubation for 24 h at 39°C (McDougall, 1948). The stability of RPLys and RPMet in the abomasum was evaluated by measuring either Lys or Met released from 1.0 g RPAA in 200 ml of KCl buffer at pH 2.5 during 1 h at 39°C (Clarks and Lubs, 1916). To estimate the Lys content of RPLys, 1 g of RPLys in 200 ml of 0.1 N HCl solution was microwave-heated for 2 min., and the amount of released Lys was measured. Met content of RPMet was measured as amount of Met released in 200 ml of 0.1 N HCl solution. Released Lys and Met from RPLys and RPMet and those remaining as RPLys and RPMet after exposure to the buffer solutions were determined with an amino acid analyzer (L-6500, Hitachi, Japan).

#### Animals and diets

Three Holstein heifers weighing 173, 190 and 205 kg were surgically fitted with a T-shape cannula at the entrance of the duodenum according to Komarec (1981) as shown in Figure 1. Post-surgical care accorded with the method reported by Robinson et al. (1990). Daily feed consumption and frequency of stool recovered by 10 days after the operation. The cows were used for the experiment 4 wk after surgery.

During the experiment, the cows were confined to tie stalls, given free access to water, and offered diet consisting of 0.4 kg timothy hay, 0.4 kg alfalfa hay cube and 0.75 kg commercial concentrate (Itochu Feed Mills Co., LTD, Japan), twice daily at 09:00 and 16:00. The cows were adapted to the experimental diet for 2 wk, followed by 48 h total abomasal outflow collection after RPLys and RPMet were supplied. After a 2 wk interval, the cows were fed RPLys and RPMet together again and total feces were collected for 72 h after administration.

#### Collection of total digesta from duodenal cannula

RPLys and RPMet, consisting of 30 g L-Lys and 30 g

DL-Met, were given to the animals mixed with 0.75 kg commercial concentrate (Itochu Feed Mills Co., LTD, Japan) at 09:00. After complete consumption of RPLys and RPMet by the animals, 0.4 kg of timothy hay and 0.4 kg of alfalfa hay cube were supplied.

The scheme of sampling is shown in Figure 1. A balloon catheter (SF-BR2605D, Termo, Japan) was inserted into the distal side of the cannula to seal the digesta from the abomasum and to divert the digesta through the stem of the cannula as described by Yamazaki et al. (1987). Abomasal digesta were sampled for 1 h prior to feeding of RPLys and RPMet to determine basal Lys and Met concentrations. For 48 h after feeding of RPLys and RPMet, total outflow of digesta from the abomasum was collected through the duodenal cannula continuously for determination of the concentration of free Lys and Met derived from RPLys and RPMet. Total abomasal outflows of digesta were pooled hourly and fractionated into liquid and solid fractions by centrifugation at 1,600×g for 7 min. In order to determine post-ruminal release of Lys and Met from RPLys and RPMet, the volume of liquid samples was measured and a portion of 25 ml was frozen (-20°C) immediately until amino acid analysis. The remainder of the liquid sample was warmed to 39°C in a water bath and returned to the duodenum through the balloon catheter by a bellow pump (PA-25/26, Master Flex) as described by Yamazaki et al. (1987).

Solid fractions were grounded mechanically with a blender (MX-9100, Toshiba, Japan) for 4 min with a two-fold volume of water by weight to determine rumen-bypassed Lys and Met that were retained as RPLys and RPMet and reached the duodenum. The slurry was centrifuged at 1,600×g for 7 min for separation into liquid and solid fractions. The volume of the liquid fraction was measured and a 25 ml portion of sample was taken for amino acid analysis. To compensate for moisture in the solid fractions, approximately 50 g of solid fractions was dried overnight in a forced air oven at 55°C. Free Lys and Met were determined with an amino acid analyzer (L-8500, Hitachi, Japan). For deproteinization, 1.5 ml of sulfosalicylic acid solution (10% w/v) was added to 1.5 ml of thawed sample and mixed well with a vortex mixer. The mixture was then transferred to a refrigerator kept at 4°C and centrifuged for 20 min at 10,000 rpm. The supernatant was removed and filled up to 3 ml with 0.02 N HCl buffer.

Hourly recovery rates of RPLys and RPMet from abomasal outflow were determined by dividing the amount of Lys or Met in the liquid and solid fractions of the abomasal outflow by the total amount of Lys and Met in RPLys and RPMet ingested by the animals.

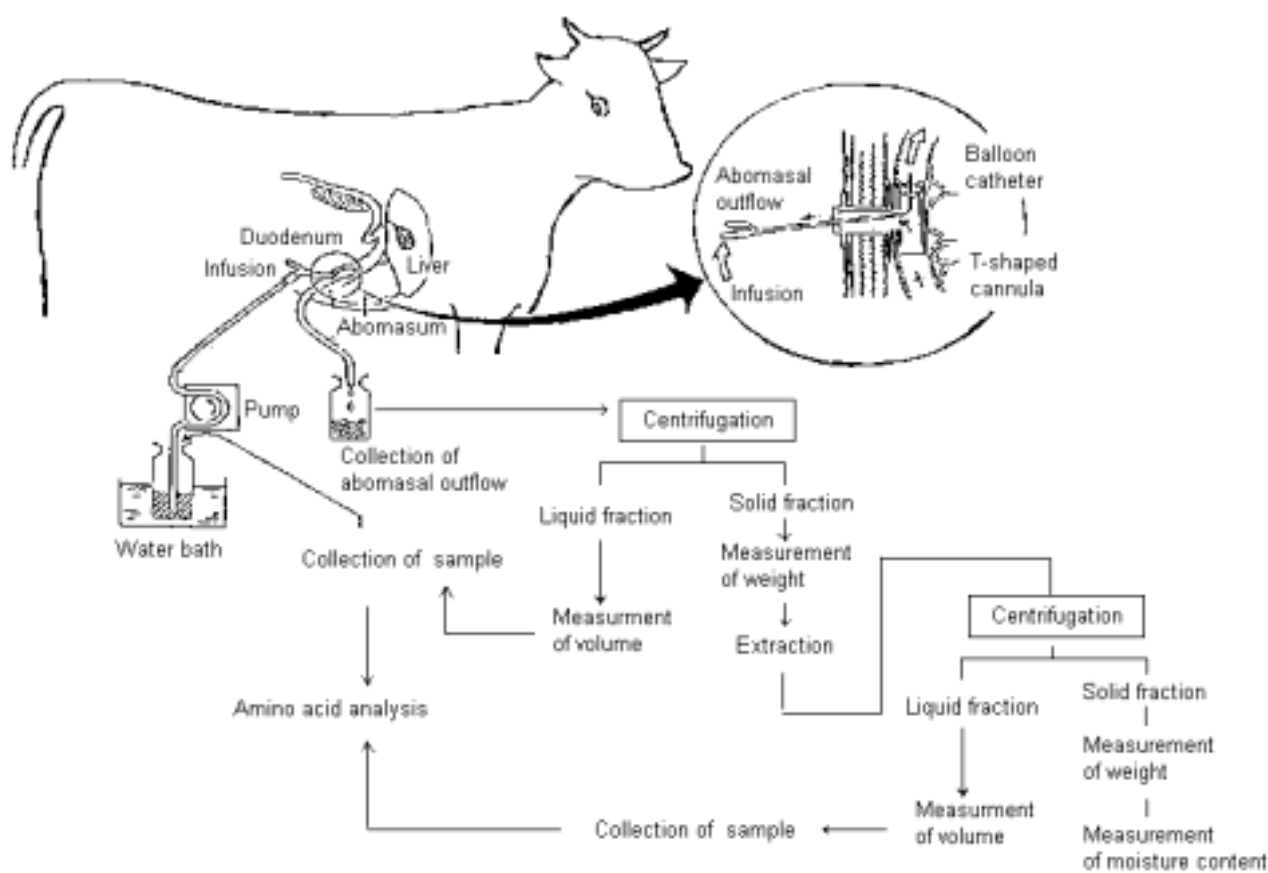
#### Collection of feces

On d 14 after total abomasal outflow sampling, RPLys

**Table 1.** Characters of RPLys and RPMet

	RPLys	RPMet
Specific gravity	1.1-1.2	1.1-1.2
Lys or Met content (%)	36	64
RPAA recovered after incubation	75	100
in a buffer (pH 7.0) for 24 h (%)		
Destroyed RPAA during incubation	2	99
in a buffer (pH 2.5) for 1 h (%)		

Each values is means of duplicate analysis.



**Figure 1.** Scheme of sampling procedure of total abomasal outflow from heifers with a T-duodenal cannula.

and RPMet were administered to determine the recovery of Lys and Met in feces. The management of cows, experimental feed and RPLys and RPMet administration were performed in the same way as for the duodenal digesta sampling experiment, with the exception that a rubber mat (110 cm×150 cm) was spread under the hind legs for ease of collection of feces. Feces were collected immediately after animals defecated and pooled every 12 h for 72 h. Pooled feces were ground mechanically with a blender (MX-9100, Toshiba, Japan) for 4 min with a two-fold volume of water by weight. The slurry was centrifuged at 1,600×g for 7 min. Volumes of separated liquid fractions of the slurry were measured and a 25 ml portion of liquid phase was used for amino acids analysis. After determination of moisture in the separated solid fractions, and deproteinization, amino acids in the separated solid fraction were determined.

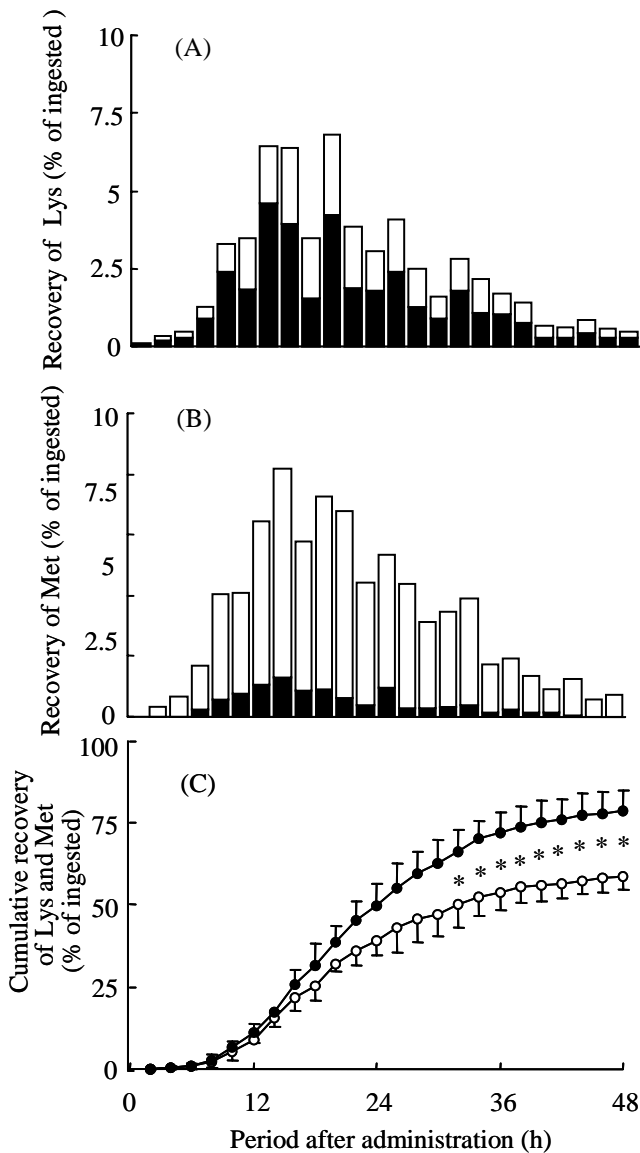
#### Statistical analysis

Student's *t*-test was used to compare recovery rates and cumulative recoveries from abomasal outflow, recovery

rates from feces, and intestinal disappearance of RPAA (Zar, 1984).

## RESULT AND DISCUSSION

The stabilities of RPLys and RPMet during 24 h incubation in the buffer, which simulated conditions in the rumen, were 75 and 100%, respectively. During a 1 h incubation period in KCl buffer at pH 2.5, 2% of Lys and 99% of Met were released from RPLys and RPMet, respectively. During the experimental period, there was no material failure of the duodenal cannula. Judging from the feed intake during the experiment for measuring duodenal outflow and fecal excretion, normal passage of digesta was maintained. The patterns of time course of recovery of Lys and Met in the abomasal outflow closely coincided (Figure 2). The recovery rates of RPLys and RPMet peaked from 12 to 16 h after administration, and then decreased gradually until 48 h.



**Figure 2.** Time course of recovery percentage of Lys (A) and Met (B) in solid ( ) and liquid fraction ( ) and cumulative recovery percentage of Lys ( ) and Met ( ) from total abomasal outflow of heifers during 48 h (C). Values are means of three observations with their standard errors represented by vertical bars. Significant differences between values for cumulative recovery of Lys and Met (Student's *t*-test): \*  $p < 0.05$ .

Since the specific gravities of RPLys and RPMet were the same, at 1.1 to 1.2, passage of them through the rumen to the duodenal cannula were probably almost the same, since it has been suggested that specific gravity plays a key role determining time of passage through the digestive tract of ruminants, and has been shown that specific gravity determines time of passage through the reticulo-rumen (Hooper and Welch, 1985; Katoh et al., 1988; Katoh et al., 1991).

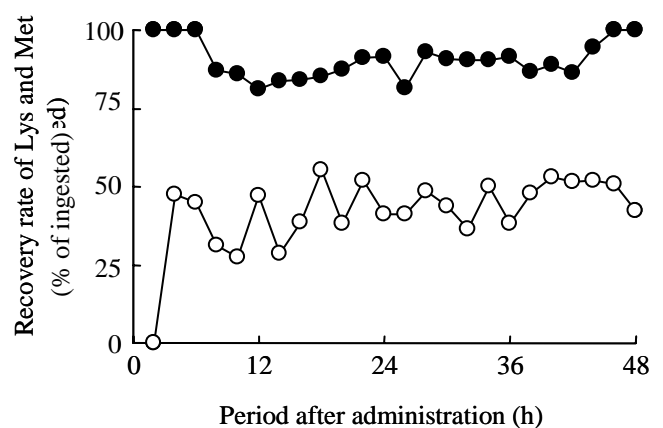
As shown in Figure 2 (C), Cumulative recovery rates of RPLys and RPMet had nearly plateaued by 48 h after

administration, indicating that 48 h was sufficient as a sampling period. Cumulative recovery rates in the abomasal outflow for 48 h were  $58.3 \pm 4.1$  and  $78.3 \pm 6.4\%$  ( $p = 0.058$ ) for RPLys and RPMet, respectively. The lower cumulative recovery rate of Lys than for Met might be due to lower stability of RPLys in the stomachs, specifically the rumen, and consequent consumption of it by microbes. Since the same pattern of cumulative recovery rate was observed for the first 16 h after feeding, protection of RPLys in the rumen for the first 16 h appeared to be similar to that of RPMet, but rapidly decreased after 16 h.

The time courses of recovery of Lys (A) and Met (B) from the liquid fractions of duodenal outflow of heifers over 48 h after a single feeding of RPLys and RPMet are shown in Figure 3. As also shown in Figure 2, constantly higher recovery rates of Lys were observed in the duodenal liquid fractions, although they were lower than those for Met ( $41.9 \pm 13.8$  and  $90.4 \pm 5.8$  of RPLys and RPMet, respectively). Free Lys in the duodenal fluid may be derived from degradation of RPLys by rumen microbes since RPLys is stable in the abomasal environment, as shown in Table 1. The findings of higher Met recovery in the liquid than in the solid fractions was in agreement with the result of *in vitro* evaluation with low pH buffer (Table 1). These results indicated that Met coated with a pH-sensitive braking polymer is effective in releasing Met in the abomasum.

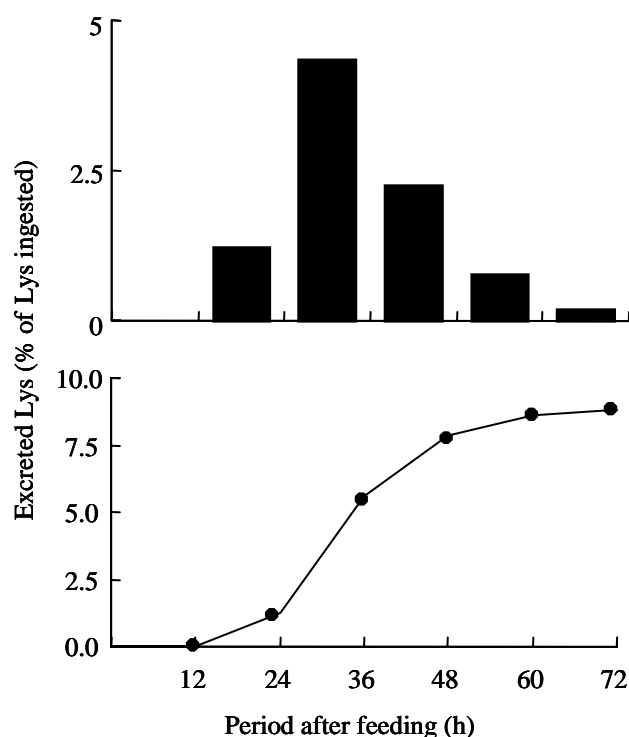
Ingested particles suffered not only from enzymatic digestion by rumen microorganisms but also physical digestion by rumination. DesBordes and Welch (1984) demonstrated that 20 to 30% of ingested particles were ruminated, even though the specific gravity of particles was appropriate for prompt passage from the rumen. In this study, 21.7% of RPMet was lost in the rumen, mainly due to rumination. Effects of rumination and other physical damage on availability of RPLys and RPMet might be considerable, but were not taken into account by other traditional *in vivo* and *in situ* measurements.

The time courses of recovery rate of Lys (A) and cumulative recovery rate in feces of heifers (B) over 72 h after RPA administration are shown in Figure 4. Met was not detected, indicating that all RPMet that reached the duodenum was absorbed or degraded in the small and large intestine. Apparent intestinal disappearance, expressed as



**Figure 3.** Time course of recovery of Lys ( ) and Met ( ) rate in liquid fraction of duodenal outflow of heifers for 48 h.

the difference between the amino acid recovered from the



**Figure 4.** Time course of recovered Lys rate (A) and cumulative recovery of Lys in feces (B).

abomasal outflow was  $49.5 \pm 2.6$  for RPLys and  $78.2 \pm 6.5\%$  for RPMet ( $p=0.015$ ) (Table 2).

Berthiaume et al. (2000, 2001) measured the availability of RPMet with the mobile nylon bag technique. They placed RPAA in nylon bags and inserted them into the duodenum through a cannula following incubation in the rumen. RPAA, especially fat-coated RPAA, became fragile following incubation in the rumen (our unpublished data). Physical damage induced by the procedure following the incubation process may increase breakability of the coating and lead to overestimation of disappearance of RPAA in the small intestine.

Okine (1991) reported passage rate from the ruminoreticulum increased with intake in nonlactating Holstein dairy cows. In these days, heifers and lactating cows are reared under very high intake levels, it means

**Table 2.** Recovery rate from abomasal outflow and feces, and intestinal disappearance of RPLys and RPMet

	RPLys	RPMet	P
Recovery rate from abomasal outflow (%)			
Liquid phase	$23.9 \pm 8.3$	$68.6 \pm 3.6^{**}$	0.008
Solid phase	$34.3 \pm 12.1$	$9.7 \pm 4.9$	0.133
Total	$58.3 \pm 4.1$	$78.3 \pm 6.4$	0.058
Recovery rate from feces (%)	$8.8 \pm 2.9$	$0.0 \pm 0.0$	-
Intestinal disappearance (%)	$49.5 \pm 2.6$	$78.2 \pm 6.5^*$	0.015

Values are means  $\pm$  SE for three observations.

\*\*\*  $p < 0.05$ ,  $p < 0.01$ .

different passage rate compared to the present study. Probably passage rate affect the availability of RPLys more and that of RPMet less, because of different existence phase in abomasal out flow. We should take account of the influence of passage rate when we apply the result from this evaluation technique to a lactation cow.

In conclusion, quantitative measurements of amino acids in abomasal outflow and fecal excretion are useful for precise determination of the availability of ruminally-protected amino acids. They are also useful when fat or a pH-sensitive polymer is used to bypass supplemented amino acids through the rumen in dairy cattle.

## ACKNOWLEDGMENT

The authors thank Dr. S. Hayashi of Itochu Feed Mills Co., LTD, for surgery and care of the animals; and T. Suzuki for sample preparation and organization of this experiment.

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