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N^T METHYL HISTIDINE CONTENT OF FEEDSTUFFS
FED TO RUMINANTS

By

Phoebe Wei-Tsu Gur-Chiang

A THESIS

Submitted to
Michigan State University
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ABSTRACT

N^T METHYL HISTIDINE CONTENT OF FEEDSTUFFS FED TO RUMINANTS

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The N^T-methylhistidine content of 12 feedstuffs, duodenal digesta from steers fed alfalfa haylage and 4 different muscles obtained from young and finished steers was determined. Degradation of N^T-methylhistidine by ruminal microorganisms was studied in vitro. N^T-Methylhistidine was quantitated using ion-exchange chromatography. Results indicate that feeds consumed by ruminants contain little or no N^T-methylhistidine. Steers, consuming dry matter at 2-3% of bodyweight would consume 5-15 μmol N^T-methylhistidine daily. In vitro degradation of N^T-methylhistidine by rumen microbes was 14.5% while leucine (control) was totally degraded. Skeletal muscle N^T-methylhistidine content was higher in the older steers (3.5 μmol /g protein ; 2.8 μmol /g protein) while red muscles contained less N^T-methylhistidine than predominantly white muscles (2.9 μmol /g protein ; 3.4 μmol /g protein).

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INTRODUCTION

There has been a growing interest in recent years in the study of protein turnover in ruminants (McCarthy, 1981). To assess the role of dietary and endocrine status on protein turnover (breakdown rate; BR) in large domestic animals it is convenient to employ noninvasive procedures utilizing a muscle protein turnover marker; one amino acid N^T -methylhistidine is such a marker.

Fifteen years ago, N^T -methylhistidine (3-methylhistidine; 3-MeHis) was identified as a constituent of both actin (Asatoor & Armstrong, 1967) and myosin (Johnson et al., 1967). The available evidence indicates that at a stage during the synthesis of the polypeptide chains of actin and myosin, some of the peptide-bond histidine residues are methylated to give N^T -methylhistidine (Haverberg, 1975; Ward and Buttery, 1978). N^T -Methylhistidine cannot be reutilized for protein biosynthesis when released by protein breakdown and is excreted quantitatively in the urine. Asatoor and Armstrong (1966) were first to suggest that the measurement of the rate of excretion of N^T -methylhistidine could provide an estimate of myofibrillar degradation or turnover. Since

then, many researchers have applied this concept and utilized N^T-methylhistidine to assess skeletal muscle BR in man and animals (Young and Munro, 1978).

To utilize N^T-methylhistidine as a skeletal muscle protein turnover marker, it is essential that all nonendogenous (i.e. exogenous) sources of N^T-methylhistidine be eliminated. It had been suggested that forages and other feeds fed to ruminants may contain sizable levels of N^T-methylhistidine. This research was initiated to determine the concentration of N^T-methylhistidine in 12 different feedstuffs, to assess the ruminal microbial metabolism of free N^T-methylhistidine and to assess duodenal flow of N^T-methylhistidine in cattle. To obtain these data, extensive work had to be done on analytical techniques for N^T-methylhistidine.

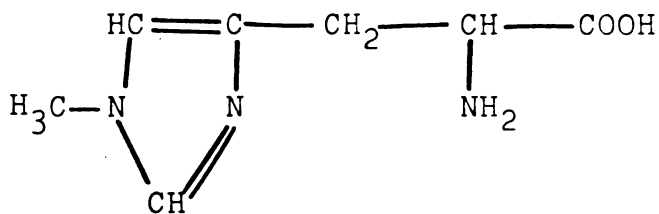
REVIEW OF LITERATURE

I. N^{τ} -Methylhistidine Historical Background

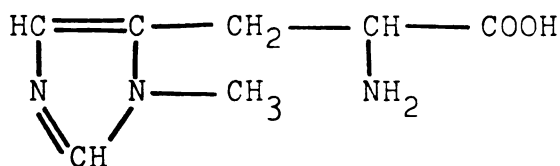
A. Nomenclature and Structure

Alpha amino- β -(1-methyl-4-imidazole) propionic acid was assigned the name tele-methylhistidine (N^{τ} -methylhistidine) by the IUPAC-IUB (1972) to remove confusion as to which nitrogen on imidazole-ring the methyl group of N^{τ} -methylhistidine is attached.

N^{τ} -Methylhistidine is an isomer of N^{π} -methylhistidine (1-methylhistidine; α -amino- β -(1-methyl-5-imidazole) propionic acid). The chemical structure of N^{τ} -methylhistidine and N^{π} -methylhistidine are as follows:



N^{τ} -Methylhistidine (3-methylhistidine)



N^{π} -Methylhistidine (1-methylhistidine)

B. Initial Work

Urinary excretion of N methyl derivatives of histidine had been observed by early workers. Searle and Westall (1951) showed that 1-methylhistidine is a normal urinary constituent. In normal man, amino acids containing the imidazole ring account for 30% of the free amino acid α -amino nitrogen found in the urine. N^T -Methylhistidine is usually found in smaller amounts than the 1-methylhistidine (Tallan et al., 1954). 1-Methylhistidine has long been known to be a constituent of anserine and had been isolated in the free form from urine (Searle and Westall, 1951). During this time Tallan et al. (1954) were also working on the isolation of N^T -methylhistidine from cat urine. Both laboratories demonstrated that N^T -methylhistidine from man or cat were indistinguishable when (co)chromatographed on paper and on Dowex 50.

N^T -Methylhistidine has been synthesized from phthaloylhistidine and from L-histidine. Synthetic N^T -methylhistidine, identical with the natural product in respect to elementary analysis, optical rotation, chromatographic behavior, and infra-red spectrum, was isolated from the reaction mixture by chromatography (Tallan et al., 1954).

C. Further Developments

a. Site of Histidine Methylation

Datta and Harris (1951) showed that after the ingestion

of large amounts of rabbit meat by humans (rabbit muscle contains high amounts of anserine) there was a measurable rise in the excretion of 1-methylhistidine but not for N^τ-methylhistidine. Tallan et al. (1954) could not locate the source of the urinary N^τ-methylhistidine both in the cat and in the human. Asatoor and Armstrong (1967) demonstrated that in samples of blood and urine collected under fasting conditions N^τ-methylhistidine was present and therefore urinary N^τ-methylhistidine was thought to be of endogenous origin. Asatoor and Armstrong (1967) presented evidence that N^τ-methylhistidine is a component of the muscle protein actin (now we know that actin is found in all cells). All acid hydrolysates of actin preparations (actin was prepared by the procedure of starch gel electrophoresis (Carsten and Mommaerts, 1963)) contained N^τ-methylhistidine in the proportion of 1 mole per 8 to 10 moles of histidine.

Asatoor and Armstrong (1967) also showed that histidine and the methyl group of methionine serve as precursors for N^τ-methylhistidine, and that urinary 1-methylhistidine and N^τ-methylhistidine come from pools with markedly different turnover rates. Johnson et al. (1967) provided evidence for the presence of N^τ-methylhistidine as part of the primary structure of actin and myosin. Their work also confirmed the Asatoor and Armstrong (1967) findings on the presence of N^τ-methylhistidine in actin hydrolysates.

N^τ-Methylhistidine occurs as a constituent of skeletal

and cardiac muscle actin and in the myosin of skeletal muscle consisting predominantly of white fiber types (Lobley and Harris, 1977). N^T -Methylhistidine is present in actin from a number of species of adult and fetal skeletal muscle, whereas it is only found in myosin shortly after birth in the rabbit (Johnson et al., 1969). Trayer et al. (1968) also pointed out that the ninhydrin-positive material corresponding to N^T -methylhistidine could not be detected in significant amounts in acid hydrolysates of myosin isolated from skeletal muscle of the 28 day old fetus. Actin from red skeletal muscle and cardiac muscles of the rabbit, from smooth muscle of the cow uterus and from certain invertebrate muscle gave a N^T -methylhistidine: histidine ratio as found in purified actin from adult rabbit white skeletal muscle. Myosins from red and cardiac muscle had a much lower N^T -methylhistidine: histidine ratio than that of purified myosin from adult rabbit white skeletal muscle (Johnson et al., 1969).

Young et al. (1970) showed that N^T -methylhistidine was not bound to transfer RNA by amino acyl ligase from skeletal muscle in vitro and thus the methylation process must occur at a stage in fibrillar protein synthesis which is subsequent to the formation of histidyl-tRNA. Since methylation occurs after translation, reutilization is not possible, and that free N^T -methylhistidine cannot be directly used for the synthesis of the fibrillar proteins. And during the course of actin and myosin breakdown it is

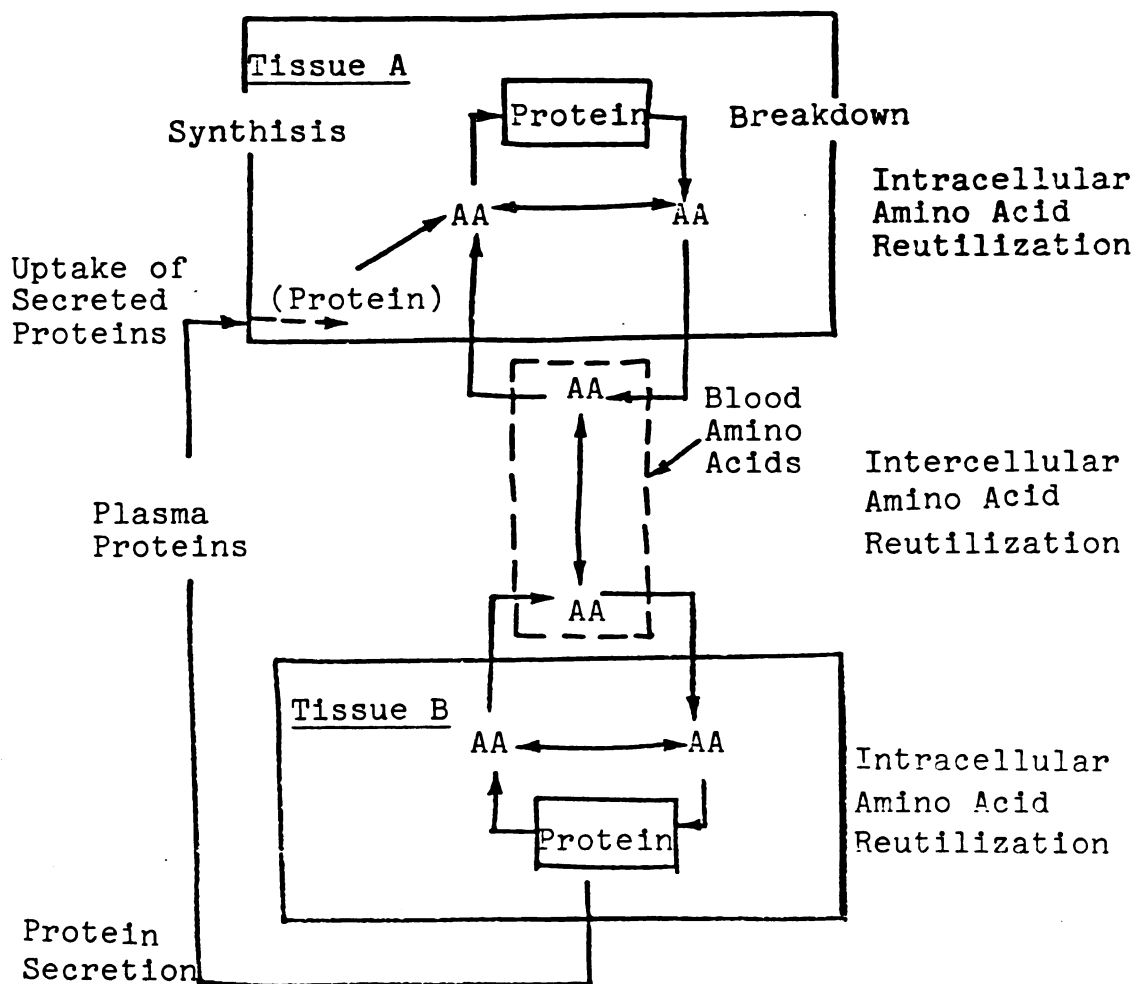
liberated into the free amino acid pool and then quantitatively eliminated from the body, chiefly via the urine (Young et al., 1972). These findings were consistent with the observations of Asatoor and Armstrong (1967) that the specific activity of uniformly ^{14}C -labeled histidine is unchanged after isolation of injected label as N^{T} -methylhistidine from muscle homogenates.

Young and Munro (1978) indicated that studies of tissue protein breakdown in vivo are difficult because of extensive amino acid reutilization as shown in Figure 1. The amino acids that are released during the intracellular breakdown of proteins can be extensively reutilized for protein synthesis within the cell (intracellular recycling) or they may be transported to other organs where they enter pathways of protein anabolism (intercellular recycling). This recycling frustrates studies of protein breakdown after administration of a labeled amino acid followed by measurement of the rate of loss of label from the tissue proteins.

b. N^{T} -Methylhistidine as an Index of Muscle Protein Breakdown

Although no rapid and satisfactory method has yet been devised for measurement of protein degradation in the whole animal the work of Young and his colleagues (1973) indicates that in vivo catabolism of muscle can be followed in the urinary elimination of N^{T} -methylhistidine (Lobley &

Figure 1. Flow of Amino Acids in the Intact Organism,
 Illustrating Inter- and Intracellular Amino Acid
 Reutilization^a



^a

Adapted from Young and Munro (1978).

Harris, 1977). Young and Munro (1978) recognized the potential importance of previous findings on myofibrillar N^T -methylhistidine content and also suggested that the urinary excretion rate of N^T -methylhistidine could potentially be used as an index of muscle protein breakdown provided that:

- (A) The N^T -methylhistidine was present exclusively in muscle protein and at a constant amount.
- (B) N^T -Methylhistidine released after protein degradation was neither reutilized for protein synthesis nor metabolized.
- (C) Free N^T -methylhistidine was rapidly and quantitatively excreted in the urine.

Initial experiments apparently confirmed all three conditions.

Young and Munro (1978) also listed characteristics of an ideal marker amino acid as shown in Table 1. Asatoor and Armstrong (1974) proposed and Young and Munro (1978) justified and clarified how N^T -methylhistidine in muscle fulfills these characteristics (Table 1). Young et al. (1973) first discussed the potential use of N^T -methylhistidine excretion as an index of progressive reduction in muscle protein catabolism during starvation in obese human patients. Similar studies were done by Rao and Nagabhushan (1973) in men suffering from protein-calorie malnutrition. These two groups observed a progressive decrease in urinary N^T -methylhistidine with

Table 1. Characteristics of Amino Acid for In Vivo
Measurements of Muscle Protein Breakdown^a

Amino Acid is modified chemically after peptide
bond synthesis.

Chemically modified group does not undergo
exchange once it appears in the protein-bound
amino acid.

Concentration is known or constant in muscle
protein.

Amino acid not formed to an important extent in
other tissues.

Released at the same time as other amino acids
are released from the completed protein.

Not reutilized for protein synthesis.

Does not undergo further metabolism in the body.

Has a low renal threshold.

Quantitatively excreted in the urine.

a

Adapted from Young and Munro (1978).

starvation and protein-calorie malnutrition. These findings suggested that the reduced output of urinary N^T-methylhistidine coincided with decreased urinary N output due to an adaptation and decrease in catabolism of muscle proteins as starvation and protein-calorie malnutrition progressed (Millward et al., 1976).

Haverberg et al. (1975) using rats, showed a marked and progressive decrease in N^T-methylhistidine excretion in the protein-depleted group. The group restricted in both dietary protein and energy, showed an initial small increase in daily N^T-methylhistidine output but then a decrease in excretion. These results were confirmed by Funabiki et al. (1976) and Nishizawa et al. (1978), in subsequent studies. Ogata et al. (1978) and Nishizawa et al. (1978) both showed an increase in N^T-methylhistidine excretion initially in starved rats, indicating an increase in degradation of muscle. Ogata et al. (1978) did not observe a decrease in N^T-methylhistidine excretion, however in their study rats were starved for 72 hours then fed for 24 hours. Nishizawa et al. (1978) did observe a decrease in excretion of N^T-methylhistidine after 4 days of starvation. Omstedt et al. (1978) used N^T-methylhistidine excretion as an index for muscle degradation in evaluating protein quality and found that the better the protein quality the greater was the rate of growth of the rat and the higher was the total urinary N^T-methylhistidine (nonacetylated + acetylated) excretion.

N^T -Methylhistidine, unfortunately cannot be used as a protein turnover marker in all species. Harris and Milne (1980, 1981b) demonstrated that urinary N^T -methylhistidine excretion is an inadequate index of muscle protein breakdown in the pig and sheep. However, by the criterion of the rate of clearance of labeled N^T -methylhistidine from the body, the excretion of N^T -methylhistidine in urine appears to be a valid index of muscle protein breakdown in cattle (Harris and Milne, 1981a; McCarthy et al., 1983) as well as man and laboratory rodents (Young and Munro, 1978).

McCarthy et al. (1983) found that N^T -methylhistidine was quantitatively excreted by cattle at a rapid rate, thus demonstrating the N^T -methylhistidine released during breakdown of muscle proteins would be quickly removed from the body. However, two problems in the use of N^T -methylhistidine as a muscle protein turnover marker were not directly addressed by McCarthy et al. (1983). The first problem is the possibility that N^T -methylhistidine of exogenous feed origin may be absorbed and excreted in the urine and a second problem is the N^T -methylhistidine contribution from other contractile proteins of non-muscle tissue whose turnover may differ from skeletal muscle proteins (Rennie et al., 1983). McCarthy et al. (1983) assuming that only 80% of urinary N^T -methylhistidine excretion arose from skeletal muscle tissue, demonstrated that N^T -methylhistidine excretion per unit of body weight

did not differ between finishing steers of large and small frame sizes and that the fractional degradation of muscle protein did not differ between large and small cattle. The fractional rate of muscle degradation was also shown to decline with age for all steers.

c. Location of N^T- Methylhistidine in Muscle
Myofibrillar Proteins

Huszar and Elzinga (1971) determined the amino acid sequence around the single N^T-methylhistidine residue in rabbit skeletal muscle myosin by the use of the enzyme thermolysin. This enzyme usually hydrolyzed peptide bonds in which the amide bond is contributed by an amino acid with a hydrophobic side chain, such as Gly-Ile and Thr-Tyr bonds. Thermolysin does not split the Asp-Val bond. Elzinga (1971) determined the amino acid sequences around N^T-methylhistidine in both myosin and actin; his results are presented in Figure 2.

In summarizing these studies, Huszar et al. (1971) indicated that:

- 1) Actin and myosin are the only myofibrillar proteins that have been shown to contain N^T-methylhistidine; this methylated amino acid is located in the globular subfragment-1 of myosin. The only other proteins which have been reported (at that time) to contain this residue was ameba actin (Weihsing et al., 1969).

Figure 2. Amino Acid Sequences Around the Single
N^T-Methylhistidine in Actin and Myosin^a

Myosin : Leu-Leu-Gly-Ser-Ile-Asp-Val-Asp-
N^T-Methylhistidine-Gln-Thr-Tyr-Lys

Actin : Leu-Thr-Leu-Lys-Tyr-Pro-Ile-Glu-
N^T-Methylhistidine-Trp-Gly-Ile-Ile

^aAdapted from Elzinga (1971), Huszar and Elzinga
(1981).

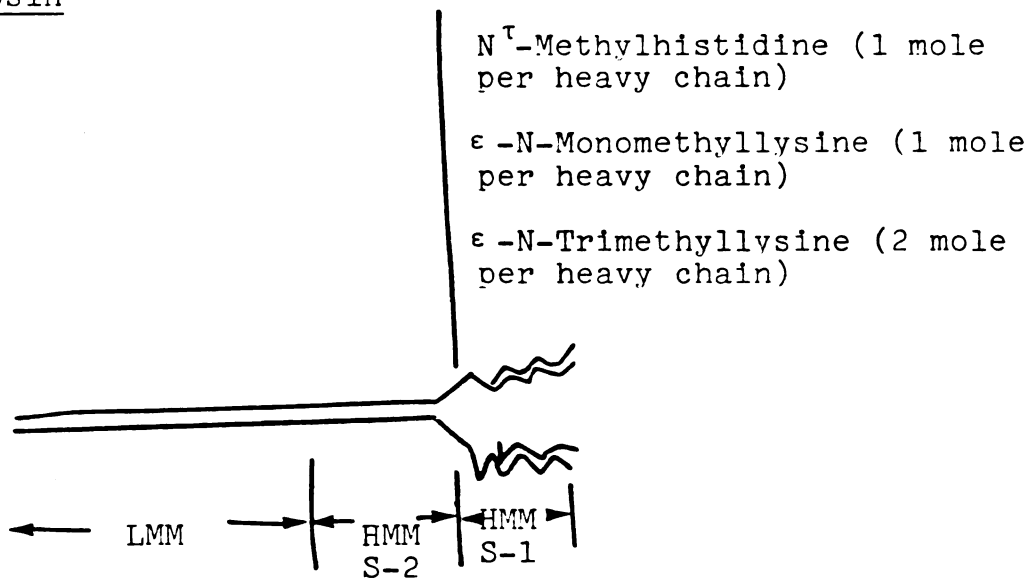
- 2) Actin and myosin subfragment-1 contain sites of interaction with nucleotides and metals.

Huszar et al. (1971) further pointed out that a feature common to both peptides is the acidic residue adjacent to N^T-methylhistidine; acidic side chains often lie at the surface of a protein, so the side chains of N^T-methylhistidine would be at or near the surface of actin and myosin. Elzinga et al. (1973) published the complete amino acid sequences of actin of rabbit skeletal muscle. The actin polypeptide chain is composed of 374 residues, including one residue of N^T-methylhistidine. Their study represented the first complete determination of the amino acid sequence of a myofibrillar protein. The schematic depiction of the location of N^T-methylhistidine in myosin and actin is shown in Figure 3 (Young and Munro, 1978).

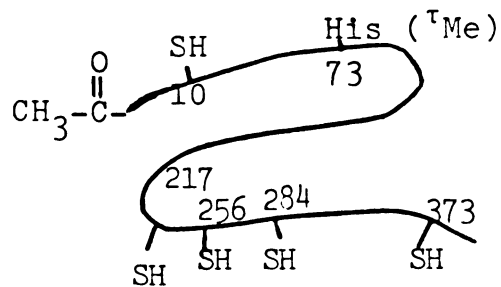
Haverberg et al. (1974) demonstrated that there were no significant age-related changes in the N^T-methylhistidine content of either actin or myosin in rats weighing from 94 to 324 g, but Young et al. (1972) reported that in rats the relative proportion of N^T-methylhistidine and its acetylated metabolite, N^T-acetyl methylhistidine, in urine changed with increasing body weight in growing rats. The N-acetyl form accounts for the major fraction of total urinary N^T-methylhistidine in the adult rat (Young et al., 1972) and at present, this acetyl form is only found in laboratory rodents (rats and mice). N-Acetylation of N^T-methylhistidine has not been observed in other animals

Figure 3. Schematic Depiction of the Location of
N^τ-Methylhistidine in Myosin and Actin^a

Myosin



Actin^b



^a Adapted from Young and Munro (1978).

^b Rabbit 374 residues.

(Young et al., 1978; McCarthy, 1981).

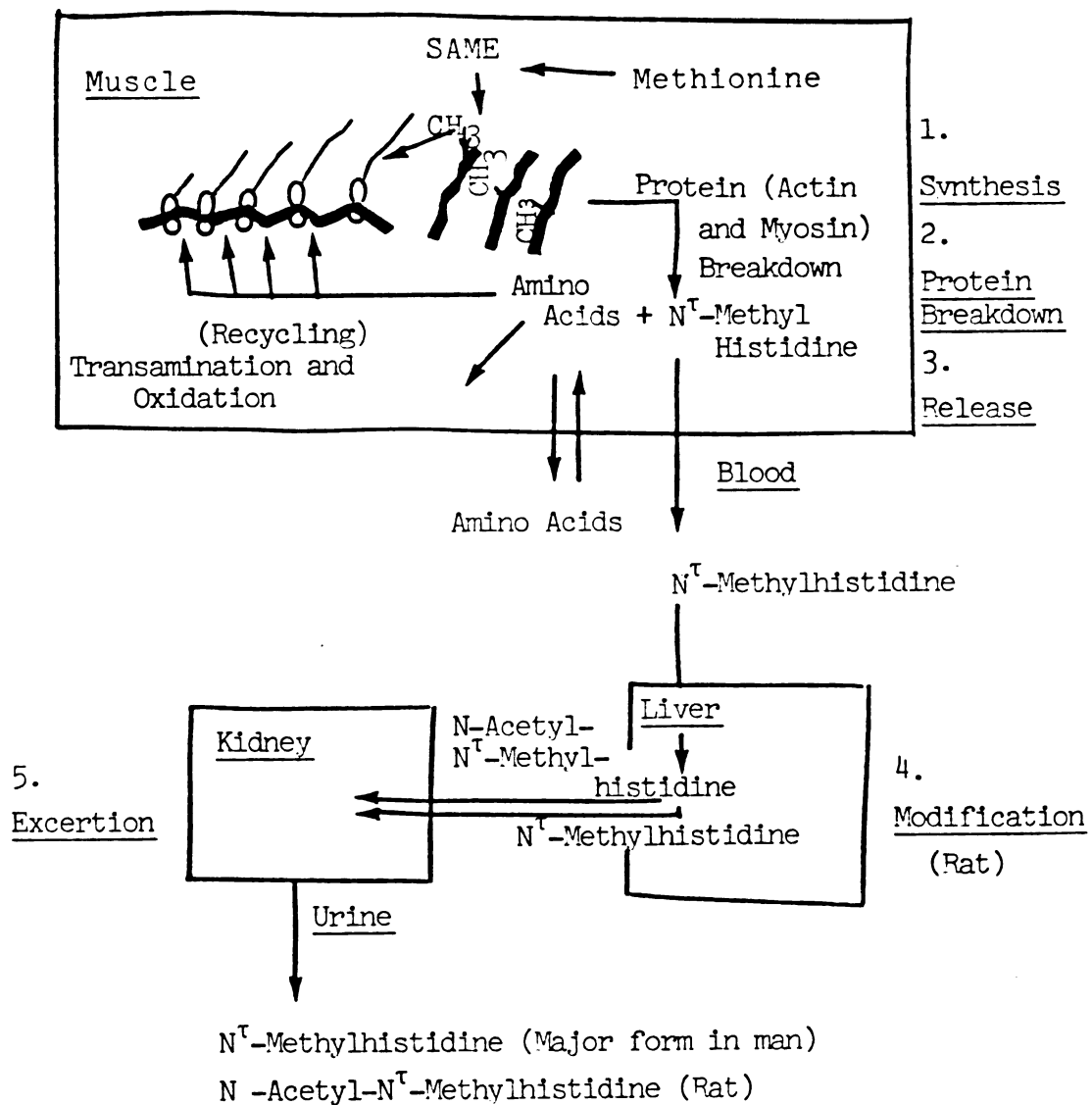
Haverberg et al. (1975) studied the concentration of N^T -methylhistidine in blood serum and in the mixed proteins of heart, brain, lung, kidney, diaphragm, spleen, testis, stomach, liver and leg skeletal muscles of approximately 400 g body weight male rats. Haverberg et al. (1975) indicated that skeletal muscle is likely to be the major source (658 nmol N^T -methylhistidine/g tissue) of urinary N^T -methylhistidine, which in turn is then a reflection of myofibrillar protein breakdown in skeletal muscle.

d. Metabolism of N^T -methylhistidine

Young and Munro (1978) presented a summary of N^T -methylhistidine metabolism in animals (Figure 4). Figure 4 shows the relationship between the N^T -methylhistidine present in actin and myosin of skeletal muscle and urinary output of the amino acid in rats and human subjects. The N^T -methylhistidine released during protein turnover is excreted quantitatively in urine.

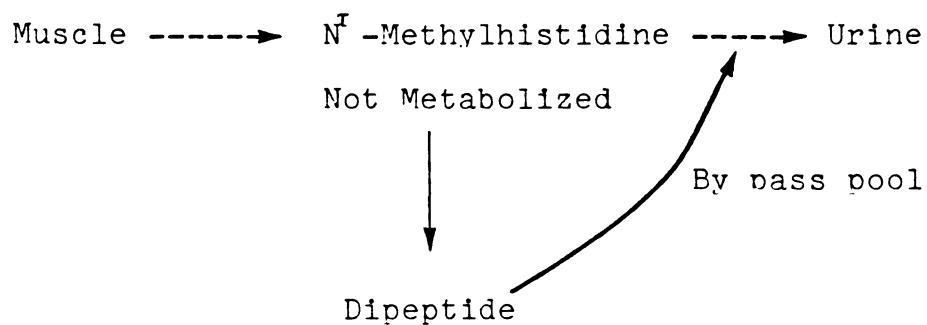
While ultimately all N^T -methylhistidine synthesized and released from muscle protein is quantitatively excreted in the urine and not catabolized within the body, the imidazole amino acids tend to form specific dipeptides that are found in the blood and tissue of the animals (Figure 5, Carnegie et al., 1984). The dipeptides present an intermediate step in N^T -methylhistidine metabolism and cause spurious and slow urinary excretion of the amino acid

Figure 4. The Origin and the Fate of
N^T-Methylhistidine in Myofibrillar
Proteins^a



^a Adapted from Young and Munro (1978).

Figure 5. Histidine Dipeptides and Metabolism of N^T -Methylhistidine^a



Histidine Dipeptides :

Anserine	β -alanly-1-methylhistidine
Balenine	β -alanly-L- N^T -methylhistidine
Carnosine	β -alanyl-histidine

^a Adapted from Carnegie et al., (1984).

such that the rate of urinary N^T -methylhistidine excretion cannot be used to assess muscle protein turnover.

In sheep, Harris and Milne (1981a, b) reported evidence of a N^T -methylhistidine dipeptide, called balenine (Figure 5), present in muscle which ties up large amounts of N^T -methylhistidine released during muscle protein degradation into a storage-bypass pool which presumably has its own kinetic properties.

Balenine (β -alanyl-L- N^T -methylhistidine) is present in skin and muscles of whales, snakes, pigs with lower amounts in chicken, cattle or sheep (Carnegie et al., 1984). Anserine (β -alanyl-L-1-methylhistidine) occurs in much higher levels in muscle of chicken, steers and cattle than pigs. The function of the histidine dipeptides is not known (Young and Munro, 1978).

Bergen and Potter (1971) pointed out that while the basic amino acids 1-methylhistidine, N^T -methylhistidine and the dipeptide carnosine (β -alanyl-L-histidine) are present in plasma of sheep, another major methylated amino acid eluted with lysine. This amino acid was identified as N methyl lysine (NML) and is present in amounts exceeding the methylated histidine. NML is not found to a great extent in cattle and cannot be used as a protein turnover marker as NML is the carnitine precursor in animals (Horne et al., 1971; Tanphaichitr et al., 1971; Horne and Broquist, 1972; Borum and Broquist, 1977). Table 2 lists

Table 2. Methylated Amino Acids in Muscle^a N^{τ} -Methylhistidine (3-Methylhistidine)

Soluble dipeptide: Balenine (β -alanyl-1- N^{τ} -methylhistidine) in skin and muscles of whales, snakes and pigs.

Protein-bound: Myosin (white or fast twitch fibers)

Actin (all muscles and cytoplasmic actins)

1-Methylhistidine (N^{π} -Methylhistidine)

Soluble dipeptide: Anserine (β -alanyl-1-methylhistidine)

Function: ATPase activators

Histamine-like action

Buffer action

ϵ -N-Monomethyl lysine and ϵ -N-trimethyllysine

N^{δ} - N^{δ} -Dimethylarginine

^a

Adapted from Young and Munro, 1978.

the methylated amino acids that have been isolated in muscle (Young and Munro, 1978).

e. Distribution of N^T -Methylhistidine in Tissue from Different Animals

Johnson and Perry (1970) were concerned with the distribution of N^T -methylhistidine in myosin and actin from various species and muscle types. Mixed longissimus dorsi and leg muscle of adult New Zealand White and Dutch rabbits, pigeon breast muscle, adult rabbit cardiac muscle, mixed skeletal muscles of white mice, smooth muscle of cow uterus, crab claw muscle and lobster tail muscles were analyzed for N^T -methylhistidine content (Johnson and Perry, 1970). In rabbits, the amounts of N^T -methylhistidine in cardiac and red skeletal muscle were significantly lower than that present in adult mixed skeletal muscle, which is mainly white. N^T -Methylhistidine contents of the mixed skeletal muscles from mice was also much lower than that of mixed skeletal muscle of the rabbit (Table 3; Johnson and Perry, 1970).

The fact that N^T -methylhistidine is present in actin and myosin isolated from seven species of vertebrates and invertebrates strongly suggests that it is a normal component of these proteins in all species. The N^T -methylhistidine content of actin, however, appears to be much more constant than is the case with myosin. The values for the N^T -methylhistidine content of myosin were much more variable than those of actin and in all cases the

Table 3. N¹-Methylhistidine Content of Actin and Myosin Prepared from Different Muscles^a

Muscle	N ¹ -Methylhistidine		N ¹ -Methylhistidine Content (Residues per mol of protein)		
	Myosin	Actin			
Type	Source	Myosin	Actin	Myosin	Actin
White striated	Adult rabbit mixed skeletal	1:44.2	1:7.6	1.63	.95
		1:79	-	.91	-
		1:178	1:10	.41	.72
Red striated	Pigeon breast	1:79	-	.91	-
	Rabbit heart	1:178	1:10	.41	.72
Smooth	Cow uterus	1:93	-	.78	-
		1:20.4	1:8.4	3.5	.86
		1:68.3	-	1.05	-
Invertebrate Striated	Crab claw Lobster tail	1:24.6	-	2.93	-
		1:25.7	-	2.80	-
		1:28.2	1:15.2	2.55	.47

^a Adapted from Johnson and Perry, 1970.

N^T -methylhistidine/histidine ratio of actin is higher. The N^T -methylhistidine/histidine ratio of myosin varies from 1:20.4 (Cow uterus) to 1:178 (Rabbit heart), however N^T -methylhistidine/histidine ratio of actin varies from 1:7.6 (Adult rabbit mixed skeletal) to 1:15.2 (Lobster tail) (Johnson and Perry, 1970; Table 3).

Johnson and Perry (1970) concluded that 1) the N^T -methylhistidine content is highest in myosin from white skeletal muscle and lower values are obtained from myosin of red skeletal and smooth muscle, and 2) the N^T -methylhistidine content of actin was similar in all of the types of muscle from which it was isolated. Haverberg et al. (1974) determined the content of N^T -methylhistidine in actin and myosin from different species (Table 4).

Other tissues contain actin and therefore have N^T -methylhistidine, at least 16.6% of the daily urinary N^T -methylhistidine excretion is released from skin and gastrointestinal tract (10.4% is from skin, 6.2% is from gastrointestinal tract). Thus, when calculating the fractional catabolic rate of myosin and actin from the urinary excretion of N^T -methylhistidine, these N^T -methylhistidine contributions to daily urine output have potential to result in erroneous breakdown rate estimates of skeletal muscle protein and hence N^T -methylhistidine contribution from these two tissues should not be overlooked (Nishizawa et al., 1977; Rennie and Millward, 1983; Ballard and Tomas, 1983).

Table 4. N^T-Methylhistidine Content of Actin and Myosin
from Different Species^a

Species	Muscle	N ^T -Methylhistidine	
		Actin*	Myosin**
Rat (growing)	Mixed skeletal	.78	.88
Rabbit (adult)	Hind leg	-	1.8
		1.3	-
		.88	1.63
		.86	-
Chicken (adult)	Mixed skeletal	.73-1.05	-
Mouse	Mixed skeletal	-	.78
Pigeon	Breast	-	.91
Lobster tail	Invertebrate Striated	.44	2.55
		-	1.8
Cow uterus	Smooth	.80	1.05
Sheep	Lateral thigh	-	1.7
Cat	Flexor hallucis longus	-	1.3
Fowl	Pectoralis	-	1.5
Cat	Soleus	.74	-

^a Adapted from Haverberg et al., 1974.

* Expressed as moles per 41,785 g of protein.

** Expressed as moles per 500,000 g of protein.

Huszar et al. (1983) found a sudden rise in N^T -methylhistidine excretion shortly after meat ingestion by man. Since free N^T -methylhistidine is excreted rapidly, this rapid rise was most likely due to rapid absorption of soluble N^T -methylhistidine from the meat.

f. N^T -Methylhistidine in Plants or Microorganism

Orchardgrass hay and concentrate (composed of corn, barley, bran, rice bran, soybean meal and salt mixture) contained 2.40 and 7.73 mg N^T -methylhistidine/kg (mean values of five determinations), respectively, according to Nishizawa et al., 1979. Since the mean intake was 1.58 kg/d for orchardgrass hay and 5.79 kg/d for concentrate, each animal (217 kg, 8-9 months) obtained approximately 49 mg N^T -methylhistidine/d from their feed (Nishizawa et al., 1979) and this is a physiologically significant quantity which will influence urinary N^T -methylhistidine output. These workers also found that rumen protozoa contained trace amounts of N^T -methylhistidine, but N^T -methylhistidine was not present in rumen bacteria. Rumen contents contained approximately .131 mg N^T -methylhistidine/kg fresh material. Feces contained $1.51 \pm .14$ mg N^T -methylhistidine/kg dry matter which suggested that cattle at 312 kg body weight voided 2.49 mg N^T -methylhistidine/d into feces. From feed intake and fecal excretion of N^T -methylhistidine, apparent digestibility of N^T -methylhistidine in the feed was calculated to be 94%. Almost 30% of the urinary

N^T -methylhistidine output was contributed by N^T -methylhistidine in the feed if N^T -methylhistidine from feed was neither degraded nor metabolized in the digestive tracts of the cattle.

More recent studies indicated that there was .21 mg N^T -methylhistidine per gram of dried mixed orchard-brome hay and no measurable amount of N^T -methylhistidine in corn silage (Wohlt et al., 1982). Previous workers found no N^T -methylhistidine in rumen bacterial hydrolysates, free N^T -methylhistidine does not occurs in the rumen liquor (Leibholz, 1968), but more recently others found .023 mg N^T -methylhistidine per gram of dry rumen bacteria (Wohlt et al., 1982). The limited results indicate that feeds can contribute significant quantities of exogenous N^T -methylhistidine to ruminants thus casting doubt on the validity of the N^T -methylhistidine procedure. To adequately determine the usefulness of N^T -methylhistidine as an index of myofibrillar protein turnover in ruminant animals, definitive studies must be conducted to determine if plants and microbes contain N^T -methylhistidine (Wohlt et al., 1982).

g. Analysis of N^T -Methylhistidine -- Early and Newer Methods

The potential of the N^T -methylhistidine measurement has been quickly realized in clinical studies (Young et al.,

1973) but with the obvious importance of lean meat in animal farming practice the technique is certain to have a vital role in agricultural research (Lobley and Harris, 1977). N^T -Methylhistidine is usually measured after separation from urinary metabolites on an ion-exchange column of an amino acid analyzer, or by high performance liquid chromatography (Ballard and Tomas, 1983). Haverberg et al. (1974) used a modification of the method of Spudich and Watt (1971) for purification of actin and consequent isolation and quantitation of N^T -methylhistidine in contractile proteins. Haverberg et al. (1974) described the procedures for isolation of purified actin and myosin from mixed skeletal muscle as well as preparative method for isolation of a concentrated N^T -methylhistidine fraction from protein hydrolysates for ion-exchange chromatography. The purity of the preparation was checked by the presence of a single band on both 5 and 10% sodium dodecylsulfate polyacrylamide gels according to the method of Weber and Osborn (1969).

Wassner et al. (1980) utilized precolumn derivatization and subsequent high-pressure liquid chromatographic separation of N^T -methylhistidine from urine and plasma, using a solvent of 10 mM sodium phosphate (pH 7.5) and an acetonitrile gradient (20-40% acetonitrile over 15 min at 1.5 ml/min using a concave curve). The fluorescence of N^T -methylhistidine decreased slightly as the concentration

of acetonitrile was increased and in the range 20-40% acetonitrile fluorescence was approximately 80% of maximal. This elution can be performed isocratically and requires less than 10 min. Both fluorescent and ultraviolet detection may be utilized. Wassner et al. (1980) also pointed out that this method is at least 10^3 times more sensitive than conventional ion-exchange chromatography using ninhydrin. Wassner et al. (1980) demonstrated that the use of this precolumn derivatization method and high-pressure liquid chromatographic separation of N^T -methylhistidine has several advantages over current ion-exchange chromatography techniques: First, only a single solvent is required, second, there is no need for column regeneration, and finally each run is several times faster than ion-exchange chromatography. The need for a second reagent pump is also eliminated when using precolumn rather than postcolumn derivatization. The specificity of the fluorescamine derivatives reaction eliminates the problems of coelution of other competing basic amino acid peaks when using ion-exchange chromatography and post-column derivatization. This method is extremely sensitive using fluorescamine detection with the limits of detection less than 600 fmol (600×10^{-15} mol).

Mattews et al. (1981) utilized a simple, sensitive method for measuring N^T -methylhistidine in biological

samples using a deuterated internal standard and methane chemical ionization gas chromatography-mass spectrometry. After sample preparation, a single analysis can be completed in 3 min, bringing the total time to 15 min per sample. Nanomole amounts of N^T-methylhistidine in urine or plasma sample are determined with a precision of .5%. Picomole amounts are measured with a precision of 10%. Matthews et al. (1981) pointed out that the primary advantage of the gas chromatographic-mass spectrometry method for measuring urinary N^T-methylhistidine is speed of analysis. Changing from ion-exchange chromatography to gas chromatography-mass spectrometry selected ion detection can improve the measurement of a small N^T-methylhistidine peak surrounded by other large peaks. The gas chromatography-mass spectrometry assay is ideal for measuring N^T-methylhistidine concentration in urine, plasma and muscle samples.

Murray et al. (1981) used a modification of the method of Nakamura and Pisano (1976); a modification of the reaction of fluorescamine with amines, which renders it specific for certain imidazoles. Interference due to histidine and histamine is selectively removed by prior reaction with aldehydes. The concentration of N^T-methylhistidine in human urine as determined by this technique correlate well with those determined by ion-exchange chromatography. The technique is rapid (6-7

samples/h), is reproducible, requires no prior treatment of the sample, and can be implemented with widely available diverse equipment (Murray et al., 1981).

Ballard and Tomas (1983) pointed out that since creatinine in urine is also measured conveniently with a relatively straightforward and rapid technique, the Technicon Auto-Analyzer, is usefull for quantifying the fractional rate of muscle protein breakdown both for clinical assessment of patients and for research purposes. A radioimmunoassay procedure has now been developed for N^T-methylhistidine (Bachmann et al., 1984). This should improve speed and specificity of the analytical scheme.

MATERIALS AND METHODS

The procedures used were modifications and combinations of several methods. First column chromatography was conducted according to Haverberg et al. (1974) to determine the qualitatively N^T-methylhistidine elution volume using pyridine/H₂O mobile phase. Second ion exchange chromatography analysis and total analytical system recovery after pyridine chromatography was used to quantify N^T-methylhistidine and establish the practical recovery method. Upon successful application of the methodology, feedstuffs analysis of N^T-methylhistidine followed. The feed samples were analyzed with and without external addition of N^T-methylhistidine. N^T-Methylhistidine in duodenal digesta from steers, was also determined, to assess the potential of ruminal microbes and bypass feed protein to contribute physiologically significant amounts of N^T-methylhistidine toward the urinary excretion. Further ruminal degradation of N^T-methylhistidine was evaluated using an in vitro rumen fermentation. Finally, red and white bovine muscles from young and finished cattle were analyzed for N^T-methylhistidine to assess muscle type and developmental effects on skeletal muscle N^T-methylhistidine content.

I. Pyridine-Column Chromatography Procedure

A 1.5 x 7.5 cm column of Dowex 50 W -X 8 (strongly cationic) resin of 200-400 mesh, was washed with 50 ml deionized H₂O to desalt the resin and equilibrated with 40 ml .2 M pyridine. These washings were discarded.

N^T-Methylhistidine elution from the Dowex 50 column with pyridine was evaluated by the following modification of the method of Haverberg et al. (1974). Two ml 1.0 mM N^T-methylhistidine (Sigma Chemical Company) in .01 N HCl diluted in 8 ml .2 M pyridine (final volume 10 ml) or 1 ml 1.0 mM N^T-methylhistidine in 4 ml .2 M pyridine (final volume 5 ml) were applied to columns as samples. Next, 100 ml .2 M pyridine were washed through the column (under N₂ pressure) and ten 10 ml fractions of column elutate were collected. This was followed by 125 ml 1 M pyridine elution, and hence 25 fractions of 5 ml were collected. A further 100 ml 1 M pyridine was then used as eluent and 10 tubes of 10 ml were collected. One ml aliquots from each collected fraction were checked for amino acid content by adding 2 ml ninhydrin solution (Appendix Table A. 1.). The samples were then boiled immediately for 15 min, cooled and visually inspected for dark purple color; the product of a α amino acid - ninhydrin reaction.

II. N^T-Methylhistidine Analysis by Ion Exchange

Chromatography and Total Analytical System Recovery

Various concentrations and samples of

N^T-methylhistidine and histidine were subjected to pyridine column chromatography, and the collected samples were analyzed by an amino acid analyzer to determine the pyridine elution pattern and recovery of N^T-methylhistidine.

Standard amino acid solutions were mixed in three varying combinations and applied to Dowex 50W - X8 columns as follows (Table 6) :

- 1) 1 ml 1.0 mM N^T-methylhistidine, 1 ml 1.0 mM histidine and 8 ml .2 M pyridine
- 2) 2 ml 1.0 mM N^T-methylhistidine, 1 ml 1.0 mM histidine and 7 ml .2 M pyridine
- 3) .5 ml 1.0 mM N^T-methylhistidine, 2 ml 1.0 mM histidine and 7.5 ml .2 M pyridine

The pyridine column chromatography procedure as used for samples above was repeated twice for each of the samples. After discarding the first 100 ml of .2 M pyridine from each column, 60 ml of eluent (1 M pyridine) were collected in a beaker. After air evaporating off the pyridine-H₂O, 1 ml αGPA (α-Guanidino Propionic Acid; Pierce Chemical Company) and 9 ml lithium citrate sample buffer (lithium citrate sample dilution buffer: .2 N Li⁺ pH 2.20 ± .01 contains 1% thiodiglycol and .1% phenol; Pierce Chemical Company) were added to the beaker. The solutions were recovered, filtered through a .2 μm metrical membrane filter (Gelman Science Inc., Ann Arbor, MI), and

samples were then applied onto the amino acid analyzer. A total of .1 ml sample was applied to the amino acid analyzer. The visible detector (570 nm) was at a setting of .5 AUFS (Absorbance Units Full Scale), the range for the analysis was 1-20 nmoles. The conditions of N^T -methylhistidine analysis were : Column, 26.5 cm by 3.2 mm i.d. containing DC 4A resin (Dionex Co.) with a mobile phase flow at 12 ml/h at 60°C. A four buffer step gradient was used to elute basic amino acids as follows: .25 N Li citrate, pH 2.7, 1 minute; .6 N Li citrate, pH 3.2, 5 minutes; 1.0 N Li citrate, pH 4.2, 40 minutes; and 1.3 N Li citrate, pH 5.1, 65 minutes. Postcolumn derivatization was achieved with ninhydrin added at 6 ml per hour.

III. Analysis Technique for Feedstuffs and

N^T -Methylhistidine Recovery Procedure

Twelve feed samples, alfalfa (*Medicago sativa* L.), beet pulp (*Beta vulgaris* L.), birdsfoot trefoil (*Lotus corniculatus* L.), corn silage (*Zea mays* L.), tall fescue (*Festuca arundinacea* Schreb.), high moisture corn (*Zea mays* L.), Kentucky bluegrass (*Poa pratensis* L.), orchardgrass (*Dactylis glomerata* L.), Italian ryegrass (*Lolium multiflorum* Lam.), soybean meal (*Glycine max* Merrill) and timothy (*Phleum pratense* L.) were analyzed. Except alfalfa, beet pulp, corn silage, high moisture

corn, soybean meal, the other 7 feeds were all cut by a competent person on the university farm in Fall 1983. All samples were dry and ground in a Wiley mill through a medium fine screen.

Initially crude protein content was analyzed for each sample with micro Kjeldahl digestion followed by NH_3 detection using the Nessler (hypochlorite) reaction. Twenty-five mg protein and 50 mg protein samples of each feed were weighed in duplicate for acid hydrolysis.

Hydrolysis of feedstuff samples was accomplished with 10 ml 6 N HCl at 121°C for 16 hours in sealed tubes previously flushed with nitrogen gas for 30 seconds. Following hydrolysis and cooling, hydrolysates were filtered through No. 1 filter paper (Whatman Inc, Clifton, NJ.) and washed with deionized H_2O ; the filtered samples were collected into 250 ml round bottom flasks and evaporated to dryness under vacuum with a rotary evaporator. The residues were resuspended 10 ml .1 N HCl. The filtrates were decolorized with a charcoal column (Appendix Table A.2.) as follows:
Samples were applied to the column and washed twice with 15 ml deionized H_2O , and the filtrates (elutates) were evaporated to dryness under vacuum with a rotary evaporator at 50°C .

The dried hydrolysates in the 250 ml round bottom flasks were then dissolved in 5 ml of .2 M pyridine and

applied to a Dowex 50W - X8 column (1.5 x 7.5 cm; 200-400 mesh, Bio-Rad Laboratories, Richmond, CA.). These column had been prewashed, desalted and equilibrated (50 ml H₂O; 40 ml .2 M pyridine) as describes above. The acidic and neutral amino acids were eluted with 100 ml of .2 M pyridine and the N^T-methylhistidine fraction was then eluted with 60 ml of 1 M pyridine. Other amino acids remaining on the column were eluted with an additional 125 ml of 1 M pyridine; this fraction was discarded.

The N^T-methylhistidine fraction was evaporated by rotary evaporator (50°C water bath) to near dryness and the residue was dissolved in 1 ml lithium citrate sample buffer, filtered through a .2 µm filter and then analyzed by ion-exchange chromatography.

The N^T-methylhistidine content and recovery of externally added (spiked) N^T-methylhistidine from alfalfa hay and hydrolysis was determined using the procedures outlined above with 6 various combinations of treatments in duplicate as follows:

- 1) Samples containing 25 mg protein of hydrolyzed alfalfa hay and mixed with 1 ml .1 mM N^T-methylhistidine
- 2) Samples containing 25 mg protein of hydrolyzed alfalfa hay
- 3) Samples containing 50 mg protein of hydrolyzed alfalfa hay and mixed with 1 ml .1 mM N^T-methylhistidine
- 4) Samples containing 50 mg protein of hydrolyzed alfalfa hay

- 5) Samples containing 100 mg protein of hydrolyzed alfalfa hay

The hydrolyzed samples were exposed to charcoal cleanup and pyridine chromatography.

- 6) 1 ml .1 mM α GPA, 1 ml .1 mM N^T -methylhistidine

Two further combinations of amino acids were used (7 and 8) to obtain recorder response values to quantitate combinations 1-6.

- 7) 1 ml .1 mM α GPA, 1 ml .1 mM N^T -methylhistidine were mixed, was injected into analyzer directly

- 8) 1 ml .1 mM α GPA, 1 ml .1 mM N^T -methylhistidine were mixed and diluted with 2 ml .01 N HCl and injected on the amino acid analyzer directly.

IV. N^T -Methylhistidine Analysis of Duodenal Digesta from Steers

The duodenal samples were obtained from Dr. William Vernon Rumpler's research samples, which were obtained by the following collection procedure (Rumpler, 1984):

At each sampling time a duodenal digesta sample (350 ml) was obtained and frozed until composited. Compositing of duodenal samples involved homogenization of the whole digesta sample in a large blender after thawing. Equal amounts (100 g) of each wet homogenate were then added to a composite sample at the end of the four day collection period. Duodenally fistulated Holstein steers

were fed four different diets, as followed:

- A. 31% DM alfalfa haylage
- B. 31% DM alfalfa haylage plus high moisture corn (HMC)
- C. 45% DM alfalfa haylage
- D. 45% DM alfalfa haylage plus HMC

These four duodenal digesta samples represent each of the four diets from block 1 of a 4 x 4 latin square. It was not felt to be necessary to analyze all 16 duodenal samples for all 4 blocks. The duodenal samples were weighed into hydrolysate tubes and N^T-methylhistidine content determined as detailed for the feed samples except recovery studies were not performed.

V. Ruminal Degradation of N^T-Methylhistidine in vitro

Ruminal catabolism in vitro of leucine and N^T-methylhistidine was determined with rumen fluid collected before and 4 hours after feeding. The rumen fluid donor was a rumen fistulated crossbred steer (640.9 kg) fed once daily 16 kg corn silage with .69 kg of supplement 8201 (Table 5).

Rumen fluid was collected once weekly over a four week period. Two samples were obtained before feeding and two samples four hours after feeding. After collection, the rumen contents were strained and squeezed through 2 layers of cheesecloth into a thermos. Approximately 200 ml of strained rumen fluid (SRF) was collected every time.

Table 5. 8201 Supplement Composition

Item	International Reference No.	%
Soybean meal	5-04-604	84.7
Dical (Ca_2PO_4)	6-01-069	7.0
Trace mineral salt	-	4.0
Limestone (CaCO_3)	6-02-632	1.7
Selenium 90	-	2.2
Vit A ^a	-	>
Vit D ₃ ^b	-	
Vit E ^c	-	

^a Vit A palmitate, 60,000 IU/kg of final supplement.

^b Vit D₃, 4,500 IU/kg of final supplement.

^c Vit E, 500 IU/kg of final supplement.

The in vitro rumen fermentation was initiated by mixing 50 ml SRF and 50 ml Ohio buffer (prepared according to Appendix Table A. 3.) and subsequently adding N^T -methylhistidine and leucine to a final concentration of 1 mM. The SRF-Ohio media mixture was then bubbled with CO_2 for 15 minutes and incubated with a Bunsen stopper at $39^{\circ}C$ in a water bath. Samples were obtained from the fermentation at 0, 1, 2, 4, and 8 hrs. Samples were deproteinized with .1 volume of 50% sulfosalicylic acid (SSA), stored in ice for 30 min, centrifuged at 15,000 xg for 30 min and the protein free filtrate was collected. The filtrates were diluted 10 x with .01 N HCl, then 1 ml sample was mixed with 1 ml .1 mM α GPA and filtered through a .2 μ m membrane filter. Leucine and N^T -methylhistidine were determined with the amino acid analyzer as outlined above.

VI. N^T -Methylhistidine Content of Bovine Muscles

Four different muscles were taken from three veal calves (38.6 kg, 1 wk of age; 70.9 kg, 17 wks of age; 227.3 kg, 33 wks of age) and three finished steers (518.2 kg, 18 months; 559.1 kg, 18 months; 587.3 kg, 18 months) after slaughter. The muscles removed were longissimus dorsi (LD, white muscle), gluteus medius (GM, white muscle), gluteus profundus (GP, red muscle), and vastus intermedius (VI, red muscle). Muscle tissues were finely sliced; .5 g

of each sample was weighed in duplicate for HCl hydrolysis followed the pyridine-column chromatography procedure and the N^T-methylhistidine analysis as outlined above. A total of 48 muscle samples were analyzed for N^T-methylhistidine content.

VII. Statistical Analysis

To evaluate the statistical differences among muscles, a one way analysis of variance was conducted (Gill, 1978). Bonferroni t statistic was used to compare the difference of N^T-methylhistidine between veal and steer muscles. In addition, it was used to compare the difference of N^T-methylhistidine concentration between red and white muscles; if $P < .1$ the level of statistical significance was reported. $P < .05$ was determined as being significant. Statistical analysis (ANOVA) was achieved with a program written for the Hewlett Packard 9825A.

RESULTS AND DISCUSSION

I. Pyridine Column Chromatography

Figure 6 shows Dowex 50 W - X 8 chromatography of N^T -methylhistidine, histidine and α GPA with pyridine-water eluents. N^T -Methylhistidine was visualized in the tubes 5-8 or in 20-40 ml elution volume of initial 1 M 125 ml pyridine fraction. No ninhydrin-amino acid reaction was seen in the initial 100 ml .2 M pyridine elution and in the additional 1 M pyridine elution.

In the next experiment, 2 ml 1 mM histidine was dissolved in 8 ml .2 M pyridine and applied to the Dowex column and the pyridine elution was followed as for N^T -methylhistidine. Figure 6 also shows histidine was found in the tubes 17-22 or in elution volume 85 to 110 ml of the 1 M pyridine eluent. Likewise, 2 ml 1.0 mM α GPA was dissolved in 8 ml .2 M pyridine and applied to Dowex column and eluted with pyridine as above. α GPA was found in tube 25 or in elution volume 120 to 125 ml of 1 M pyridine eluent (Figure 6).

II. Amino Acid Analysis and N^T -Methylhistidine Recovery

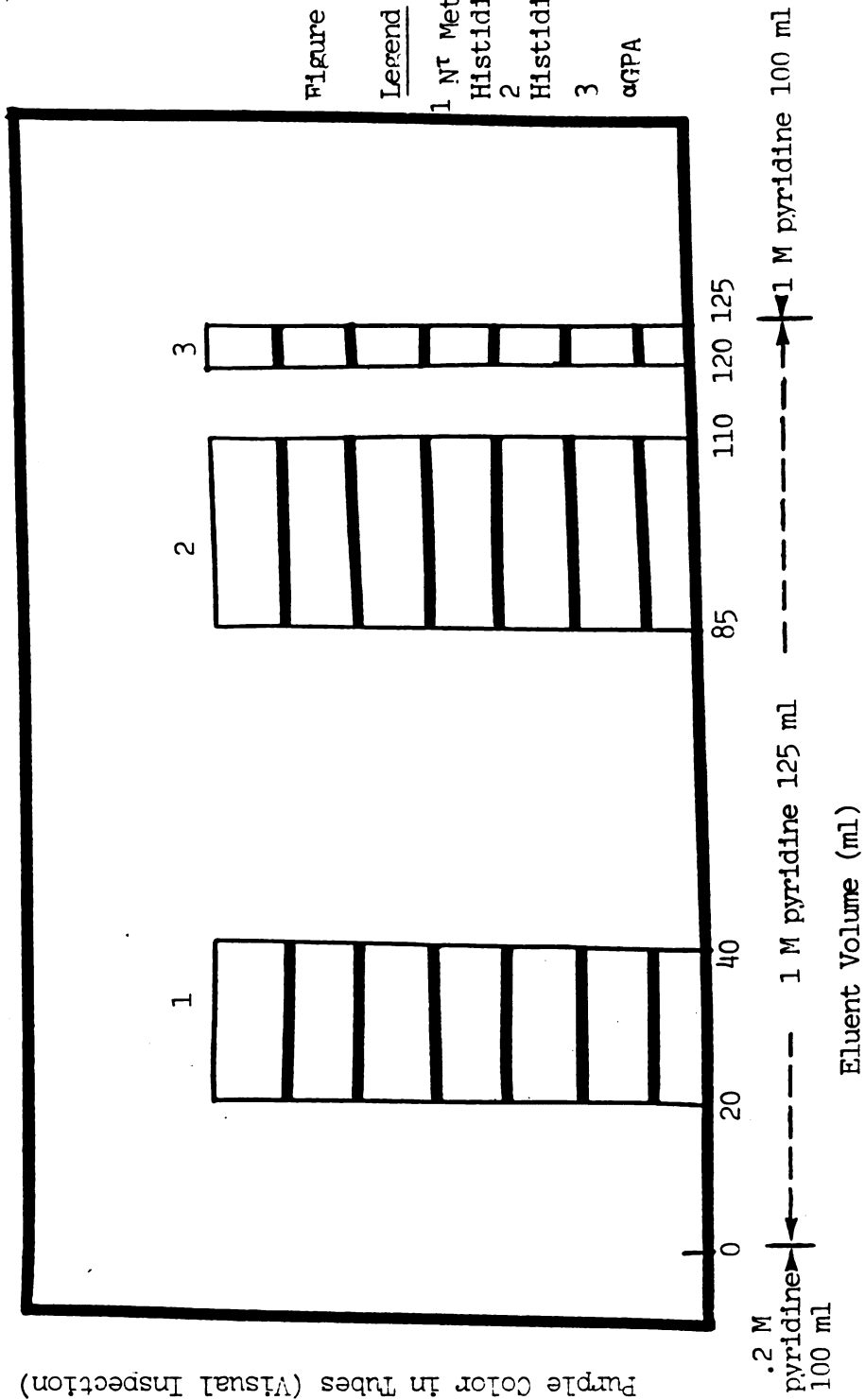
Ion-exchange chromatography was utilized to confirm that the N^T -methylhistidine elution volume indicated in

Figure 6. Dowex 50 W - X8 Chromatography of
N⁺-Methylhistidine, Histidine and αGPA
with Pyridine-Water Eluents.

Figure 6.

Legend

- 1 N⁺ Methyl
Histidine
- 2 Histidine
- 3 α GPA



the preliminary chromatography; that is, N^T -methylhistidine was found in the first 60 ml of elution after the 1 M pyridine was started.

Figure 7 shows that no amino acid was present in the 100 ml .2 M pyridine fraction, only the internal standard α GPA added after the pyridine chromatography is seen on the amino acid chromatogram.

Figure 8 shows that N^T -methylhistidine was in the first 60 ml 1 M pyridine fraction, and Figure 9 shows histidine was in the 80 to 125 ml of the 1 M pyridine fraction. There were no further ninhydrin positive compounds (amino acids) in the additional 100 ml of the 1 M pyridine elution volume (Figure 10).

N^T -Methylhistidine recovery from the Dowex-pyridine chromatography in three combinations of standards is shown in Table 6, the recovery range was 89-102 percent. The actual chromatogram of standards (1 : 1 : 1 of .1 mM N^T -methylhistidine, .1 mM α GPA and 10 mM histidine analyzed with the amino acid analyzer) is shown in Figure 11. The chromatogram of standards (1 : 1 of .1 mM α GPA and 10 mM histidine analyzed with the amino acid analyzer) is shown in Figure 12, and the chromatogram of standards (1 : 1 of .1 mM N^T -methylhistidine and .1 mM α GPA analyzed with the amino acid analyzer) is shown in Figure 13.

Figure 7. The Chromatogram of 100 ml .2 M Pyridine Elution
in Amino Acid Analyzer.

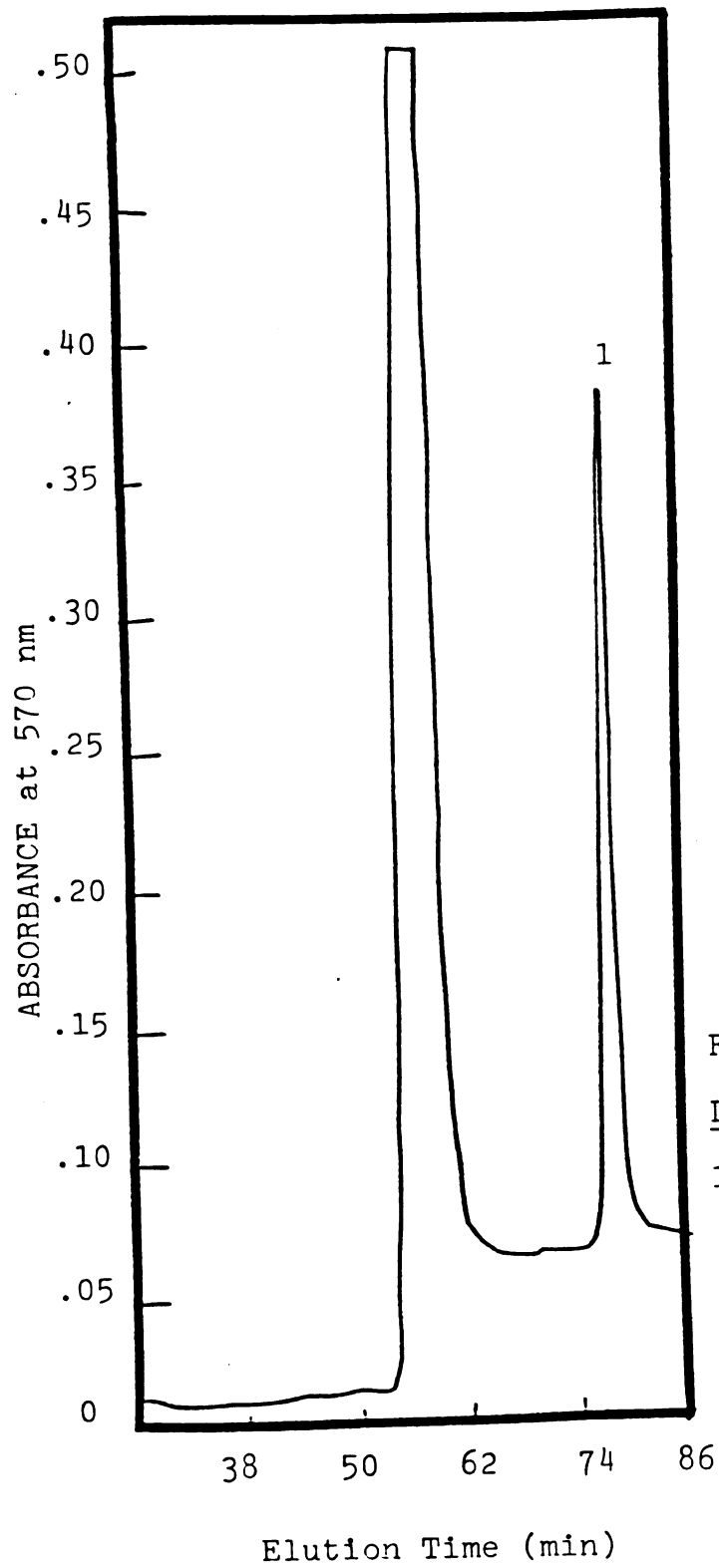


Figure 8. The Chromatogram of Initial 125 ml 1 M Pyridine
Elution with N^T-Methylhistidine Elutes in
40-60 ml Fraction.

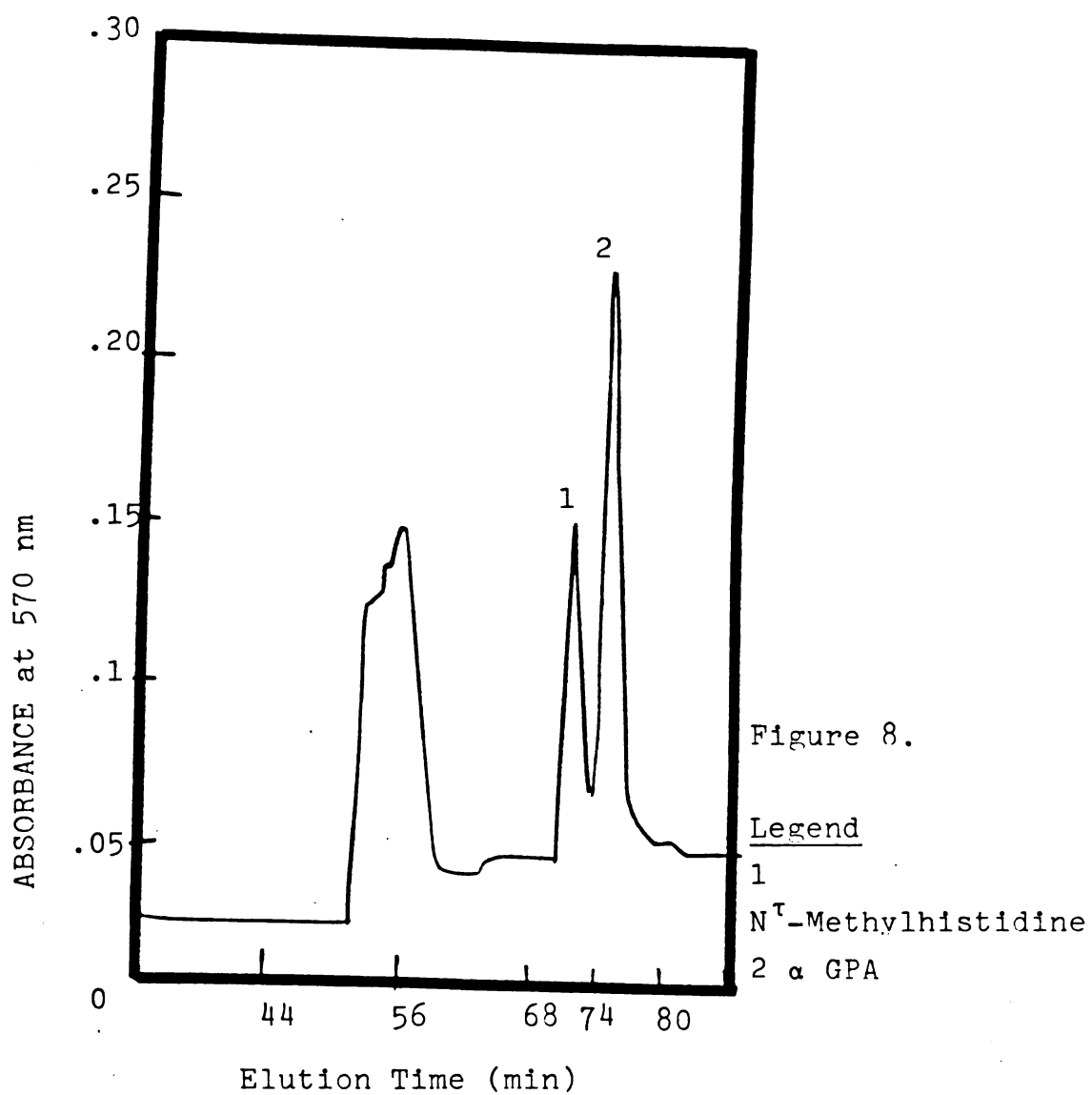


Figure 9. The Chromatogram with Histidine Elutes in
80-125 ml Fraction of 125 ml 1 M Pyridine
Elution.

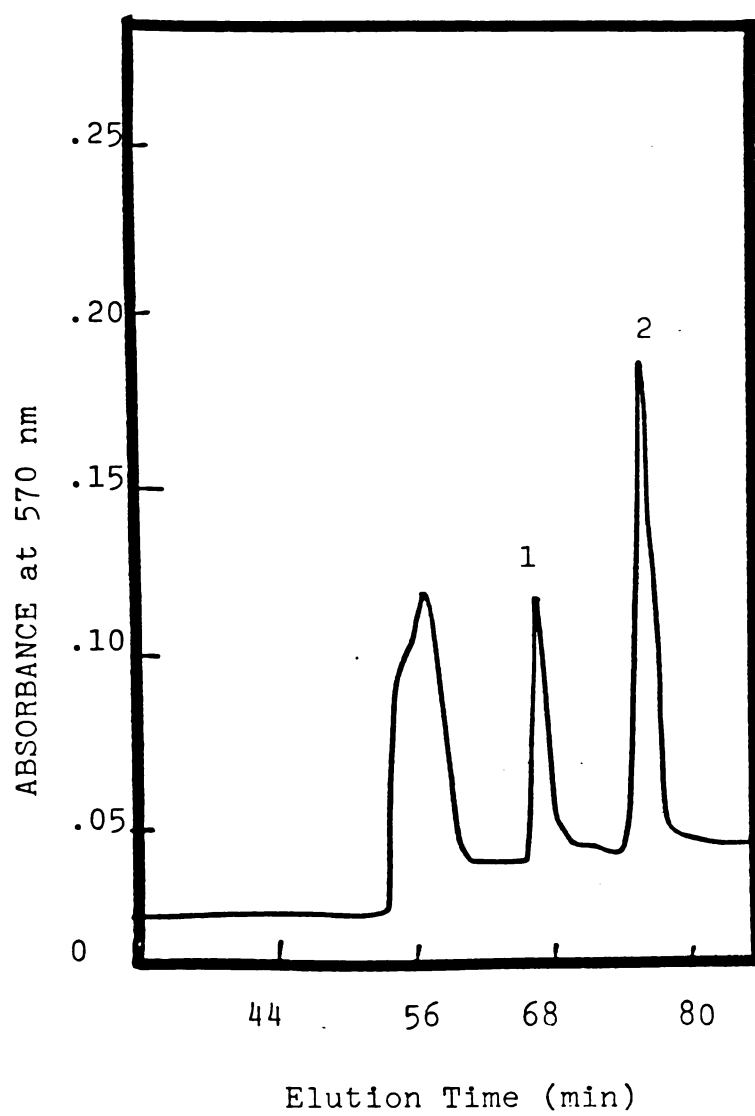


Figure 9.

Legend

1 Histidine

2 α GPA

Figure 10. The Chromatogram of an Additional 100 ml 1 M
Pyridine.

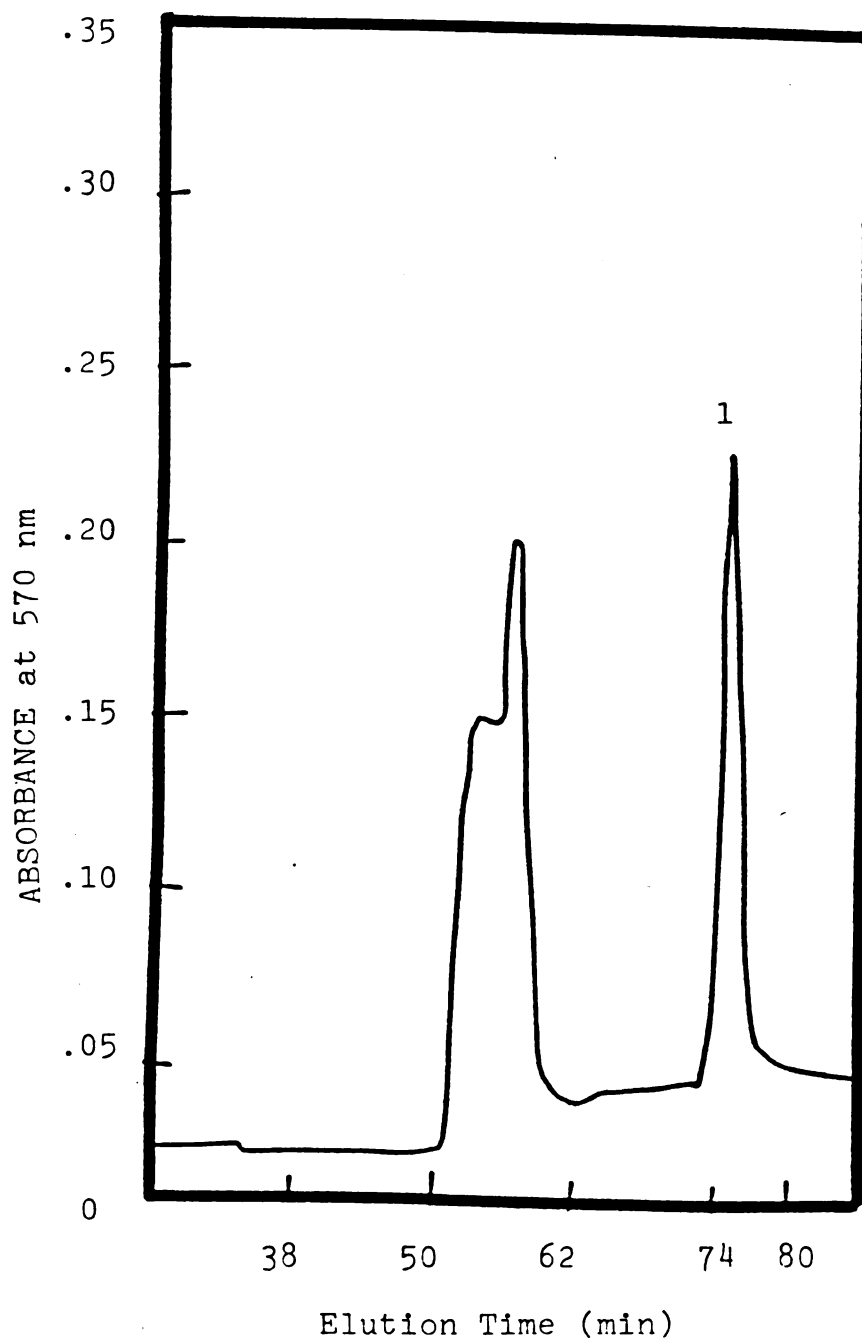


Figure 10.

Legend

1 αGPA

Table 6. N^T -Methylhistidine Recovery from Dowex 50 W - X 8
Column^a

Standard Concentration ^b		Three Combinations		
		1	2	3
		ml		
N^T -Methylhistidine	1.0 mM	1	2	.5
Histidine	1.0 mM	1	1	2
Pyridine	.2 M	8	7	7.5
Recovery (%)		95	102	89

^a N^T -Methylhistidine quantitated by ion exchange chromatography with amino acid analyzer.

^b See experimental procedures on page 34.

Figure 11. Chromatogram of Combination of .1 mM
N^τ-Methylhistidine, .1mM αGPA and 10 mM
Histidine Mixed 1/1/1 (V/V/V).

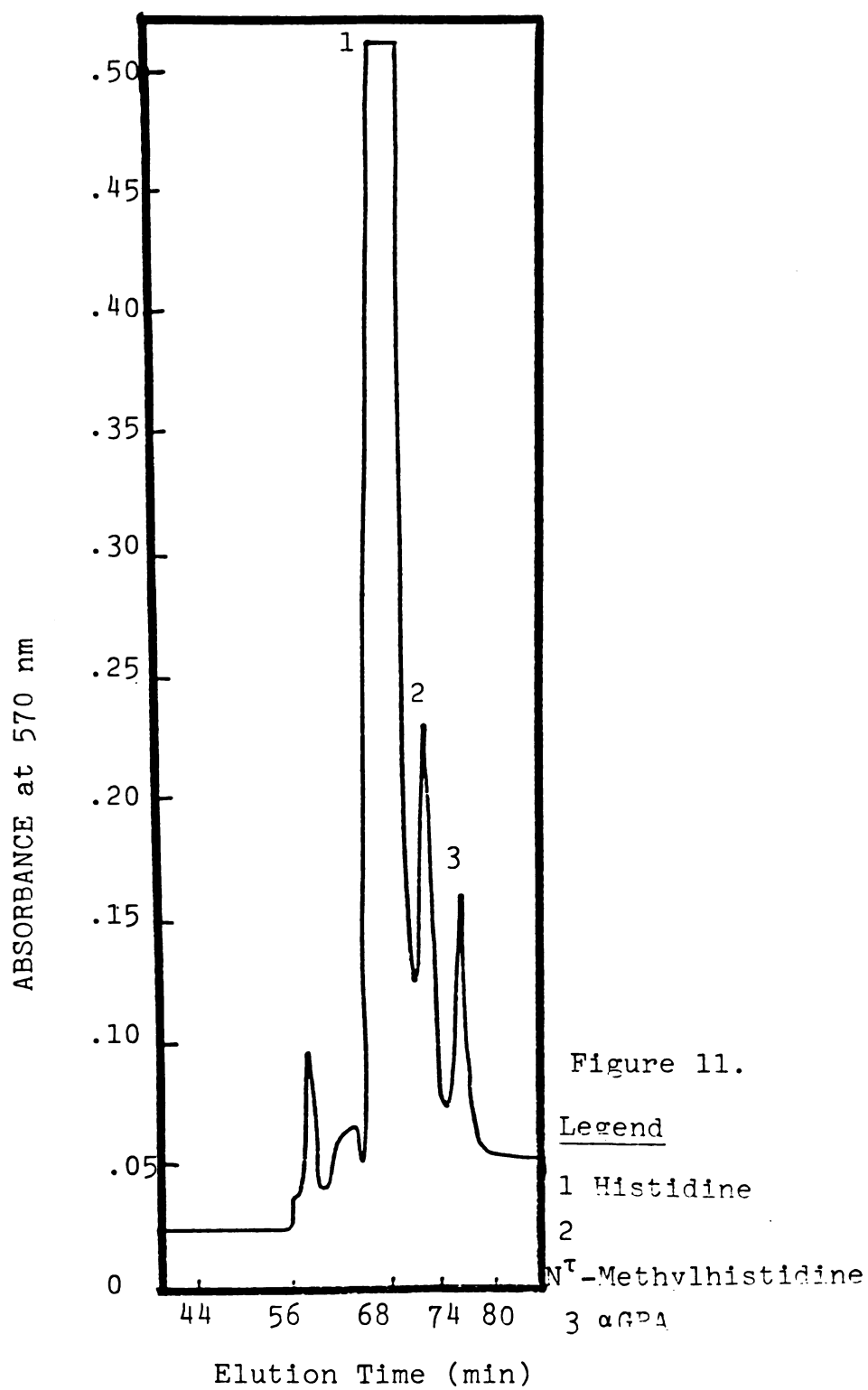


Figure 12. Chromatogram of .1 mM α GPA and 10 mM Histidine.

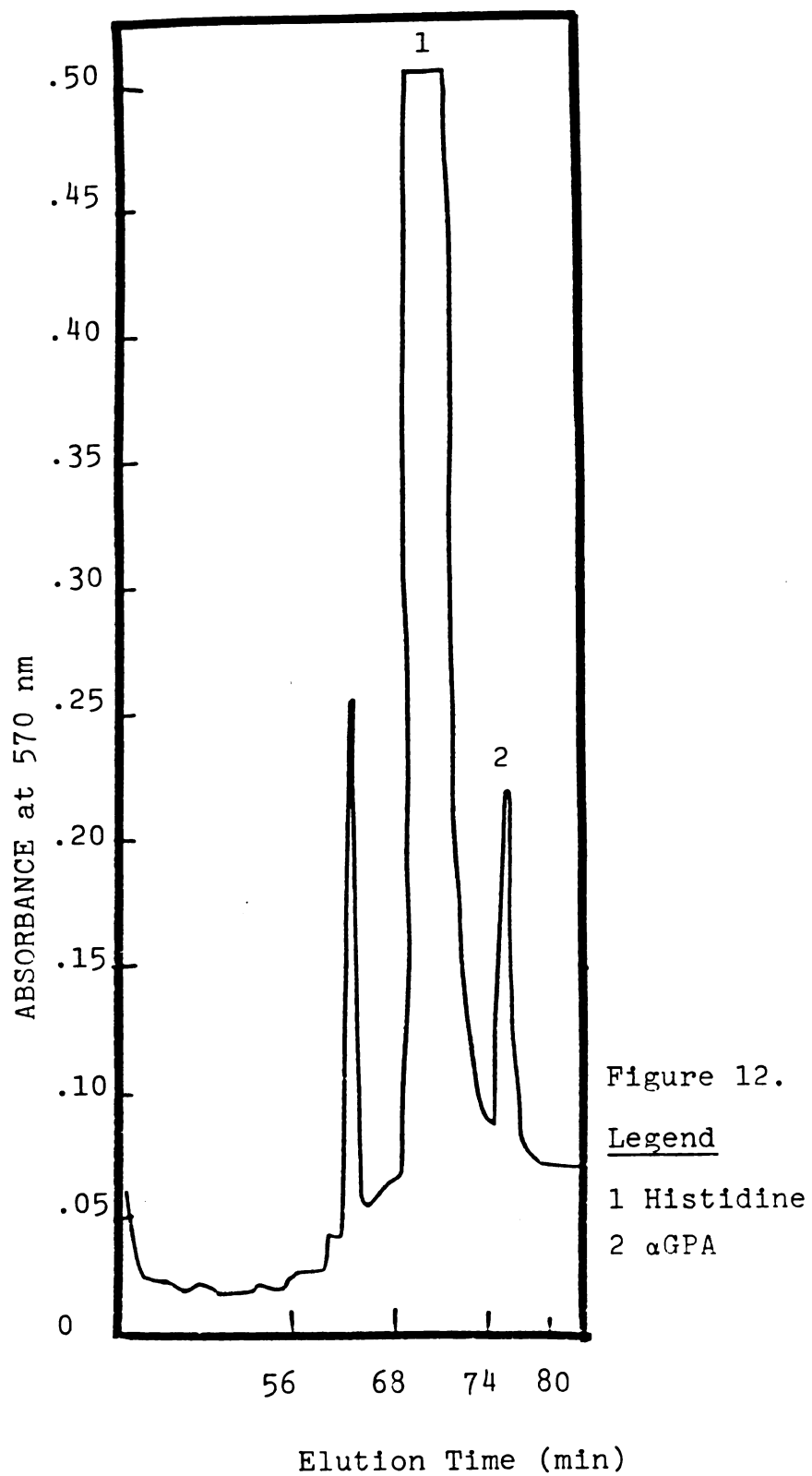


Figure 13. Chromatogram of .1 mM N^T-Methylhistidine and
.1 mM αGPA.

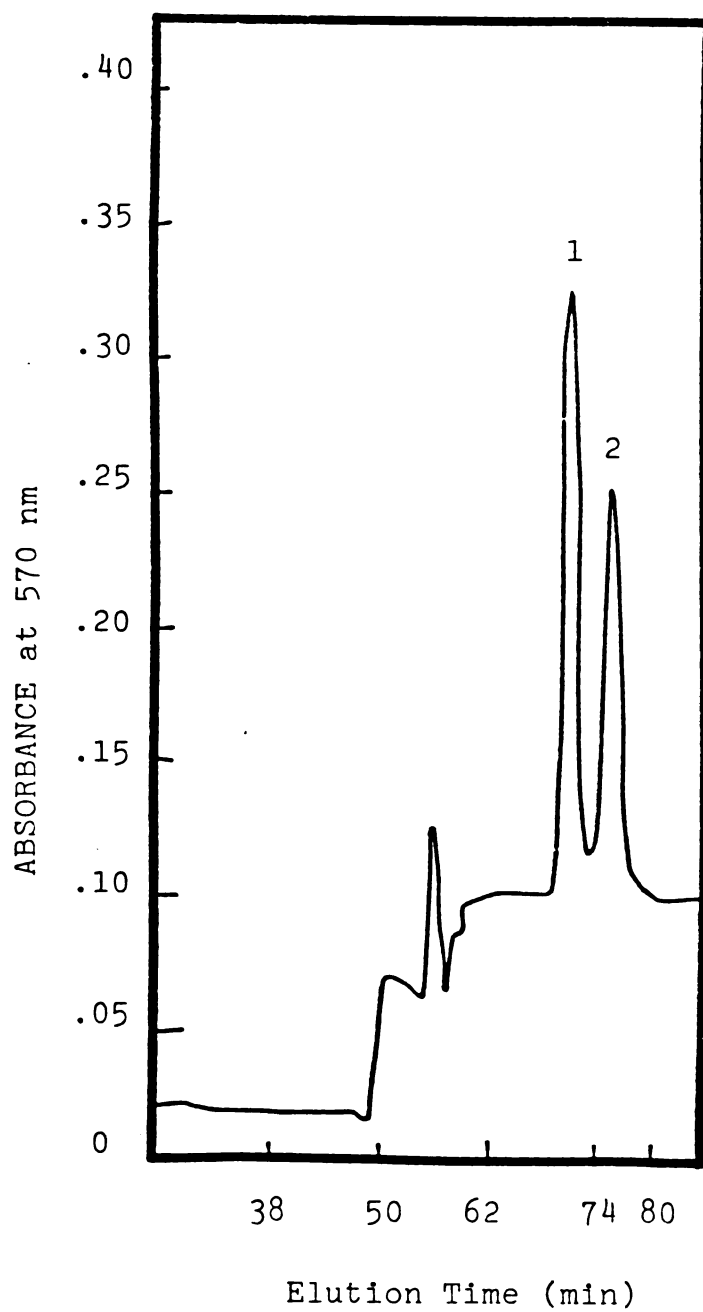


Figure 13.

Legend

- 1
N^T-Methylhistidine
2 αGPA

III. The Concentration of N^T-Methylhistidine in Feedstuffs

A. N^T-Methylhistidine Recovery in Alfalfa Hay Samples

The N^T-methylhistidine recovery from alfalfa hay was determined with 8 different experimental approaches as presented in Table 7. For experimental approaches - combinations 1, 3, 6, 7, 8, N^T-methylhistidine recovery ranged from 95-102%. In the second, fourth and fifth combinations, which were alfalfa hydrolysates without external addition of N^T-methylhistidine, the peak noted for N^T-methylhistidine was either nondetectable or very small.

B. N^T-Methylhistidine in Feedstuffs

The N^T-methylhistidine content for 12 of feedstuffs was determined and the results are given in Table 8. Alfalfa, beet pulp, birdsfoot trefoil, corn silage, high moisture corn, orchardgrass, soybean meal and timothy all contained very small amounts of N^T-methylhistidine (range from .4 to 2.15 nmole per gm of DM). N^T-Methylhistidine was not detected in brome grass, fescue, Kentucky bluegrass and ryegrass.

The potential daily contribution of dietary N^T-methylhistidine intake (assuming no degradation) to the animal can be estimated from the following formula:

$$\begin{aligned} \text{Daily N}^T\text{-methylhistidine intake} &= \text{Daily feed intake (g)} \\ &\quad \times \text{N}^T\text{-methylhistidine} \\ &\quad (\text{nmol/g DM}) \end{aligned}$$

Table 7. N¹-Methylhistidine Recovery of Alfalfa Hay

Sample	Concentration	Experimental Approaches "Combination"							
		1	2	3	4	5	6	7	8
		"Spike" ^a		"Spike" ^a					
Alfalfa hay	25 mg protein	x	x	-	-	-	-	-	-
hydrolysate	50 mg protein	-	-	x	x	-	-	-	-
	100 mg protein	-	-	-	-	x	-	-	-
N ¹ -Methylhistidine	.1 mM	1	-	1	-	-	1	1	1
αGPA	.1 mM	-	-	-	-	-	1	1	1
HCl	.01 N	-	-	-	-	-	-	-	2
Pyridine	1 M	60	60	60	60	60	60	-	-
Recovery (%) of N ¹ -Methylhistidine		95	-	102	-	-	100 ^b	100 ^b	100 ^b

^a "Spike" N¹-Methylhistidine was added to hay hydrolysate prior to pyridine column chromatography.

^b Standards, assumed to 100%.

Table 8. Crude Protein and N¹⁵-Methylhistidine of Feedstuffs

Item	Scientific Name	International Reference No.	Crude Protein	N ¹⁵ Methyl histidine	
				- % -	-nmol/g DM-
Alfalfa	Medicago sativa L.	1 08 868	19.34		1.96
Beet pulp	Beta vulgaris L.	4 00 669	7.50		1.45
Birdsfoot trefoil	Lotus corniculatus L.	1 08 853	18.40		.87
Bromegrass	Bromus inermis Leyss.	1 05 679	16.25		ND ^a
Corn silage	Zea mays L.	3 02 822	8.00		1.27
Fescue, tall	Festuca arundinacea Schreb.	1 01 904	11.83		ND ^a
High moisture corn	Zea mays L.	4 02 915	9.77		1.13
Kentucky bluegrass	Poa pratensis L.	1 00 802	8.77		ND ^a
Orchardgrass	Dactylis glomerata L.	1 07 716	15.70		.39
Ryegrass, Italian	Lolium multiflorum Lam.	2 04 070	20.04		ND ^a
Soybean meal	Glycine max Merrill	5 04 604	48.56		2.15
Timothy	Phleum pratense L.	1 05 024	11.43		.55

^aND = Not Detected.

For a 300 kg steer consuming 2% of body weight of soybean meal.

$$300 \text{ kg} \times 2\% = 6 \text{ kg} = 6,000 \text{ g}$$

$$6,000 \text{ g} \times 2.15 \text{ nmol/g DM} = 12,900 \text{ nmol} = 12.9 \text{ } \mu\text{mol}$$

Since normal urine N^T-methylhistidine excretion range from 1-4 mmol/d (Benner , 1983; McCarthy et al., 1983), the potential contribution from feedstuffs is less than 1.5% of urinary excretion.

Nishizawa et al. (1978) reported that they found significant amounts of N^T-methylhistidine in maize, soybean meal, oats, wheat, rice and lucerne meal but did not report the actual values in their paper. Wohlt et al. (1982) showed that dried mixed orchard-brome grass hay contained .021 mg N^T-methylhistidine/gm but no measurable amounts of N^T-methylhistidine were found in corn silage.

Using the same assumptions about a 300 kg steer consuming 2% body weight of feed, as above, the Wohlt et al. (1982) values for orchard-brome grass hay N^T-methylhistidine content would result in an increase of urinary N^T-methylhistidine excretion of .72 mmol daily or a 18-72% equivalent of the normal daily N^T-methylhistidine excretion (Table 9).

A standard chromatogram of 1 : 1 mix, .1 mM N^T-methylhistidine and .1 mM αGPA is shown in Figure 13. The chromatogram of a 25 mg protein hydrolysate from timothy is shown in Figure 14. Figure 15 shows the

Table 9. Calculation of Urinary N^T-Methylhistidine

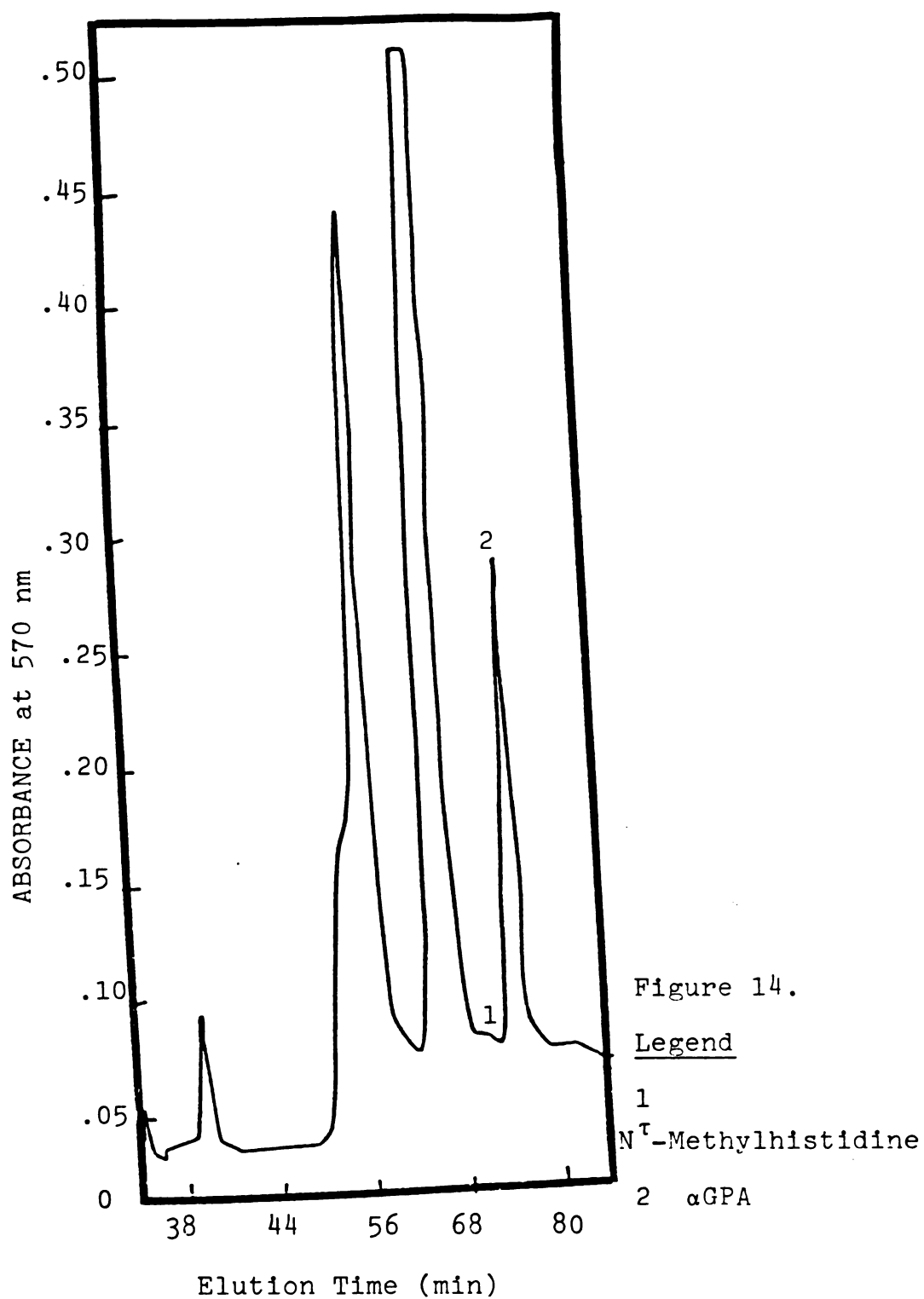
Excretion in a Steer Fed Dried Mixed Orchard-Bromegrass
Hay^a

-
-
1. Steer, body weight = 300 kg
 2. Feed intake, 2% body weight = 6,000 g
 3. N^T-Methylhistidine .12 μ mol/g dry feed
 4. 6,000 g x .12 μ mole/g dry feed = .72 mmol /d
 5. Normal urinary N^T-methylhistidine excretion = 1-4 mmol
per day
 6. Contribution from orchard-bromegrass = 18-72 % of normal
urinary N^T-methylhistidine excretion
-

^a

Based on values from Wohlt et al. (1982).

Figure 14. Chromatogram of 25 mg Protein Timothy Hay.



timothy with a external spike of 1 ml of .1 mM N^T -methylhistidine. Further the N^T -methylhistidine was also not appearing in basic amino acid chromatograms at another retention time (Figure 15) confirming that N^T -methylhistidine was not missed. It can be noted that .55 nmol/g DM of N^T -methylhistidine was detected in the original feedstuff (Table 8). According to the calculation of these two experimental data, Wohlt et al. (1981) result .72 mmol N^T -methylhistidine per day for 300 kg steer consuming 2% body weight orchard-brome grass is 56 times higher than the result of present study 12.9 μ mol N^T -methylhistidine per day for 300 kg steer consuming 2% body weight of soybean meal, as well as 218 times higher than feeding 300 kg steer of timothy hay. Hence it is clear that the feedstuff of present study does not contribute significant amounts of N^T -methylhistidine.

IV. N^T -Methylhistidine Content in Duodenal Digesta of Steers

N^T -Methylhistidine was determined in four composite duodenal samples from block 1 of a 4 x 4 latin square digesta passage study (Rumpler, 1984). The results of these analyses are presented in Table 10.

The duodenal samples contained 16-24 % crude protein and N^T -methylhistidine was detected only for the 45% DM alfalfa haylage-high moisture corn diet, the

Figure 15. Chromatogram of External "Spike" of
N^r-Methylhistidine 25 mg Protein Timothy Hay
Hydrolysate.

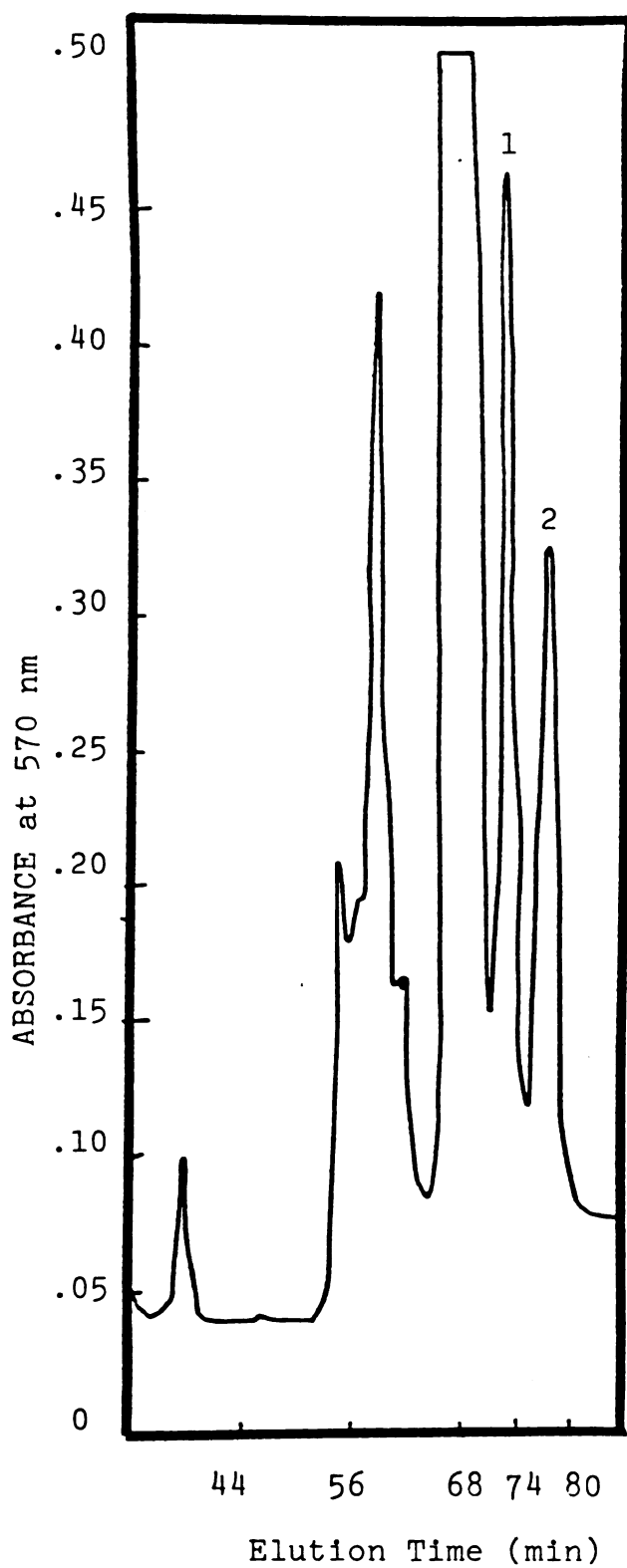


Figure 15.

Legend1 N^T -Methylhistidine2 α GPA

Table 10. N^r-Methylhistidine, Nitrogen and Crude Protein Content in Duodenal Digesta from Steers^a

Duodenum Sample	Diet Combination	Nitrogen	Crude Protein	N ^r -Methylhistidine
		— % —		— nmole/g —
1	31% DM alfalfa haylage	2.6	15.9	ND ^b
2	31% DM alfalfa haylage + HMC	3.2	19.8	ND ^b
3	45% DM alfalfa haylage	3.8	23.8	ND ^b
4	45% DM alfalfa haylage + HMC	3.6	22.2	1.2

^a Dry matter basis.

^b ND = Not Detected.

N^T-methylhistidine was below detection limits (.2 nmol) for the other three digesta samples (Table 10). The daily dry matter flow for steers fed the 45% DM alfalfa-HMC diet was 3,269 g and hence the upper level of N^T-methylhistidine flow was 3.9 μ mol/d (Rumpler, 1984; Appendix Table A. 5.). This value is far below the daily urinary excretion of N^T-methylhistidine. The conclusion can thus be reached that, at least for alfalfa haylage-HMC diets, neither ruminal microbes nor the feeds contribute a physiological significant amount of N^T-methylhistidine to cause increased urinary N^T-methylhistidine excretion which in turn result in overestimated fractional breakdown rates of skeletal muscle (McCarthy et al., 1983).

The duodenal digesta chromatogram confirms that no significant amount of N^T-methylhistidine is present (Figure 16) and internal standard α GPA is appeared in the same retention time as above.

V. N^T-Methylhistidine Degradation by Ruminal Microorganism In Vitro

Rumen fluid obtained before and 4 hours after feeding and N^T-methylhistidine and leucine (at 1 mM) were incubated with rumen fluid and a buffer for 0 and 8 hours. Analysis for both amino acids was aided by the fact that the rumen fluid alone (diluted 1:20) contained no detectable amino acids (Hungate, 1966; Bergen, unpublished

Figure 16. Chromatogram of Duodenal Digesta Sample.

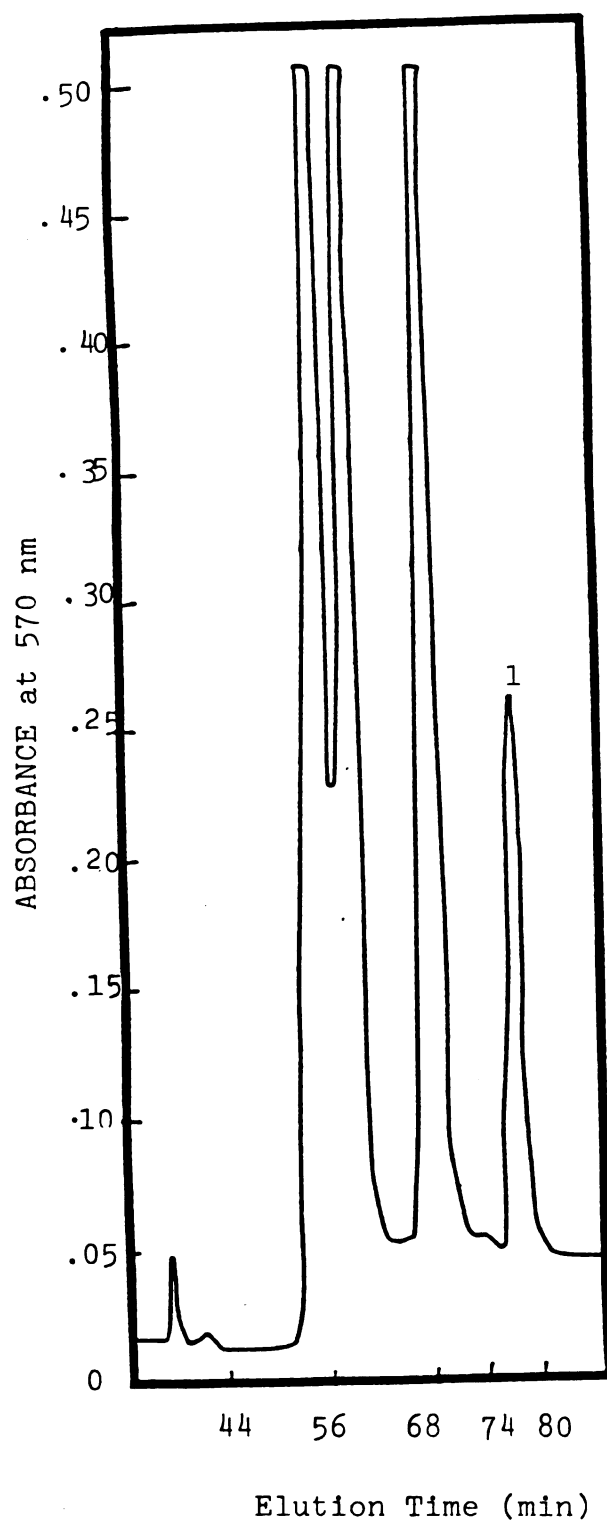


Figure 16.

Legend1 α GPA

observation) and hence dilute SRF blanks were not run.

Amino acid degradation was calculated from the formula:

$$\frac{\text{Initial (0 hour) Conc.} - \text{Final (8 hour) Conc.}}{\text{Initial (0 hour) Conc.}} \times 100\%$$

Results of this experiment are given in Table 11.

After an 8 hour incubation, leucine was not detected on the chromatograms and its degradability was therefore 100% (Table 11). The rate of degradation for N^T-methylhistidine was slight and not influenced by the source of rumen inocula. These results indicate that if a feedstuff contains high levels of N^T-methylhistidine this amino acid would escape ruminal catabolism in vivo, be absorbed from the small intestine and could thus lead to errors in the calculation of FBR based on urinary N^T-methylhistidine excretion.

Previous workers reported that free N^T-methylhistidine probably does not occur in the rumen liquor (Leibholz, 1965) or in hydrolysed rumen bacteria (Bergen and Potter, 1971). Our preliminary work with blank-diluted rumen liquor also detected no N^T-methylhistidine. This study shows that N^T-methylhistidine degradation in rumen fluid is only 14.5%, while typical protein bound amino acids, like leucine, not surprisingly are completely degraded under the same incubation conditions.

Table 11. The Degradation of BCAA-Leucine and
N^T-Methylhistidine After Incubation for 8 Hours
of Rumen Fluid Collected Before Feeding and
4 Hours After Feeding^a

Amino Acid	Degradation	
	Source of Rumen Fluid	
	Before Feeding	4 Hours Post Feeding
	%	
N ^T -Methylhistidine	14.6	14.3
Leucine	100	100

^a

Each value is the mean of two separate in vitro
fermentations.

VI. N^T-Methylhistidine in Bovine Muscles

The results for this experiment are presented by age of cattle and across muscle types (red or white) in Table 12 and strictly as a mean concentration of the four muscles analyzed for each age group in Table 13.

The LD and GM muscles (white) of calves and slaughter (beef) steers had an overall mean N^T-methylhistidine content of .68 $\mu\text{mol/g}$ fresh tissue. The N^T-methylhistidine content of GP muscle of the veal was significantly lower ($P < .05$) than the value for the slaughter steers (Table 12), but for the other red muscle (VI), veal only showed a marked numerically lower, but nonsignificant, N^T-methylhistidine content than that observed in steers.

When all four muscle samples were grouped for the young and finished slaughter steers, the mean total muscle N^T-methylhistidine content tended to be significantly lower in the young than the finished steers, however the samples from the 1 week old veal calf gave average N^T-methylhistidine value near typical of the older cattle (Table 13).

Table 14 shows that N^T-methylhistidine concentration in adult human skeletal muscle has been established at approximately 4.2 μmol per g mixed muscle protein and 3.2 $\mu\text{mol/g}$ protein for infants, but probably considerably less in the fetus due to the lack of methylation of fetal

Table 13. The N¹-Methylhistidine Content at Different Ages of Cattle

Age	Body Weight	N ¹ -Methylhistidine Content ^{**}	
	-kg-	μmol/g Protein -	μmol /g fresh tissue -
Veal			
1 week	38.6	3.25 _b	.65 _b
17 weeks	70.9	2.49*	.50*
33 weeks	227.3	2.69	.54
SEM		.34	.07
Beef			
18 months	518.2	3.69 _a	.74 ^{a*}
18 months	559.1	3.61 _a	.72 ^a
18 months	587.3	3.40	.68
SEM		.13	.03

^a ^b Means within column with different superscripts differ (P< .05).

* P < .1 .

** Values are means and SEM.

Table 14. N¹-Methylhistidine Content of Mixed Proteins in Muscles from Human^a

Human	Sample No.	Muscle Type	N ¹ -Methylhistidine
Fetus (20-40 weeks)	14	Mixed quadriceps	1.7
Infant	9	Psoas	3.2
Adult	1	Gastrocnemius	1.6
Adult	7	Lateral dorsalis, Psoas, Left intercostal	4.2

81

^a Adapted from Young and Munro, 1978.

myosin and to the lower proportion of myofibrillar to total protein in muscle at this stage than in later neonatal life (Young & Munro, 1978).

Table 15 shows that N^T-methylhistidine content of myosin in rabbit; N^T-methylhistidine was not detected in the mixed skeletal muscle of 28 day fetus rabbit. In the mixed skeletal muscle of 23 day old rabbit 1.15-1.22 residues of N^T-methylhistidine per mole myosin was detected. An average of 1.5 N^T-methylhistidine residues per mol myosin was found in adult rabbit muscle. According to the present study, finishing steers at 18 months of age had an average of 3.5 μ mol N^T-methylhistidine per g protein, while an average of 2.8 μ mol N^T-methylhistidine per g protein was found in 1-33 weeks old veal calves.

Hence, the data from the present study agree with the results of Trayer (1968) for rabbits, and the observation of Young & Munro (1978) for humans. Adult animals usually have higher concentration of N^T-methylhistidine than young animals.

As expected, red and white skeletal muscle samples contained significant amounts of N^T-methylhistidine (Figure 17). During muscle myofibrillar protein (actin or myosin) turnover, the N^T-methylhistidine is released and will not be metabolized or be reutilized in protein synthesis. The sole metabolic fate of N^T-methylhistidine

Table 15. N¹-Methylhistidine Content of Myosin^a

Animal	Muscle	Age of animal	N ¹ -Methylhistidine - residue/mol -
Rabbit	Longissimus dorsi	adult	1.63
	Heart	adult	1.35
	Mixed skeletal	28 day fetus	Nil
	Mixed skeletal	23 day	1.15 - 1.22

^a Adapted from Trayer, 1968.

Figure 17. Chromatogram of Bovine Muscle Sample.

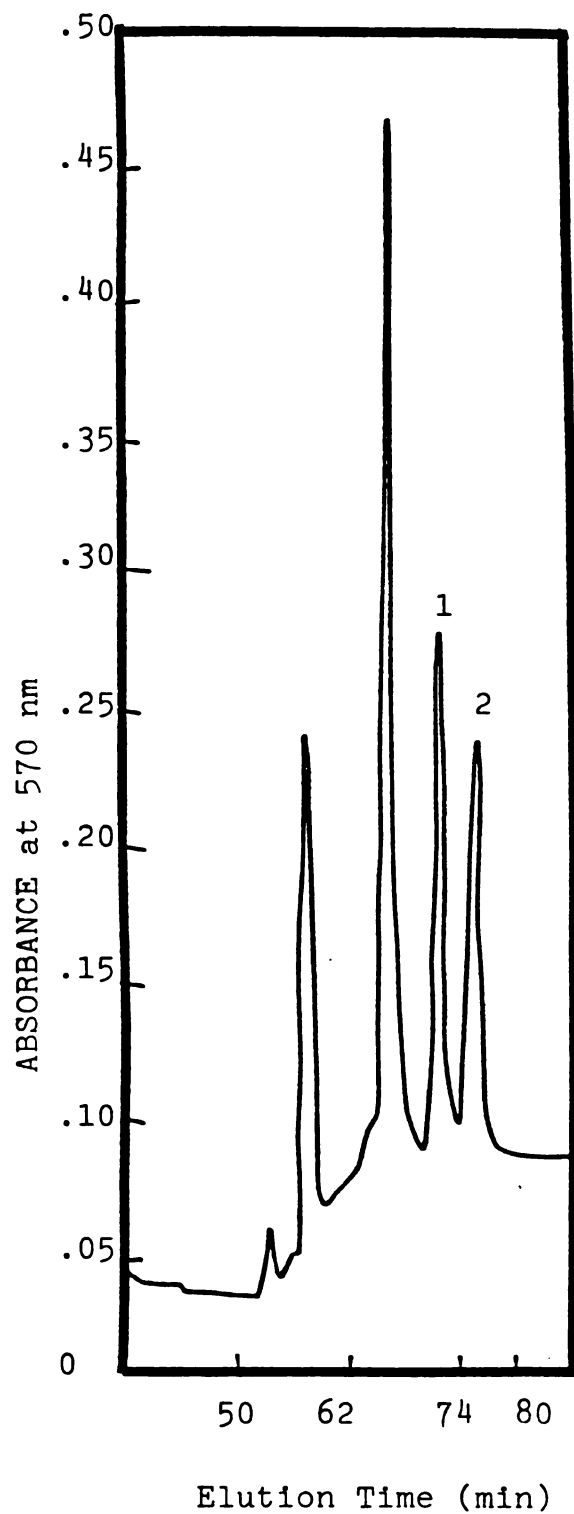


Figure 17.

Legend

- 1 N^T -Methylhistidine
2 α GPA

is then to be excreted in urine (Young and Munro, 1978).

In some species, the N^T -methylhistidine combines with β alanine to form balenine, a histidine dipeptide, and the N^T -methylhistidine is not quantitatively excreted immediately in the urine (Harris, 1977); however, ultimately all N^T -methylhistidine must be excreted as this compound and is thus not metabolized. This is in contrast to ϵ -N-Methyl Lysine (trimethyl lysine) which is the precursor of 4-trimethylaminobutryate for carnitine synthesis and can thus not be used as a muscle protein turnover marker (Bergen & Potter, 1971). Earlier workers (Leibholz, 1968) appeared to have confused N Methyl Lysine with N^T -methylhistidine on their chromatograms of sheep plasma. This is especially interesting in that most N^T -methylhistidine is found as balenine in sheep plasma, but sheep have very high plasma N Methyl Lysine and tissue carnitine levels. .

Generally dietary N^T -methylhistidine intake is of no physiological significance in ruminants. Under circumstance where the dietary intake were to be significant, however, it would be expected that N^T -methylhistidine would ostensibly escape ruminal catabolism, be absorbed in the small intestine, and excreted in the urine thereby causing an error in the estimation of muscle protein turnover rate.

CONCLUSION

This thesis work was initiated to determine the role of typical feeds and the ruminal fermentation on N^T-methylhistidine metabolism in ruminants.

It is clear, from this study, that the N^T-methylhistidine content of feedstuffs commonly fed to ruminants is very low. Of the twelve common feedstuffs chosen for this research, soybean meal N^T-methylhistidine content 2.15 nmol/g was the highest level, but the actual intake of N^T-methylhistidine from soybean meal is negligible and hence it can be concluded that feedstuffs are not a source of urinary N^T-methylhistidine. This same conclusion can also be drawn for rumen fluid and duodenal digesta.

The primary source of urinary N^T-methylhistidine is skeletal muscle and the amino acid arises during protein turnover. Based on the in vitro ruminal fermentation studies, N^T-methylhistidine is quite resistant to microbial catabolism and would "bypass" the rumen if fed in significant quantities. This thesis finally details some convenient procedures to quantitate N^T-methylhistidine in animal tissue and feed samples.

APPENDICES

APPENDIX TABLE A. 1.

Ninhydrin Solution Preparation

Combine 750 ml methyl cellusolve (Ethylene Glycol Monomethyl Ether; Pierce Chemical Company) 250 ml NaAc concentration, pH 5.51 (Pierce Chemical Company), 20 g ninhydrin, and 3.5 ml Triton x 100 and bubble with N₂ gas while stirring. Continue to bubble for 30 min, then add .45 g SnCl₂ and bubble with N₂ gas at least 3 further hours.

APPENDIX TABLE A. 2.

Charcoal Column Preparation Procedure

Glass columns, 7 mm i.d., were prepared with glass wool plugs and .5 cm filter aid (ash-free analytical filter pulp, Schleicher & Schnell, Inc., Keene, N.H. 03431) on top of plug. Slurry a mix of .5 g filter aid and .5 g charcoal and apply to the column. Continue by adding additional .5 cm filter aid on top of filter aid charcoal mix. Finally, wash columns as follows:

2 ml 4 N HCl; 2 x with 5 ml 20% EtOH : H₂O (V/V); and then with 5 ml of H₂O. Use a little N₂ gas pressure (.14 -.22 kgs/cm²) for good flows.

APPENDIX TABLE A. 3.

Composition of Ohio Media

Item	Concentration	Amount
Na_2CO_3	.2 g/ml	10 ml
Biotin	10 $\mu\text{g/ml}$	10 ml
Para-Amino Benzoic Acid (PABA)	100 $\mu\text{g/ml}$	2.5 ml
H_2O		45 ml
FeCl_3	2.64 mg/ml	7.5 ml
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	5.29 mg/ml	(FeCl_3 and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ are in one solution)
H_2O		7.5 ml
Mineral Mix "O" ^a		175 ml
Add deionized water to		875 ml

To stabilize the pH bubbled with CO_2 gas 1-2 h. Final pH adjustments to 6.7 are made with .1 N H_2SO_4 or .1 N Na_2CO_3 .

^aMineral Mix "O" composition is in Appendix Table A. 4.

APPENDIX TABLE A. 4.

Composition of Mineral Mix "O"

Item	Concentration
Na_2HPO_4	5.65 g/l
NaH_2PO_4	5.45 g/l
KCl	2.15 g/l
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	.582 g/l
Na_2SO_4	.750 g/l

APPENDIX TABLE A. 5.

Intake, Flow of Dry Matter^a

Item	60% DM Haylage + HMC
Intake (g/d)	5807.0
% of Intake Duodenal DM Flow	56.3

^a
Adapted from Rumpler, 1984.

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