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DEGRADATION OF TYROSINE BY
RUMINAL MICROORGANISMS

presented by

Kristen A. Johnson

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of the requirements for

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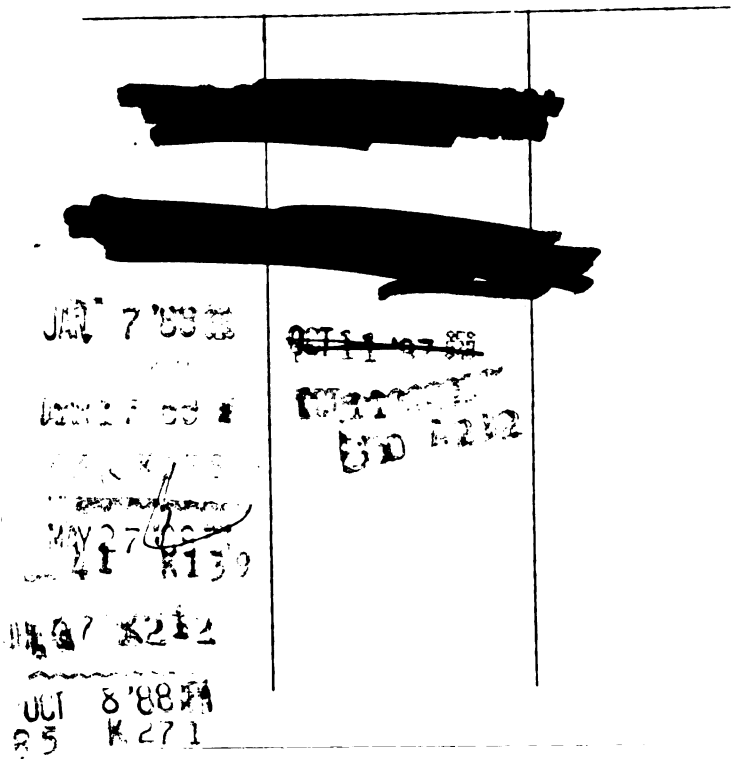

DEGRADATION OF TYROSINE BY RUMINAL MICROORGANISMS

By

Kristen A. Johnson

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ABSTRACT

DEGRADATION OF TYROSINE BY RUMINAL MICROORGANISMS

By

Kristen A. Johnson

The role of diet composition on L-tyrosine degradation by rumen microorganisms was investigated in an in vitro system. Rumen samples were collected from sheep fed a hay, hay-concentrate and concentrate diet and were incubated with L-tyrosine for 0, 4, 8, 12 and 24 hours. Primary metabolites identified were p-cresol, p-hydroxyphenylacetic acid and 7-phenylpropionic acid. There were no significant effects of diet composition on L-tyrosine degradation although the presence of hay in the diet enchanced tyrosine degradation to phydroxyphenylacetic acid. Another trial was conducted to examine rumen degradation of L-tyrosine in vivo. Sheep were fed a hay diet and were given 0.35 g L-tyrosine/kg BW. Intraruminal administration had no effect on rumen pH, volatile fatty acid concentrations and urinary 3-phenyl-propionic acid concentrations. It did increase rumen ammonia, rumen urinary p-cresol ($P<.001$), rumen 3-phenylpropionic acid and rumen and urinary p-hydroxyphenylacetic acid ($P<.001$).

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INTRODUCTION

Ruminants have the ability to utilize both conventional and non-conventional feedstuffs. As a result nutritional manipulation can serve to reduce feed costs, promote conservation of grains and, in some instances, change the composition of gain. This ability to utilize roughages and by-products arises from the metabolism of the microflora found in the rumen. Carbohydrates and proteins are fermented and serve as sources of energy and metabolic intermediates essential for the bacterial growth and protein synthesis which later function as nutrients for the host animal.

Dietary manipulation of the rumen microbial population is an approach which can be used to increase efficiency of feed utilization. Addition of carbohydrates maximizes volatile fatty acid production in the rumen which promotes growth of amylolytic and lactate utilizing bacteria as well as rumen protozoal numbers (Osborn et al., 1970; Schwartz and Gilchrist, 1975; Stewart, 1977; Abe and Iriki, 1978). Protein is digested in the rumen to constituent amino acids which may be deaminated, with the amino group serving for microbial protein synthesis, or utilized in processes such as Stickland reactions in which the amino acid may act as a hydrogen donor or acceptor for the production of branched chain keto acids (Scott et al., 1964; Oltjen, 1971).

Ruminal metabolism of individual amino acids have been investigated by many workers. Lewis and Emery (1962 a,b) investigated the catabolic intermediates of L-arginine, L-lysine and D, L-tryptophan both in vitro

and in vivo. D,L-tryptophan metabolism intraruminally was also investigated by Schatzman and Gerber (1972) and Yokoyama and Carlson (1974). Lysine catabolism was further investigated by Onodera and Kandatsu (1975) and DL-methionine by Dole and Adams (1980). Each of these workers were investigating the production or role of the catabolic intermediates formed by ruminal digestion of the individual amino acids. Tryptophan degradation in the rumen has been shown to yield skatole and idoleacetic acid, which has been implicated in bovine pulmonary edema and emphysema (Yokoyama and Carlson, 1975), and tryptamine, a biologically active amine. Lysine has been shown to be degraded to metabolic intermediates such as δ-amino-valeric acid and cadaverine (Lewis and Emery, 1962a) and eventually volatile fatty acids (Onodera and Kandatsu, 1975).

Tyrosine and phenylalanine degradation was investigated by Scott, Ward and Dawson (1964). Their findings indicate that tyrosine is degraded to three phenyl-substituted fatty acids. In an artificial rumen system 3-phenylpropionic acid was found to be the predominant metabolite with small amounts of p-hydroxyphenylacetic acid and 3 (p-hydroxyphenyl) propionic acid also detectable. The role of these secondary fermentation products in the rumen has largely been uninvestigated. Recently Hungate (1982) has shown that 3-phenylpropionic acid is stimulatory to certain cellulolylic bacteria in pure culture.

The purpose of the in vitro study was to evaluate the effects of different diet composition on the degradation of incubated tyrosine and to detect any differences in the metabolites produced as a result of the different diets. Another trial was conducted to investigate in vivo the effect of a large amount of tyrosine added intraruminally on bacterial tyrosine degradation.

LITERATURE REVIEW

The rumen microflora exhibit adaptive responses to changes in diet composition. Altering diet composition affects the rumen bacterial and protozoal population, rumen ammonia, rumen pH, volatile fatty acid production and production of secondary fermentation products.

High Roughage Diets

A diet containing 60-100% roughage is considered to be a high roughage diet (Kaufman et al., 1980). The predominant cellulolytic bacteria present in the rumen under high roughage feeding conditions are *Bacteroides succinogenes, Ruminococcus albus, Ruminococcus flavefaciens* and *Butyrivibrio fibrisolvens* (Hungate, 1966; Schwartz and Gilchrist, 1975). These bacteria by virtue of their rate of fiber digestion can affect rate of digesta passage and the other bacterial species present in the rumen that are dependent on the cellulose and hemicellulose digestion for substrate (Schwartz and Gilchrist, 1975).

Rumen cellulolytic bacteria are very pH sensitive (Ørskov, 1982). Normal rumen pHs under high forage feeding regimes range from 6.0-7.0 (Kaufman et al., 1980). This pH range is a result of fibrous feed consumption which leads to considerable buffer recycling through enhanced salivary secretions (Ørskov, 1982).

Lawler et al. (1966) measured parotid salivary secretions of sheep fed each of three diets: hay, roughage-concentrate, or a semipurified diet. Hay diets resulted in mean secretion rates of 8.1 liters of parotid

saliva per kilogram of food. The roughage-concentrate diet measured 2.9 liters and the semipurified diet 4.6 liters. Salivary composition of sodium, potassium, bicarbonate, phosphoric acid and chloride did not change. This increase in salivary secretions along with the increase in rumen volume, characteristic of high roughage rations, combine to increase rate of liquid passage out of the rumen (Warner, 1965).

Characteristic volatile fatty acid molar ratios found under high roughage feeding regimes are 70:20:10 (acetate:propionate: butyrate respectively).

Low Roughage: High Concentrate Diets

As the composition of the diet is altered to include a higher level of concentrate the rumen microfloral population shifts. The population of amylolytic bacteria is enhanced and the cellulolytic bacterial numbers decline (Osbourn et al., 1970; Leedle et al., 1982). Typical starch digesters include *Streptococcus bovis*, *Bacteroides amylophilus*, *Bacteroides ruminicola* and *Selenemonas ruminatium*. Some cellulolytic strains may also digest starch. These include some strains of *Bacteroides succinogenes* and *Butyrivibrio fibrisolvens* (Hungate, 1966). Protozoal numbers also increase substantially (Schwartz and Gilchrist, 1975; Abe and Iriki, 1978) particularly *Isotricha* and *Entodinum spp* (Hungate, 1966; Eadie and Mann, 1970; Abe et al., 1973). This shift in the rumen microflora is solely a result of pH effect on the microflora and not a result of pH optimum on the activity of cellulose or amylase (Kaufman et al., 1980). Recent data from Therion et al. (1982) minimizes the pH effect and suggests the population changes seen with increased amounts of concentrate fed is more a result of shifts in the concentration

and types of nutrients available in the rumen.

As the rumen pH approaches 6.0 the lactate producing species increase in number with concommitant increases in D and L-lactate (Mann, 1970; Huber et al., 1976). D-Lactate tends to accumulate over time and is slowly metabolized while L-lactate is rapidly absorbed. Lactate utilizers such as *Megasphaera elsdenii* and *Selenomonas ruminantium* increase in number and transform lactate into acetic acid thus preventing a pH decline. (The pk_a of lactic acid is 3.08 compared with acetic, propionic and butyric acids - pk_a 4.75-4.81). Rumen protozoa also help to prevent a further pH decline by sequestration of starch (Kaufman et al., 1980). Ørskov (1982) identified his reasons for the pH decline on high concentrate rations. First less saliva is produced due to less time eating and ruminating. Therefore the rumen is less buffered. Second, the amount of fermentable matter present in the rumen increases causing the volatile fatty acid production per feed unit to increase. Molar increases in propionate and butyrate are characteristically seen (Hungate et al., 1961).

Rumen Protein Digestion

Peptides and amino acids arise in the rumen as a result of bacterial and protozoal protease action on feed and microbial protein. This process is generally regarded to be the rate limiting step in rumen protein degradation (Nugent and Mangan, 1978). The rate of protein degradation is directly related to the solubility of the protein in the rumen liquor (MacDonald, 1952; Blackburn, 1965; Crawford et al., 1978; Craig and Broderick, 1981). Recent work on factors affecting rate of proteolysis demonstrated the importance of secondary and

tertiary structures of the protein. Studies done with bovine serum albumin (BSA) illustrate this point. BSA alone has a very slow and limited amount of hydrolysis. Treatment with dithiothreitol, which breaks some disulphide bridges, causes several fold increases in rates of rumen proteolysis (Nugent and Mangan, 1978; Wallace and Kopecney, 1983). The number of disulphide cross-linkages within the protein decreases the rate of degradation and may help protect the protein from rumen hydrolysis (Wallace and Kopecny, 1983). Also of importance in this process is the composition of the diet (Nugent and Mangan, 1981; Hazlewood et al., 1983). Nugent and Mangan (1981) demonstrated rates of proteolysis for fresh lucerne to be three to nine times that found on a hay-concentrate diet. This is contrary to the work of Blackburn and Hobson (1960a,b) and Annisor (1956) who found dietary composition to have little effect on rumen proteolysis rates.

Blackburn and Hobson (1960b) fractionated whole rumen contents and demonstrated proteolytic activity in the protozoal fractions, large and small bacterial fractions, and the supernatant. The greatest proportion of the proteolytic activity is associated with particle-bound bacteria (Brock et al., 1982). Rumen protozoa are also highly proteolytic (Blackburn and Hobson, 1960b; Ørskov, 1982), but bacterial proteases have specific activities seven to ten time higher (Brock et al., 1982). Specific proteolytic protozoal species include *Entodinium spp* and *Isotricha spp* (Allison, 1970). Little is known about specific protozoal proteases.

Bacterial proteases are predominately cell bound, constitutive enzymes (Allison, 1970; Armstrong and Weekes, 1983) and are not thought to be subject to metabolic control. Russell and Baldwin (1978) have identified catabolite regulatory mechanisms for carbohydrate metabolism in rumen bacteria. This demonstration indicates a possible mechanism

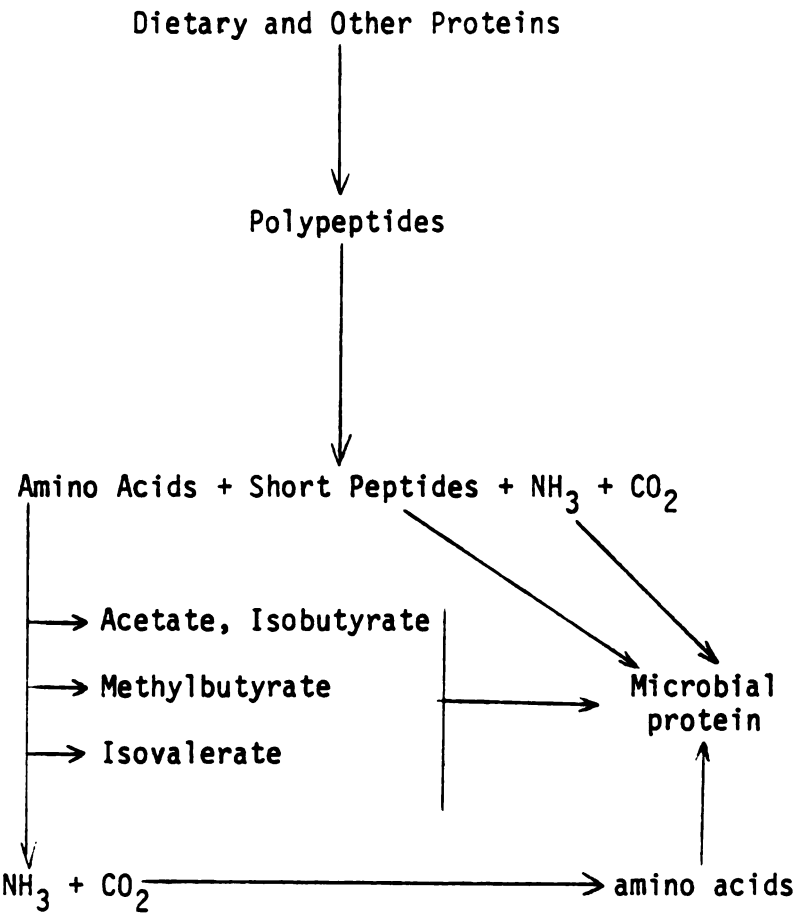


Figure 1: General scheme of rumen protein metabolism (Russell and Hespell, 1981).

for protease control as well.

Rumen pH has been demonstrated to be of importance in protease activity as low pH's have been shown to decrease proteolysis (Erfle et al., 1942). The protease of *Bacteroides amylophilus* was found to be unaffected by amino acid, protein, and peptide concentration in the medium (Allison, 1970) but protease production by the bacteria did change with growth rate. *Bacteroides ruminicola* has also been reported to have proteolytic activity. Serine, cysteine and aspartic-like proteases have been demonstrated for this bacterial species (Hazlewood and Edwards, 1981). Russell et al (1981) and Hazlewood et al. (1983) have reported proteolytic activity in *Streptococcus bovis* and *Butyrivibrio spp.* No individual bacterial species seems to be more important in proteolytic activity than any other (Armstrong and Weeks, 1983).

Rumen Amino Acid Concentrations

Amino acids appear in the rumen as a result of dietary protein breakdown, de novo synthesis by rumen bacteria, and microbial protein degradation. The concentrations of individual amino acids in the liquid phase of the rumen are low presumably because of rapid degradation or rapid uptake (Annison, 1956; Portugal and Sutherland, 1966). The values obtained by Portugal and Sutherland were probably higher than would actually be present. Wright and Hungate (1967a) demonstrated large differences in measured amino acid concentrations in acidified rumen contents and diffusate-ultrafiltrates. Acidified rumen contents were consistently higher reflecting total amino acids present in the rumen, both extracellular and bacterial. Diffusate-ultrafiltrates reflect more accurately extracellular concentrations (Wright and Hungate,

1967a). Some typical concentrations are as follows: aspartic acid - 26 µM, glutamic acid - 24 µM, glycine - 30 µM, alanine - 102 µM, leucine - 18 µM.

Amino Acid Uptake

Rumen bacteria may obtain the amino acids necessary for protein synthesis by either ammonia uptake and subsequent de novo synthesis or by uptake of preformed amino acids. Much of the older literature available in this field minimizes the contribution of direct incorporation of amino acids. Bryant and Robinson (1962) found that 82% of the rumen bacteria grown on a non-selective media could utilize ammonia as a sole source of nitrogen. Fifty-six percent could utilize either ammonia or amino acids and 25% need ammonia for growth. Similar results were demonstrated by Pilgrim et al. (1970) and Al-Rabbat et al. (1970).

In a more specific study examining amino acid and ammonia nitrogen incorporation of pure strains of selected rumen bacteria, Bryant and Robinson (1963) demonstrated that strains of *Lachnospira multiparus*, *Streptococcus bovis*, *Selenomonas ruminantium*, *Butyrivibrio fibrisolvens* (strain 1) and *Succinivibrio dextrinosolvens* preferentially incorporated exogenous amino acids. *Megasphaera elsdenii* is another amino acid dependent organism (Miura et al., 1980).

Eubacterium ruminantium (strain 6A 195), *Butyrivibrio fibrisolvens* (strain A 38), *Eubacterium sp*, *Ruminococcus albus* and *Ruminococcus flavefaciens*, were shown to have a reduced ability to take up amino acids and therefore preferentially incorporate ammonia.

The composition of the diet may have a marked effect on the amount of exogenous amino acid intake versus de novo synthesis from nonprotein

nitrogen. Maeng et al. (1976) determined the optimum ratio of urea-N to amino acid-N. In an in vitro system 75% urea-N and 25% amino acid-N supported maximal microbial growth. At this ratio 53% of the added amino acids were incorporated into bacterial cells, 14% fermented directly to CO_2 and VFA's and 33% were left in the supernatant.

In vivo studies using preformed protein sources or nonprotein nitrogen (urea) supplementation demonstrate many of the same results. Nolan and Leng (1972) using ^{15}N and lucerne chaff diets demonstrated approximately 29% of the nitrogen incorporated directly as amino acid N. Experiments with labeled sulfur illustrate the same results. With diets containing appreciable preformed amino acids bacteria preferentially incorporated preformed S-containing compounds such as cystine-S (McMeniman et al., 1976). Daneshuar et al. (1976) reported results that are contrary to Nolan and Leng and McMeniman and coworkers, but reversed themselves in a later paper (Salter et al., 1979). Their most recent conclusion is that with diets adequate in protein, amino acids such as proline, arginine, histidine, methionine, and phenylalanine were preferentially taken up.

Much of the research done on nitrogen (amino acid, oligopeptide and ammonia) uptake in rumen bacteria has been done with *Bacteroides ruminicola*. Pittman and Bryant (1964) examined peptides, amino acids and ammonia as nitrogen sources needed for the growth of *B. ruminicola*. Using a medium adequate in energy (glucose), minerals, vitamins, VFA's, heme and sulfur (methionine and cysteine) Pittman and Bryant demonstrated that the organism could use peptide nitrogen and ammonia nitrogen but not amino acid nitrogen. Further work from this group (Pittman et al., 1967) elucidated a transport system for oligopeptides. This indicates that

B. ruminicola uses this transport system to supply the organism with a readily hydrolyzable source of amino acids. Stevenson (1979) was able to demonstrate in continous culture amino acid uptake systems in this same organism. Alanine, asparagine, glycine, serine, threonine and tyrosine uptakes were limited (2.3 - 7.1 %). Competition tests demonstrated six different uptake systems.

There are two distinct energy coupled modes of amino acid transport in bacteria (Oxender et al., 1980). The first of these is an ATP-dependent process. It utilizes ATP or an ATP derived metabolite as a source of energy. The second are protein-motive force-dependent systems. These systems are dependent upon ion gradients generated during respiration or ATP hydrolysis.

Amino Acid Degradation

Anaerobic bacteria degrade amino acids in two ways. The first of these is through deamination to carbon dioxide, ammonia and volatile fatty acids. Normally, with rumen pH near neutrality, this is the predominant route (Prins, 1977). When rumen pH drops decarboxylation reactions are favored with a concomittant increase in rumen amines (Hungate, 1966; Prins, 1977).

The rate of deamination of amino acids is slower than the rate of proteolysis (Annison, 1956; Blackburn and Hobson, 1960a). The difference in these two processes results in increased concentrations of amino acids and small peptides in the rumen immediately after feeding. Within approximately 3 hours, peak ammonia concentrations can be found indicating deamination of virtually all amino acids. (Some of the amino acids may be decarboxylated or taken up intact).

Differences do exist among amino acids in rates of degradation. Lewis and Emery (1962c) placed amino acids in three categories based on rates of deamination. Serine, aspartic acid, cysteine, threonine, and arginine were readily dissimilated. The intermediate amino acids included glutamic acid, phenylalanine and lysine. Tryptophan, σ-aminovaleric acid, methionine, alanine, valine, isoleucine, ornithine, histidine, glycine, proline and hydroxyproline were those that were very slowly dissimilated. Similar results were found by Cooper et al. (1959), Lewis and Emery (1962b), Chalupa (1976), Maeng et al. (1976) and Scheifinger et al. (1976).

Amino acid mixtures have been shown to be more rapidly metabolized. Alanine and proline when incubated together were more rapidly metabolized (El-Shazly, 1952). Alanine is oxidized to acetate, CO_2 and ammonia, while proline is reduced to α-amino valeric acid. This is evidence of a Stickland reaction. A Stickland type reaction is an oxidation-reduction reaction in which the amino acid involved acts as an electron acceptor or donor. The oxidation step may involve transaminases, α-ketooxidases or oxidative deaminases (not a major pathway). The reduction reaction is distinctive depending on the organism involved (Barker, 1981). Products of these reactions include volatile fatty acids (VFA's), σ-amino valeric acid and molecular hydrogen (Barker, 1981).

Actual rates of degradation were measured in vitro and in vivo by Chalupa (1976). His data reflects that found by Lewis and Emery (1967c). In vitro Vmax values ranged from 0.88 mM/hr for arginine to 0.09 mM/hr for methionine. Intermediate values were obtained for threonine, lysine, phenylalanine, leucine, isoleucine and valine. In vivo degradation rates were approximately 1.5 times higher. This is quite understandable because

in vitro conditions present in rumen incubations are less optimum for normal bacterial growth and metabolism. Degradation rates in both cases exhibited zero order kinetics (Chalupa, 1976).

Bacteroides ruminicola, Butyrivibrio sp., Clostridia sp. and some *Selenomonas sp.* have been shown to possess deaminating activity as has *Megasphaera elsdenii* (Lewis and Elsden, 1962).

Individual Amino Acid Degradation

Several workers have examined the degradation of individual amino acids by rumen microorganisms in vitro. El Shazly (1952) identified the major products from casein hydrolysate degradation to be ammonia, carbon dioxide and volatile fatty acids (VFA's). In the same study, ruminal washed cell suspensions (from a sheep fed a casein:hay:concentrate diet) were incubated with L-proline and DL-alanine. The disappearance of L-proline with the subsequent appearances of σ-amino valeric acid was demonstrated. This is evidence of a Stickland reaction with proline acting as the oxidizing agent. Some studies have subsequently verified this conclusion (Barker, 1981).

Aspartic acid incubated with mixed cell suspensions of rumen bacteria was shown to be degraded in a series of steps beginning with deamination to succinate followed by decarboxylation to propionate (Sirotnak et al., 1954). A later study with radiolabelled aspartic acid demonstrated many of the same results with only eight percent of the total radioactivity found in the bacteria. Only four percent of this bacterial radioactivity was present as aspartic acid (Huber et al., 1958, Portugal and Sutherland, 1966). D-L lysine alone or D-L lysine, glycine and D-L alanine were not shown to be fermented to volatile fatty acids in vitro but glycine and

and D-L alanine were deaminated in vivo (Hueter et al., 1958). Later work with glycine metabolism by rumen bacteria was done by Wright and Hungate (1967b). U^{14}C-glycine was incubated in rumen contents for 120 seconds then stopped with addition of 10N H_2SO_4. Bacterial glycine accounted for twelve percent of the label, carbon dioxide twenty five percent and volatile fatty acids twenty two percent. These results demonstrate a rapid conversion of glycine to breakdown products. As would be expected 89.8 percent of the radioactivity in the volatile fatty acids was acetate, 6.1 percent propionate and 4.1 percent butyrate and higher volatile fatty acids.

Later work on the degradation of L-lysine, arginine, and proline with both washed cell suspensions and whole rumen contents revealed that all of these three amino acids were metabolized to σ-aminovaleric acid (Lewis and Emery, 1962a). L-Arginine also yielded ornithine and putrescine and L-lysine also was degraded to cadavarine. Media pH had a marked effect on the extent of these degradation reactions. No amines were produced by any of the incubated amino acids (histidine, lysine, phenylalanine, tryptophan and arginine) at pH's of 4.5 and 6.5. As would be expected maximal ammonia production for all amino acids was seen at pH 6.5. Again this is optimum for deamination to occur (Prins, 1977). Phenylalanine was deaminated equally well at pH 5.5. At a higher pH (6.5) maximal ornithine production from arginine was seen.

In vivo administration of single amino acids has also been investigated. Addition of L-arginine and L-lysine to the rumen of alfalfa fed steers, with analysis to identify degradation products, showed good agreement with in vitro studies. Arginine yielded σ-aminovaleric acid and ornithine while lysine produced only σ-aminovaleric acid (Lewis and

Emery, 1962b). This is contrasted by Heuter et al. (1958) who failed to demonstrate L-lysine catabolism in vivo. Lewis and Emery's data is also contradictory to that found by Onodera and Kandatsu (1975) who (in an in vitro system) did not detect σ-aminovaleric acid or cadaverine. When radiolabeled lysine was added to rumen contents no labeled amines were detected, whereas ninety six percent of the label was found in the VFA's. Acetate and butyrate were both heavily labeled.

Methionine degradation in the rumen has also been investigated (Emery, 1971; Merricks and Salsbury, 1975; Doyle and Adams, 1980). Degradation rates are low (Emery, 1971) at high concentrations which may result in large amounts leaving the rumen undegraded. At low methionine concentrations rapid uptake is seen (Merricks and Salsbury, 1975). Compounds formed by rumen bacteria from L-methionine or cysteine are volatile sulfur compounds such as dimethyl sulphide and s-methyl-L-cysteine (Prins, 1977)

The metabolic pathways for the utilization of the aromatic amino acids also have been studied. Tryptophan is dissimilated to indole, skatole (Lewis and Emery, 1962a; Yokoyama and Carlson, 1974) and indoleacetic acid (Scott et al., 1964) and tryptamine (Schatzman and Gerber, 1972). The conversion of tryptophan to skatole in the rumen is a two step reaction sequence. The first reaction, tryptophan to indoleacetic acid, has been shown to be a Stickland reaction (Figure 2) with tryptophan acting as a hydrogen donor in a coupled deamination of a pair of amino acids (Scott et al., 1964).

The second reaction in the sequence, indoleacetic acid to skatole, is a decarboxylation reaction (Yokoyama and Carlson, 1974). The rate of tryptophan disappearance in the rumen (Lewis and Emery, 1962b)

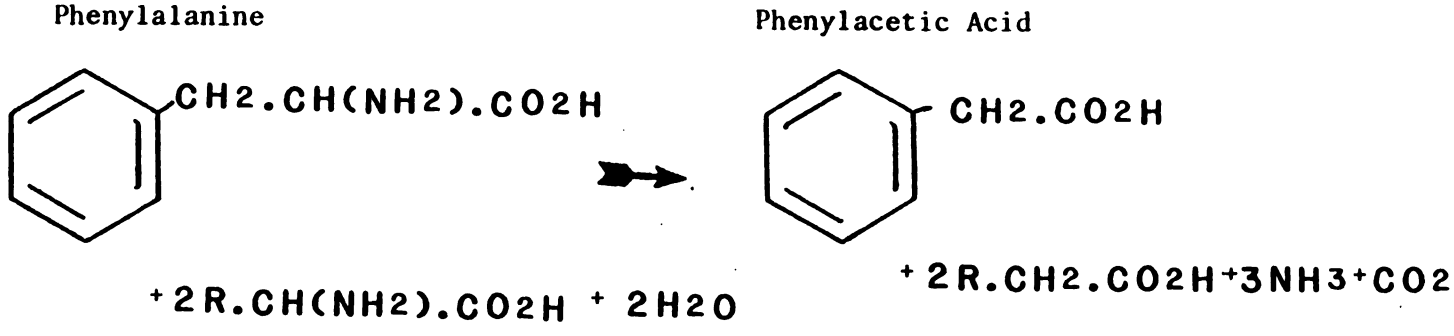


FIGURE 2. Stickland Reaction: Phenylalanine

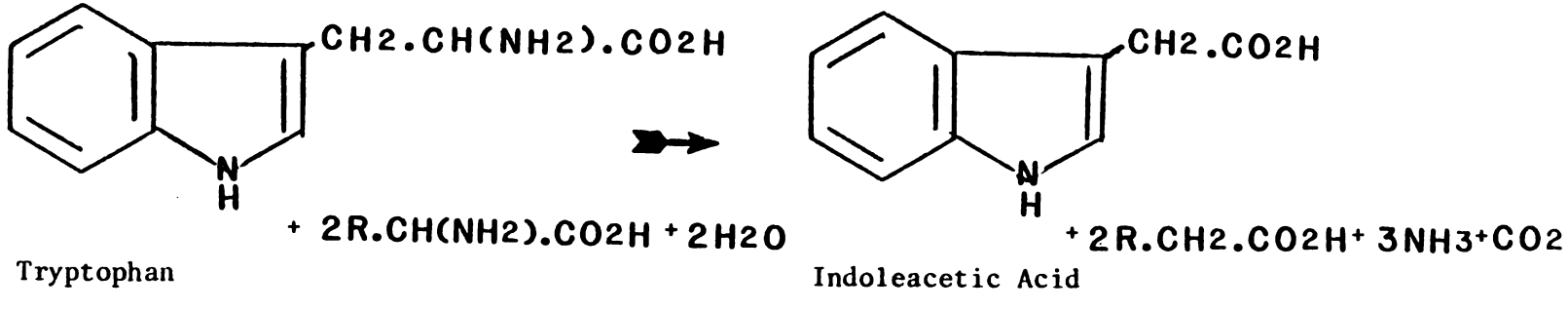


FIGURE 3. Stickland Reaction: Tryptophan

and in vitro (Scott et al., 1974; Yokoyama and Carlson, 1974) is very slow.

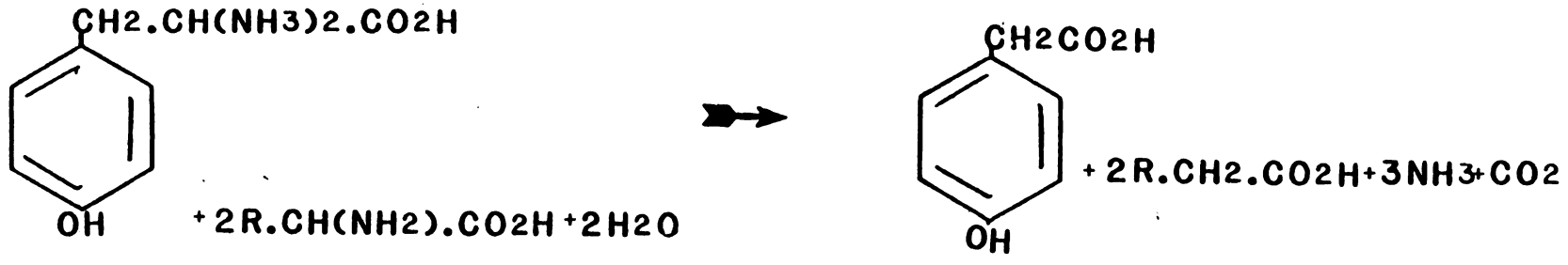
Yokoyama and coworkers (1977) isolated a specific *Lactobacillus sp* from the rumen that decarboxylates indoleacetic acid to skatole. This isolate cannot, however, use tryptophan directly for the conversion to skatole and therefore requires another species to carry out the Stickland reaction.

Phenylalanine has been shown to be degraded almost exclusively to phenylacetic acid (Scott et al., 1964; Patton and Kessler, 1967). The reaction mechanism here has also been proposed to be a Stickland reaction (Figure 3) with phenylalanine acting as a hydrogen donor (Scott et al., 1964; Patton and Kesler, 1967).

Tyrosine metabolism intraruminally is slightly different than that of the other aromatic amino acids. The product with the greatest radiolabel when [U-^{14}C] tyrosine is added to rumen contents is 3-phenylpropionic acid (Scott et al., 1964; Patton and Kesler, 1967). Significant amounts of p-hydroxyphenylacetic acid are produced as well as small amounts of 3-(p-hydroxyphenyl) propionic acid. The longer the incubation the greater the concentration of p-hydroxyphenylacetic acid and the lower the concentration of 3(p-hydroxyphenyl) propionic acid (Scott et al., 1964). The formation of p-hydroxyphenylacetic acid involves tyrosine acting as hydrogen donor in a reaction sequence almost identical to that of tryptophan and phenylalanine (Figure 4).

The production of 3(p-hydroxyphenyl) propionic acid involves a Stickland reaction but in this reaction tyrosine is acting as a hydrogen acceptor instead of donor (Figure 5).

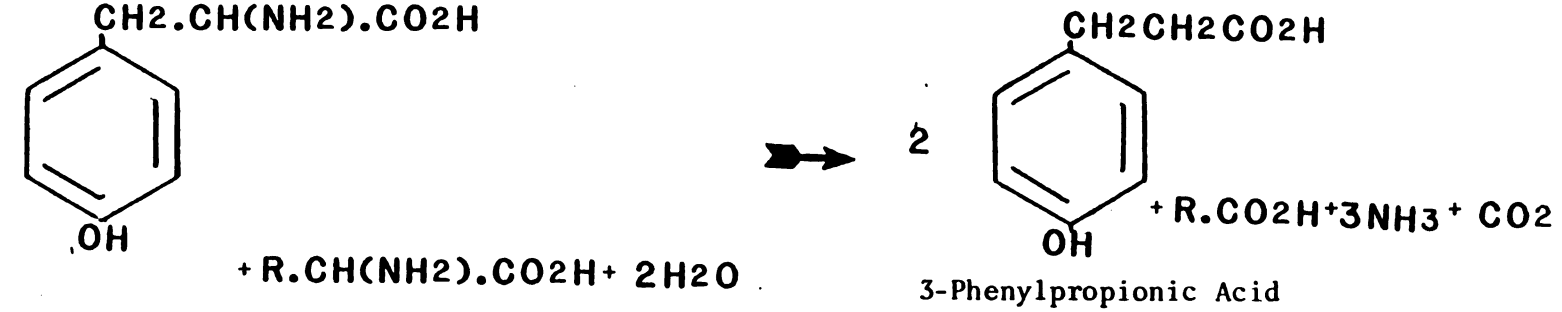
The 3(p-hydroxyphenyl) propionic acid is converted slowly to



Tyrosine

p-Hydroxyphenylacetic Acid

FIGURE 4. Stickland reaction: Tyrosine



Tyrosine

3-Phenylpropionic Acid

FIGURE 5. Stickland reaction: Tyrosine

3-phenylpropionic acid by the removal of the hydroxyl group and water formation (Scott et al., 1964).

The Role of Amino Acid Degradation Products

The branched and straight chain volatile fatty acids that arise through amino acid fermentation are stimulatory to some rumen cellulolytic bacteria (Hungate, 1966). Cellulose digestion by washed cell suspensions of rumen bacteria has been shown to be improved with the addition of valine, leucine and isoleucine. These amino acids are fermented to isobutyrate, isovalerate and 2-methylbutyrate respectively (Hungate, 1966). These volatile fatty acids are not only stimulatory but are essential for some bacteria. *Bacteroides succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens* require one or more of these acids (Bryant and Robinson, 1962).

Phenylpropanoic acid (3-phenylpropionic acid) has also been demonstrated to be stimulatory to *Ruminococcus albus'* growth and cellulose digestion in pure culture (Hungate and Stack, 1982). The concentration of phenylpropanoic acid in the rumen has been found to be in the range of 300 μM to 660 μM (Scott et al., 1964; Hungate and Stack, 1982). This acid may arise from tyrosine degradation, although the concentration in the rumen is higher than can be accounted for by tyrosine degradation alone (Scott et al., 1964). It has been suggested that lignin degradation may account for the rest (Hungate and Stack 1982; Martin, 1982b).

Amino Acid Biosynthesis

Since some rumen bacteria either preferentially take up ammonia when peptides and amino acids are provided or have a defined ammonia

requirement it seems likely that there is a capacity present in these organisms to synthesize amino acids (Hungate, 1966). The mechanisms of amino acid biosynthesis found in rumen bacteria were reviewed by Allison (1969). *Streptococcus bovis* has been shown to possess a (NADP)-linked glutamic dehydrogenase (Wolin et al., 1959) as have other rumen bacteria. *Ruminococcus albus* and *Megasphaera elsdenii,* rumen protozoa and rumino reticular mucosa contain an NAD-linked glutamic dehydrogenase (Hoshino et al., 1966). Glutamine synthetase, glutamate synthetase, and transaminases are also key enzymes involved in ammonia assimiliation and transfer (Prins, 1977).

The carbon skeletons for amino acid biosynthesis are provided by glycolytic intermediates and fermentation products such as volatile fatty acids (particularly acetate), carbon dioxide and isoacids (Allison, 1969; Sauer et al., 1975; Prins, 1977). The basic reaction sequence is reductive carboxylation through a complicated scheme utilizing ATP, CoASH and TPP as coenzymes, and amination of the corresponding fatty acid (Allison, 1969; Kristensen, 1974; Prins, 1977). Valine is synthesized from isobutyrate and acetate (Sauer et al., 1975; Prins et al., 1977) by *Ruminococcus flavefaciens*. Leucine is derived from isovalerate (Allison, 1966; Hungate, 1966). Isoleucine from 2-methylbutryate, phenylalanine from phenylacetic acid (Allison, 1965), and tryptophan from indoleacetic acid (Allison and Robinson, 1967). Kristensen (1974) demonstrated tyrosine biosynthesis from 4-hydroxyphenylacetic acid. The shikimic acid pathway as well as the condensation of phenol and serine were found to be slow and of lesser significance in the rumen.

The role of some rumen bacteria in production of extracellular amino acids has also been investigated (Stevensen, 1978, 1979). Alanine,

glutamic acid, valine, aspartic acid and glycine were produced by the greatest number of bacterial isolates at the greatest concentrations (Stevensen, 1978). Interestingly the concentrations produced in these in vitro studies reflect those found in rumen contents by Wright and Hungate (1967). These data suggest that production of amino acids with subsequent excretion of these amino acids to the extracellular fluid in the rumen may be a greater source than earlier suspected (Stevenson, 1979).

Urinary Aromatic Acid Excretion

Attempts have been made to use the urinary excretion of phenolic compounds by ruminants as a measure of forage utilization and digestibility and protein digestion in the rumen (Martin, 1969a,b, 1970, 1973, 1978). Ruminal protein metabolism was shown to increase urinary output of aromatic acids (Blaxter and Martin, 1962). Further investigation of urinary phenolics led to a demonstration that the quantity of phenolics excreted was quite variable with types of foods eaten (Blaxter et al., 1966). Martin (1969a), expressing aromatic acids on a benzoic acid equivalent (BAE), reported values for ruminants between 96 and 1475 mg BAE/kg $BW^{\cdot 75}$/day and nonruminants 14-115 mg BAE/kg $BW^{\cdot 75}$/day.

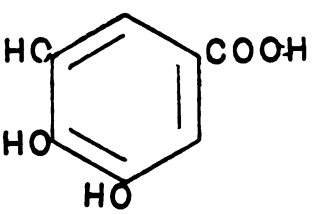
With this demonstration of large quantities of aromatic acids in ruminant urine, further studies were conducted to determine the origin of these acids. It had already been established that the excretion of large quantities of aromatic acids in herbivore urine was due to microbial fermentation of foodstuffs in the intestine (Hawk, 1947 as cited by Martin, 1978), but the identity of these compounds and the precursors were unknown.

Predominant aromatic acids identified in sheep urine include benzoic acid (2.7-16.3 g/kg food intake), phenylacetic acid (0.3-3.2 g/kg), cinnamic acid (0.2 g/kg), phenol (3-35 mg/kg), 3-phenylpropionic acid (0.1 g/kg intake) and p-cresol (600-1750 mg/kg) (Martin, 1975). These quantities are not absolute and vary with diet. Martin (1969) investigated endogenous levels of urinary organic acids. Results of this study show declines in benzoic acid (gBAE/34h) from 2.20 in fed animals to 0.19 g on day five of fasting. Phenylacetic acid remained constant at 0.43 g and hippuric acid declined from 0.72 to 0.01 g over the period of the fast. This indicates that approximately 76% of the total aromatic acid excretion of the sheep is of dietary origin. The phenylacetic acid output may have been a result of rumen fermentation even out to day four. 3-Phenylpropionic acid has been shown to arise from rumen fermentation of tyrosine (Scott et al., 1964) as has p-cresol (Martin, 1978).

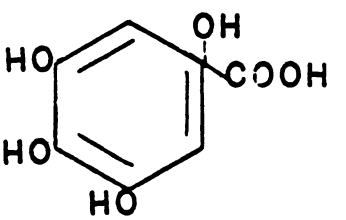
Dietary Precursors

There are many possible sources of the aromatic acids found in ruminant urine. These acids may arise as a result of rumen fermentation and microbial metabolism of precursor substances in foods, as a result of metabolism peculiar to ruminant tissues or as a combination of the above explanations (Martin, 1969). If these compounds are of dietary origin than they should be found in some form in the forages or cereal grains consumed by the animal. Both grain and plant phenolics have been studied as possible precursors.

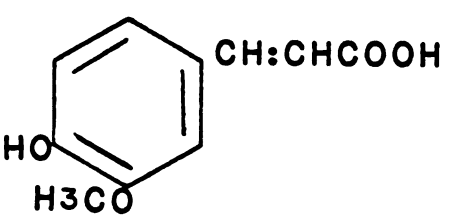
When different cuttings of hay were examined for dietary precursors of aromatic acids (Figure 6) the following components decreased with increasing maturity: chlorogenic acid, shikimic acid, quinic acid,



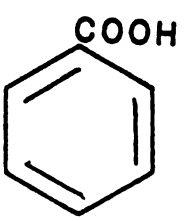
Skikimic Acid



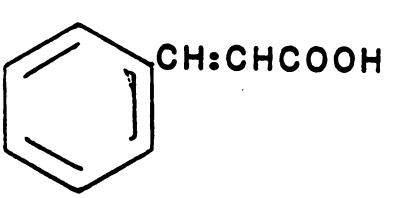
Quinic Acid



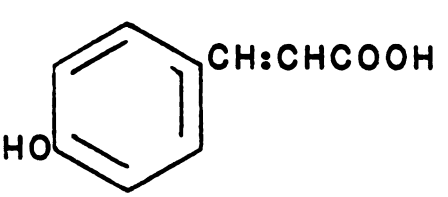
Ferulic Acid



Benzoic Acid



Cinnamic Acid



p-coumaric

FIGURE 6. Common phenolic acids present in plants.

total o-dihydroxy phenols and crude protein. Only lignin increased in quantity (Martin, 1970). This concurs with the observation that when feeding younger forages to sheep increasing amounts of urinary aromatic acids were seen (Martin, 1970, Ely et al., 1953). Lignin is known to possess 4-hydroxy and 3-methoxy derivates of 4-hydroxycinnamic acid attached to the lignin polymer by labile ester bonds (Higuchi et al., 1967 in Martin 1970 and Martin 1969). The extent to which these bonds are broken in ruminal degradation is not known, but the lignin molecules may lose methoxyl groups from the ring.

Cinnamic acids are some of these phenolic acids in plants that may be precursors of urinary phenolics. These acids, p-coumaric, caffeic, and derulic acids, are ester-linked to plant polysaccharides and are found in increasing quantities with increasing forage maturity (Theander et al., 1981; Hurrell and Finot, 1982).

The metabolism of rumen bacteria incubated with these phenolic acids has been investigated. Booth and Williams (1963) demonstrated that rumen microorganisms and rat and rabbit cecal contents can dehydroxylate caffeic acid. Metabolites present after incubation include residual caffeic acid, 3-4-dihydroxyphenylpropionic acid, m-hydroxyphenylpropionic acid and m-hydroxycinnamic acid. In vitro studies with timothy hay of different maturities demonstrated that rumen microbes were able to remove ferrulic acid residues to a greater extent than those of p-coumaric acid (Theander et al., 1981). Scheline (1968a,b) demonstrated that intestinal microflora can decarboxylate phenolic constituents if they contain a 4-hydroxy group. This, then, applies to derivatives of benzoic acid, phenylacetic acid and the cinnamic acids. Martin (1975) confirmed these results in a study in which he infused intraruminally derivatives

of benzoic, phenylacetic and 3-phenylpropionic acids. He observed metabolism of those substances that possessed 4-hydroxy groups. Phenolics resulted from decarboxylation of benzoic and phenylacetic derivatives while 3-phenylpropionic acids or cinnamic acids resulted in 80-107% BAE. Abomasal infusion of these acids did not result in any increase in benzoic acid or phenols in the urine.

Perhaps the most extensive studies of these acids and their eventual contribution to the urinary aromatic acid pool have been reported by Martin in 1982 and 1983. In his first paper (Martin, 1982a) benzoic, phenylacetic, 3-phenylpropionic and cinnamic acids were infused both intraruminally and abomasally. Urine was then collected and analyzed for phenolic constituents. Benzoic acid and phenylacetic acid were excreted quantititively unchanged. 3-phenylpropionic acid and cinnamic acid were recovered almost totally as benzoic acid. When cinnamic acid was infused none was found in the rumen fluid but the 3-phenylpropionic acid concentration was increased substantially indicating rumen bacteria can saturate the double bond present on the side chain.

In a second study (Martin, 1982b) mono-hydroxycinnamic acids, substituted 3-phenylpropionic acids and disubstituted cinnamic acids were infused continously into the rumen and abomasum. Again urine was collected and analyzed for phenolic acids. Rumen infusions of phenolic derivatives of 3-phenylpropionic acids and cinnamic acids increased rumen 3-phenylpropionic acid levels and did not effect benzoic acid concentrations in the rumen. The 2-,3- or 4-hydroxy acids, the 3,4-dihydroxy acids, the 3-methoxy and 4-hydroxy acids resulted in between 63-106% benzoic acid in the urine. Some of the dose, variable with animal, was excreted as cinnamic acid. Abomasal infusions did not result in benzoic

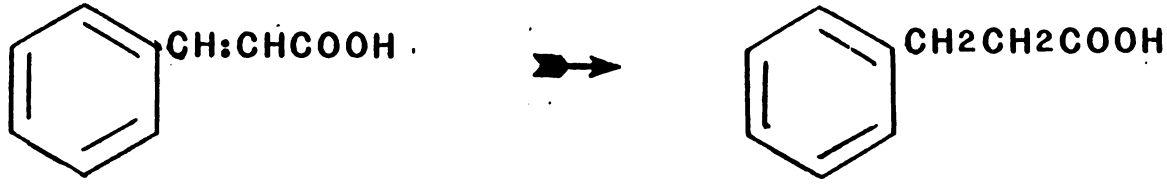
acid in the urine except for some of the substituted cinnamic acids which did result in 33-34% urinary benzoic acid. The reactions that must take place in the rumen and intestine are then demethylations, dehydroxylations and decarboxylations as well as reduction reactions on aliphatic side chains (Martin, 1982).

The role of rumen protein metabolism and dietary phenols in urinary aromatic acid output was examined in a third study (Martin, 1982c). Eighteen phenolic acids were administered in a continuous drip inter-ruminally or abomasally. Urine was collected and analyzed. Phenolic acids containing 4-hydroxy substituent groups were shown to yield large quantities of urine phenols. 4-hydroxyphenylacetic acid was shown to be decarboxylated to p-cresol in the rumen. This has been demonstrated previously to occur in the rumen by a *Lactobacillus sp* (Yokoyama and Carlson, 1981).

It has been suggested that phenolic compounds excreted in the urine could be used as an index of voluntary food intake by animals under grazing conditions. This has been investigated (Martin, et al., 1983) and found to be plausible. Orcinol output was found to be the least variable and was excreted at 99% of intake. The others; phenylacetic acid, p-cresol, catechol and phenol, were too variable and were not linearly related to intake.

The effect of phenolic acids of plant orgin upon rumen bacteria has been investigated (Chesson et al., 1982). The hydroxycinnamic acids, ferrulic acid and p-coumaric acid, were toxic to rumen bacteria and suppressed growth of *Ruminococcus flavefaciens* and *Bacteroides succinogenes*. *Ruminococcus albus* was not affected. The hydroxybenzoic acids did not inhibit growth or cellulolytic activity. All of the strains of cellulolytics

and *Streptococus bovis* demonstrated the ability to hydrogenate the hydroxycinnamic acids which may be evidence of a protective mechanism of these bacteria to the presence of hydroxycinnamic acids (Figure 7).



Cinnamic acid

3-Phenylpropionic Acid

FIGURE 7. Hydrogenation reaction

MATERIALS AND METHODS

Experiment I - In Vitro Study

Three crossbred wethers averaging 45 kg in weight were fitted with rumen cannulas and fed three diets. The first of these was composed entirely of a mixed grass hay, the second a 50:50 mixed grass hay:concentrate diet and the third a 100% concentrate diet. Diets were calculated to be isocaloric and isonitrogenous. Subsequent feed analysis however showed diet three to be slightly higher in protein and lower in energy. (Table 1). Animals were fed each diet for at least 30 days. Twenty one days served as an adaptation period and the final seven as collection days. Collections were taken on alternate days for a total of three collections per animal per diet. On each collection day, 300 ml of rumen contents was removed four hours after feeding and strained through a double layer of cheesecloth. At this time a zero time sample (50 ml) was removed, its pH taken, then acidified with concentrated HCl to a pH of 2.0-2.5. The remaining 250 ml of rumen contents was added to 500 mg of L-tyrosine (Lot 81F-0198 Sigma Co., St. Louis) sparged with CO_2, stoppered and incubated at 39^{o} C in a water bath. At 4, 8, 12 and 24 hours flasks were removed from the water bath, opened under CO_2 and a 50 ml subsample removed. Flasks were stoppered again and reincubated until the next collection was taken. The pH of the subsamples was taken, the samples acidified and frozen until later analysis.

On the day following the final rumen collection animals were placed in metabolism cages and allowed a one day adjustment period before two

Table 1. COMPOSITION OF DIETS

Ingredient	Reference No	1	2	3[1]	4
			% of the DM		
Mixed grass hay	1-02-249	100	50	---	100
Ground yellow corn	4-02-915	---	50	36	---
Soybean Meal	5-04-604	---	---	8	---
Oats	4-03-309	---	---	13	---
Dehydrated Alfalfa Meal	1-00-027	---	---	23	---
Ground corn Cob	1-02-783	---	---	20	---
Dical	1-02-783	---	---	1	---
Trace Mineral Mix[2]		ad lib	ad lib	ad lib	ad lib
Vitamin A		---	120,000 IU	55,250 IU	--
Vitamin D		---	25,000 IU	12,016 IU	--
% CP		12.2	12.5	13.9	12.2
GE (kcal/gm DM)		4.0	4.3	3.8	4.0
% Tyrosine (% CP)		--	4.5	4.3	--

[1]Fed pelleted

[2]Trace mineral mix composition: Zinc oxide .350%, manganese oxide .200%, Iron .200%, Copper oxide .030%. Cobalt carbonate .005%, Iodide .007%, Salt 96-98.5%, Sodium Chloride, Ferrous Carbonate, Calcium Iodinate.

24 hour urine collections were taken. Fifty milliliters of 2% mercuric chloride was added to the collection bucket to prevent bacterial degradation. Total urine was collected and frozen for subsequent analysis.

Incubated rumen contents and urine samples were extracted and analyzed for volatile and nonvolatile phenolic compounds by gas liquid chromatography.

Experiment II - In Vivo Study

Two ruminally fistulated mature wether sheep (68 and 72 kg) were placed on a mixed alfalfa-grass hay diet with trace mineral salt and water available ad libitum for at least thirty days prior to the days on experiment. Animals were observed for baseline levels of rumen and urine parameters on two alternate days and served as their own controls. On one of these days serial blood samples (0 time - prefeeding, 2, 4, 6, 8, 12 and 24 hours) were taken via jugular catheter. Samples were placed in heparinized tubes and frozen. Rumen contents were collected at these same times, strained through double layers of cheesecloth. The pH was taken and samples were acidified with concentrated HCl to a final pH range of 2.5-3.0. Samples were frozen for later analysis. Total urine collections were also made on these days with 50 ml of a saturated mercuric chloride solution used as a bacteriostat and samples were frozen.

On the third collection day animals received 0.35 g/kg BW tyrosine intraruminally in a slurry with distilled water. Rumen contents, blood and urine was collected as before with the addition of a 24 and 48 hour collection of each.

Rumen contents were analyzed for VFA's, ammonia and volatile and

nonvolatile aromatic phenolic compounds. Urine and blood was also analyzed for these same phenolic compounds.

VFA's. Five ml of rumen contents was added to 1 ml of 25% metaphosphoric acid allowed to stand for 30 minutes and centrifuged at 15,000 x G for 15 minutes (Erwin et al., 1961). The resultant supernatant was transfered to a GLC vial and 2 microliters of extract was analyzed by a Hewlett-Packard gas-liquid chromatograph (Model 5840A). A 180 cm x cm stainless steel Chromasorb column (10% SP 1200 and 1% H_3PO_4 80/100 WAW mesh) (Supelco Inc., Bellefonte, PA) was used. Conditions were column temperature 125°C, injection temperature 170°C, flame ionization detector temperature 175°C, carrier gas flow (Helium) 50 ml/min.

Urine Analysis. Five ml of urine was analyzed for nonconjugated volatile phenolics. In a screw cap vial 5 ml of diethyl ether and 1 ml of p-methoxyphenol as an internal standard, were added and the urine extracted. The ether layer was drawn off and 2 microliters was analyzed by GLC. Another 5 ml of urine was added to 5 ml 4 N.HCl and hydrolyzed in a boiling water bath for 60 minutes. Ten ml of diethyl ether and the internal standard were added and the urine extracted in a screw cap vial. The ether phase was then drawn off and 2 microliters was analyzed by GLC. A 180 cm x .3125 cm stainless steel Chromasorb WAF-DMCS 60/80 mesh column (21% Carbowax 4000; Supelco Inc., Bellefonte, PA) was used with column temperature, 185°, injection and FID temperature 250° and helium flow 40 ml/min.

Nonvolatile aromatic acids were analyzed with a trimethylsilylation procedure. Two ml of the diethyl ether extract was placed in a small screw cap vial and evaporated to dryness under a nitrogen gas stream in a warm water bath. Five tenths milliliters of chloroform and .02 ml of

N,N-dimethylformamide (Aldrich Chemical Co., Milwaukee, Wis) were added to each tube to redissolve phenolic acids. Two tenths milliliters of N, -bis-(Trimethylsilyl)-acetamide (# 38839 Pierce Chemical Co., Rockford, Ill.) was added with a syringe and samples were then incubated in a sealed screw cap vial for 30 minutes at 60° C. Samples were allowed to cool and two microliters were analyzed by GLC.

Samples were analyzed on a 180 cm x 0.3125 cm stainless steel Chromasorb WHP column (57 OV-210 mesh 100/120 Supelco Inc., Bellfonte, PA.) with conditions as follows: column temperature, 140° C, injection temperature, 250°; flame ionization detector temperature, 300°, carrier flow rate (helium) of 25 ml/min.

Rumen contents. Rumen contents were acidified with concentrated HCl to a pH of 2.0. The final volume was measured and placed in a separatory funnel. Diethyl ether was added three separate times at three times the volume of the acidified rumen contents. The ether layer was evaporated to a final volume of 25 ml in a hot water bath under a stream of nitrogen. This ether extract was then stored in tightly sealed screw cap vials for volatile and nonvolatile aromatic phenolics as above. The same procedure was carried out for Experiment II as well, with one exception. Acidified rumen contents were only extracted twice at three times the final volume.

Blood extraction. Whole blood was extracted twice with diethyl ether at three time final volume. The ether phase was then evaporated under nitrogen in a hot water bath and treated the same as for the rumen contents extract.

Rumen Ammonia. Three milliliters of the supernatant from the VFA procedure was analyzed for ammonia with a Technicon Auto Analyzer (AAC

#7.026-7.030).

Gross Energy of Feed Samples. Each diet was measured for its caloric content with the use of a Parr Adiabatic Oxygen Bomb Calorimeter (Parr Instrument Co., Moline, ILL.) A known amount of finely ground dry feed material from each diet was pelleted and placed in a combustion capsule. The capsule was then placed in an oxygen bomb containing 25 to 30 atmospheres of oxygen. The oxygen bomb was then placed in exactly 2000 g of water and the bomb and water allowed to equilibrate to the same temperature. After this equilibration period the sample was ignited with a fuse wire and the temperature rise was measured. The gross energy of the sample was calculated by multiplying the change in temperature times the hydrothermal equivalent of the calorimeter minus the titrated acid production plus a fuse wire oxidation correction divided by the sample weight.

Statistical Analysis

Experiment I was analyzed as a split plot and Experiment II was evaluated with Students T test (Gill, 1978a,b,c).

RESULTS

Experiment I - In Vitro Study

pH

Incubation pH values are given in Table 2. There were no statistically significant differences in control or treatment pH values for any diet. The treated hay diet had an initial pH of 6.6± and a final pH after 24 hrs of 6.3±. Control observations for the hay diet declined from 6.6± to 6.2±. The hay:concentrate diet had initial pH values of 5.8 for the control and treatment incubations and final values of 5.1± for control and 5.1± for treatment flasks. Overall the hay:concentrate diet had intermediate values in pH. The greatest reduction in incubation pH was between 0 to 4 hours in all cases. This decline continued for 8 hours for all diets. After 8 hours the all hay diet incubation pH values remained relatively constant while the hay: concentrate and concentrate incubations fluctuated. No statistical conclusion as to the difference in pH due to diet could be made due to a significant diet x time interaction. This was also the case for differences in pH with time.

Volatile Fatty Acids

Volatile fatty acid concentrations are given in Tables 3 and 4. Concentrations were not significantly affected by diet or tyrosine incubation. Acetate and butyrate concentrations could not be termed significantly different because of a significant diet x time interaction. The hay diet had the highest acetate concentration with values ranging

Table 2. In Vitro Incubation pH: Control (C) and Tyrosine Treated (T) Flasks for All Diets[1]

	Diet[2]					
	Hay		Hay:Concentrate		Concentrate[3]	
Hour	C	T	C	T	C	T
0	6.61	6.61	5.84	5.84	4.70	4.83
4	6.40	6.36	5.35	5.37	4.20	4.63
8	6.32	6.28	5.10	5.08	4.15	4.59
12	6.25	6.27	5.20	5.25	4.05	4.64
24	6.19	6.28	5.15	5.11	4.37	4.75

[1]Mean Values given for nine observations
[2]Diets are not significantly different
[3]Mean values for seven observations

Table 3. In Vitro Volatile Fatty Acid Concentration[1] (mMolar) Control Incubations

	Diet								
	Hay			Hay:Concentrate			Concentrate[2]		
Hour	A	P	B	A	P	B	A	P	B
0	127.60	36.33	22.66	180.77	32.57	33.79	243.62	66.29	25.11
4	133.71	33.83	22.60	192.17	37.87	45.03	313.07	114.50	40.55
8	134.03	34.26	23.67	173.63	44.46	35.69	266.30	67.17	36.78
12	130.92	33.34	25.42	256.60	49.14	56.78	256.43	75.76	50.15
24	153.99	39.12	31.56	293.39	51.79	54.81	309.76	93.18	45.78

Abbreviations: A - Acetate; P - Propionate; B - Butyrate

[1]Values given are means of nine observations

[2]Values are means of seven observations

Table 4. In Vitro: Volatile Fatty Acid Concentrations[1] (mMolar) Tyrosine Incubations

	Diet[2]								
	Hay			Hay:Concentrate			Concentrate[3]		
Hour	A	P	B	A	P	B	A	P	B
0	128.72	33.58	21.03	228.00	48.38	37.36	228.34	70.79	38.72
4	155.13	41.44	26.77	216.98	54.52	44.94	262.35	83.95	40.72
8	166.17	44.95	30.10	284.43	63.98	60.57	287.54	93.29	36.54
12	164.67	45.08	32.44	319.73	64.28	59.05	296.41	110.43	45.32
24	172.69	51.26	35.30	276.73	66.01	67.09	394.19	116.51	62.51

Abbreviations: A - Acetate; P - Propionate; B - Butyrate

[1]Values given are means of nine observations

[2]No significant differences exist due to diet or time

[3]Values are means of seven observations

from 62.08 molar % to 59.40 molar % at 24 hours. Propionate concentrations declined to 27.17 molar % at 12 hours and increased slightly at 24 hours. Butyrate concentrations declined to 4 hours and increased thereafter. The hay:concentrate diet incubations had lower acetate concentrations and higher propionate and butyrate concentrations. There was no trend over time with any of the volatile fatty acids. No difference existed between the hay:concentrate and concentrate incubations except for apparently higher propionate concentrations at every incubation time for the concentrate diet.

Phenolic Acid Concentrations

Tyrosine addition to rumen contents had little effect on 3-phenylpropionic acid (3-PPA) concentrations. There were no significant differences in 3-PPA between control and treated flasks in any of the diets except the hay:concentrate diet at the 0 time observation. This is likely to be experimental error despite the fact that repeat analyses demonstrated the same values. Looking at the data in Tables 5 and 6 there is an obvious trend toward increased 3-PPA with tyrosine incubations and the presence of hay in the diet. Peak 3-PPA concentration for the hay diet for the tyrosine treated incubations was 2.06 μM which occurred at 24 hours. The other diets demonstrated considerable fluctuation in 3-PPA concentrations with time. Peak 3-PPA levels for the hay:concentrate were at 4 hours for control incubations (0.74 μM) and 8 hours for tyrosine treated rumen contents (1.88 μM). The concentrate diet had initial 3-PPA concentrations which were at least 25% less than that of the other diets. Treated incubations had the highest 3-PPA concentrations at 8 hours (0.67 μM) and declined thereafter. Despite the large difference present between the diets

Table 5. In Vitro Rumen 3-Phenylpropionic (3PPA) Acid, p-Hydroxyphenylacetic Acid (PHPAA) and p-Cresol (PC) Concentrations: Control Incubations (μMolar)

	Diet								
	HAY			HAY:CONCENTRATE			CONCENTRATE		
Hour	3PPA	PHPAA	PC	3PPA	PHPAA	PC	3PPA	PHPPA	PC
0	.939 ± .03	ND	ND	.317 ± .05	ND	.009 ± .006	.308 ± .23	.187 ± .18	ND
4	.685 ± .21	.219 ± .04	ND	.744 ± .38	.543 ± .16	.139 ± .16	1.059 ±1.36	.374 ± .35	ND
8	.456 ± .35	.182 ± .92	.472 ± .02	.685 ± .56	1.223 ± .63	.037 ± .02	.638 ± .20	.069 ± .04	.028 ± .07
12	.102 ± .05	.214 ± .12	.823 ± .35	.629 ± .23	1.123 ± .51	.407 ± .13	.716 ± .30	.046 ± .04	.036 ± .02
24	.330 ± .15	.306 ± .15	1.390 ± .38	.507 ± .35	.219 ± .02	.509 ± 2.09	.685 ± .63	.179 ± .16	.162 ± .004

ND - Not Detectable

Table 6. In vitro Rumen 3-Phenylpropionic (3PPA) Acid, p-Hydroxyphenylacetic Acid (PHPAA) and P-Cresol (PC) Concentrations:[1] Tyrosine Incubations (μMolar)

	Diet[2]								
	HAY			HAY:CONCENTRATE			CONCENTRATE[3]		
Hour	3PPA	PHPAA	PC	3PPA	PHPAA	PC	3PPA	PHPPA	PC
0	1.073 ± .09	0.125 ± .003	0.026 ± .01	1.196[b] ± .15	0.019 ± .01	0.017 ± .006	0.041 ± .03	0.011 ± .01	0.010 ± .005
4	1.449 ± .21	9.646 ± 5.83	0.312 ± .20	1.215 ± .34	0.867 ± .23	0.238 ± .16	0.239 ± .11	0.340 ± .09	0.063 ± .02
8	1.627 ± .34	12.160[a] ± 2.46	1.413 ± 1.0	1.875 ± .39	1.516 ± .50	0.367 ± .17	0.668 ± .15	0.464 ± .15	0.209 ± .04
12	1.976 ± 1.17	6.696[a] ± 1.01	0.863 ± .33	1.776 ± .28	1.220 ± .41	0.544 ± .08	0.468 ± .03	0.690 ± .01	0.333 ± .04
24	2.063 ± .29	0.917 ± .30	1.898[b] ± .476	1.629 ± .33	0.155[c] ± .01	2.022 ± .74	0.610 ± .16	0.123 ± .07	0.848 ± .30

[a]Means differ significantly from control P<.10

[b]Means differ significantly from control P<.025

[c]Means differ significantly from control P<.05

[1]Values reported are means of nine observations

[2]No significant differences due to diet observed

[3]Values reported means of seven observations

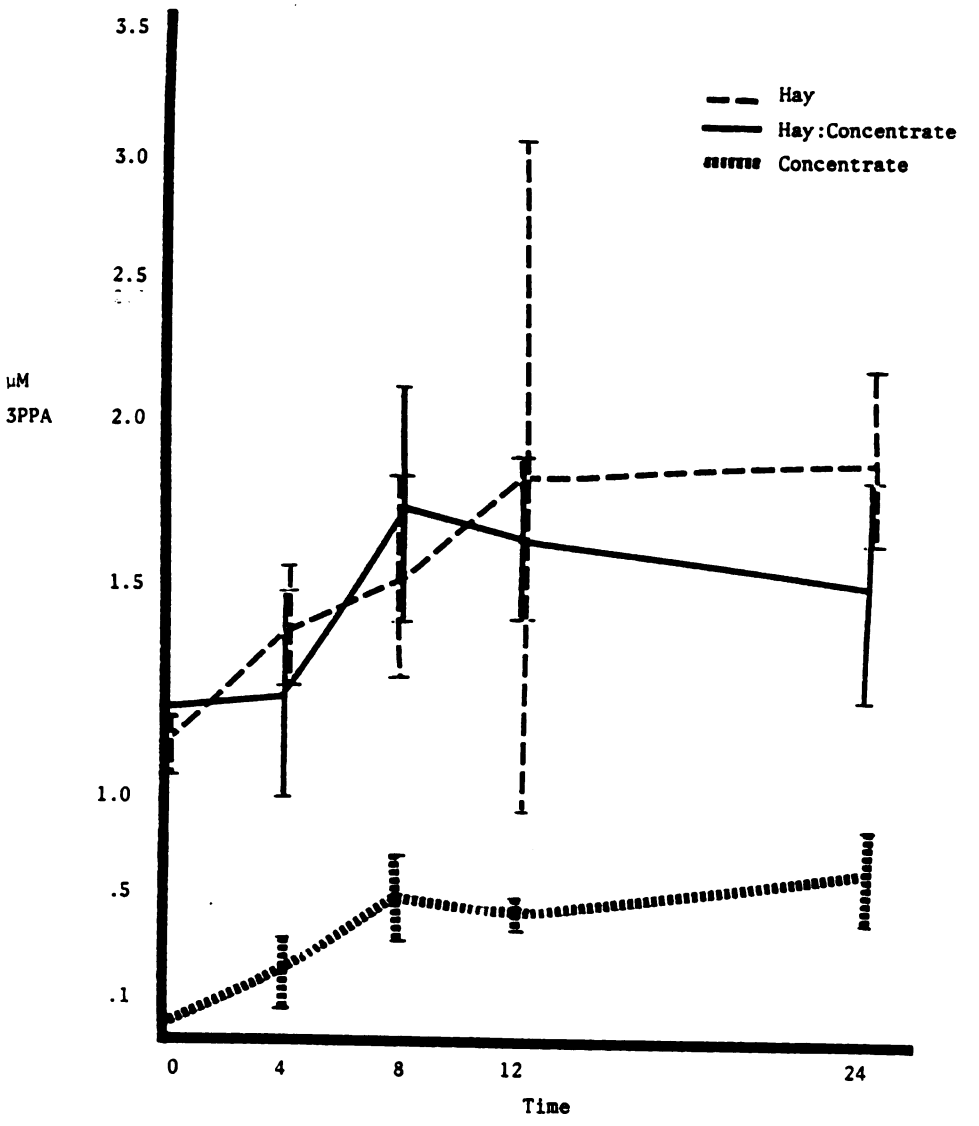


Figure 8. In vitro mean 3-phenylpropionic acid (3PPA) Concentration (µM) for tyrosine treated incubations for all diets.

containing hay and the concentrate diet no statistical significance could be shown for treated incubations. This was due to a significant interaction between diet and observation. Figure 8 illustrates the means of all diets across animals plotted with standard errors. The large standard errors illustrate the variance within and between animal observations.

In general addition of tyrosine to rumen fluid incubations increased p-hydroxyphenylacetic acid (PHPAA). Comparing the control rumen fluid incubations to the tyrosine treated incubations for the hay fed animals there were significant differences at 8 and 12 hours of incubation ($P<.10$). At 8 hours the tyrosine treated incubations had an average PHPAA concentration of 12.16 μM while control incubations only had a concentration of 0.18 μM. Concentrations declined to an average of 6.69 μM for the tyrosine incubations and increased to 0.21 μM for the control incubations. Four and 24 hour PHPAA concentrations were significantly different at the 35% confidence level but were not significantly different at the 90% confidence level.

The rumen fluid incubations from animals fed a hay:concentrate diet were not different in PHPAA concentration when tyrosine was added except at 24 hours ($P<.05$). At this time the control incubations had a PHPAA concentration of 0.21 μM while treated flasks had a lower concentration (0.15 μM). Both incubations showed increases in concentration to 8 hours with declines occurring thereafter.

The concentrate diet showed no difference between tyrosine treated incubations and control incubations with respect to PHPAA at any time. At 4 and 8 hours PHPAA concentrations were higher for treated flasks but not different due again to large variation between diet within

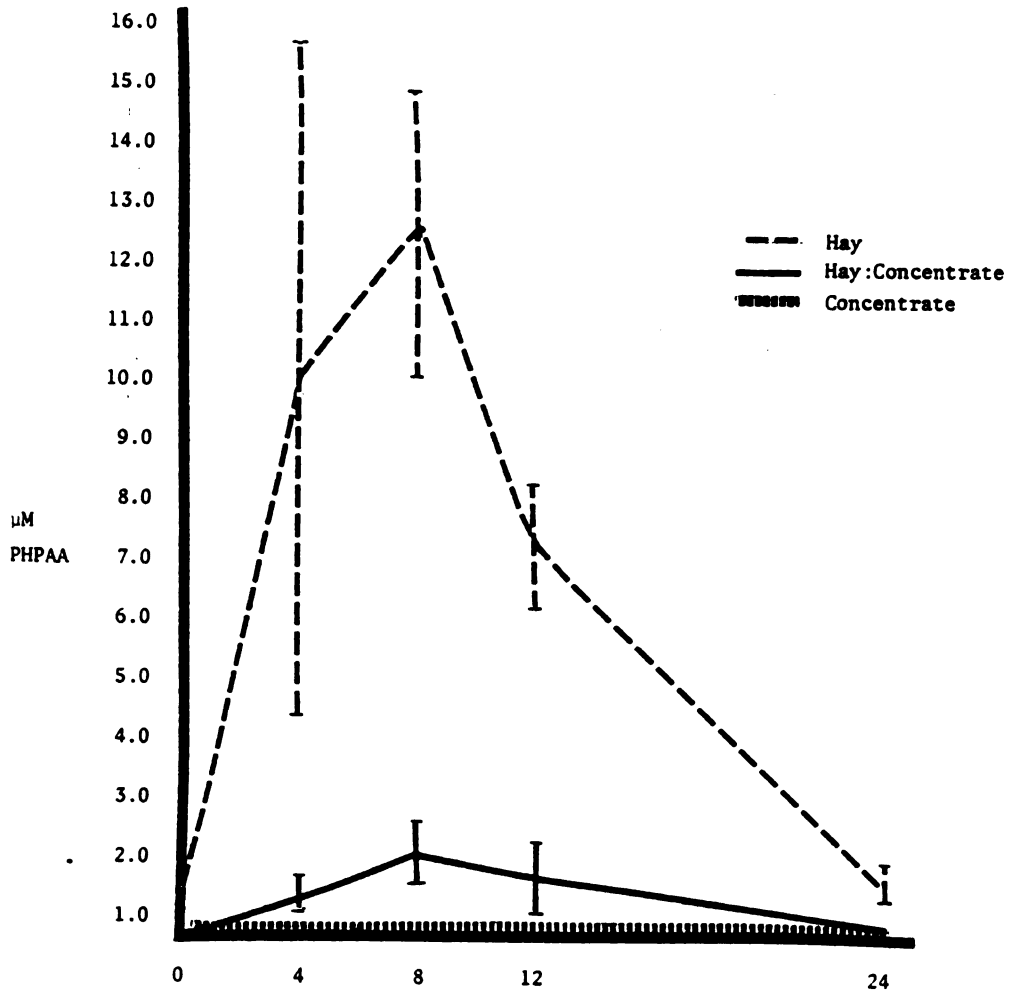


FIGURE 9. In vitro mean p-hydroxyphenylacetic acid (PHPAA) concentration (µM) for tyrosine treated incubations for all diets.

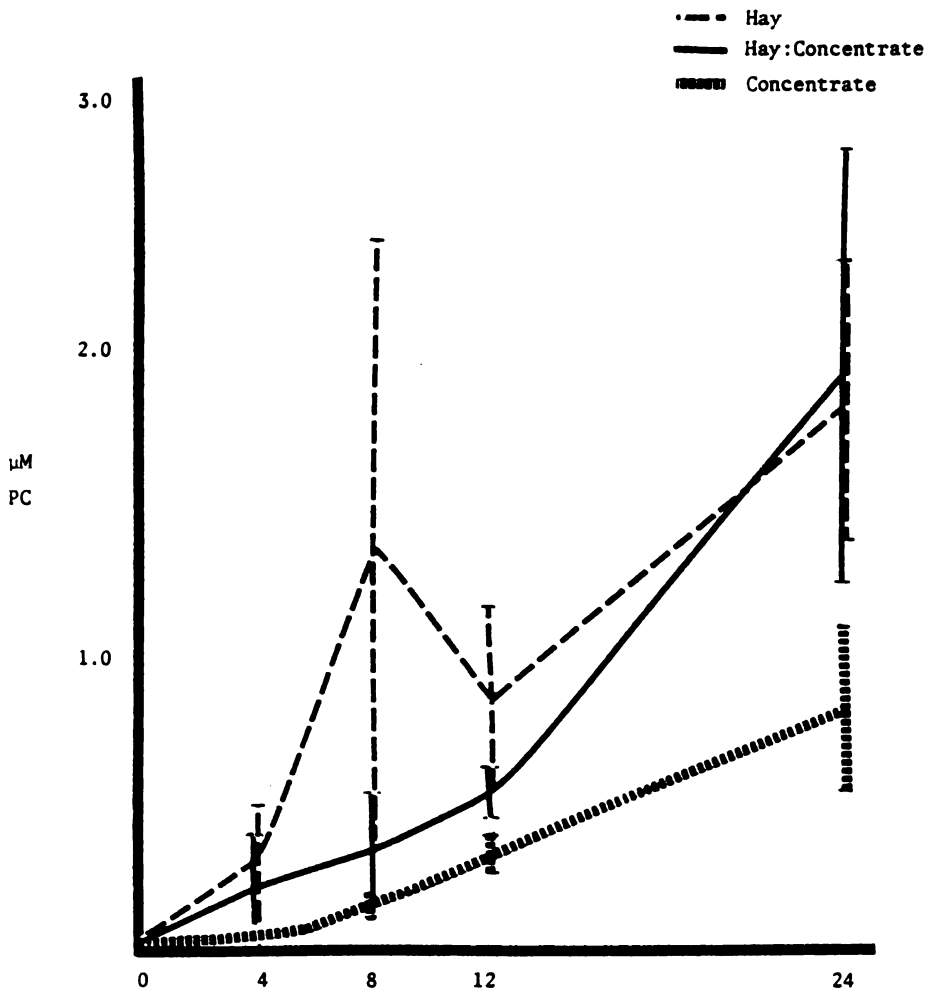


FIGURE 10. In vitro: Mean p-cresol (PC) concentration (µM) for tyrosine treated incubations for all diets.

observation.

P-cresol concentrations were not statistically different for the hay diet except at 24 hours. At all times however, the concentration in the tyrosine treated flasks exceeded that of the control flasks. Peak p-cresol concentrations occurred at 24 hours and were 1.387 μM for control flasks and 1.898 μM for tyrosine treated flasks. These values are significantly different ($P<.05$).

There was no statistical difference apparent for p-cresol levels in control and treated flasks from the hay:concentrate or concentrate diets. Control flasks always had lower p-cresol concentrations. In all incubations for diets containing concentrates (treated and control) p-cresol concentrations increased from 0 time to 24 hours with highest concentrations found at 24 hours. The control and tyrosine treated incubations for the hay:concentrate diet had higher p-cresol concentrations than did the same incubations for the concentrate diet.

Comparing tyrosine treated flasks for diet effects revealed 3-phenylpropionic acid concentrations to be higher with the presence of hay in the diet. This, however, was not significantly different due to a significant diet x observation interaction. This is not surprising due to the small sample size and large variation between observations within animal and within diet.

p-Hydroxyphenylacetic acid concentrations were also not different between diets. In this case three interactions were determined to be highly significant ($P<.005$). These were diet x observation, observation x time and diet x time. Within the tyrosine treated flasks, p-hydroxyphenylacetic acid concentrations were the greatest at all times for the hay diet and the lowest at all times for the concentrate diet.

In vitro p-cresol concentrations for the tyrosine incubations were significantly different with time of incubation ($P<.0005$). P-cresol concentrations were not different between diets due to a significant diet x observation interaction.

Urinary Phenolics

Urinary 3-phenylpropionic acid and p-hydroxyphenylacetic acid concentrations were not significantly different between diets (Table 7). This was due largely to variations in urine volume as well as large differences in concentrations excreted. There was a trend for increasing 3-PPA levels excreted in the urine when the animals were consuming a concentrate diet.

P-cresol concentrations were significantly different between diets ($P<.10$). Total p-cresol in the urine was significantly different at the 85% confidence level but not at the 90% level of confidence. The presence of hay in the diet increased the urinary p-cresol excretion over that seen with the all concentrate diet (Table 7).

Experiment II - In Vivo Study

pH

Tyrosine administration at 0.35 g/kg body weight significantly lowered rumen pH at 2 hours, 8 hours ($P<.10$) and 24 hours ($P<.05$). Rumen pH declined an average of 0.14 pH units at all times of observation after tyrosine was administered intraruminally. Actual values are given in Table 8. Because of the limited number of animals used in this study the data are presented for the individual animals.

Table 7. Concentration and Total Excretion of Urinary 3-Phenylpropionic Acid (3PPA), p-Hydroxyphenylacetic Acid (PHPAA) and P-Cresol (PC).

Diet	3PPA	PHPAA	PC
Hay			
μM	0.04 ± 0.24	0.49 ± 0.40	0.99[a] ± 0.23
Total (mg)	3.77 ± 27.37	34.68 ± 46.09	54.95 ± 19.60
Hay:Concentrate			
μM	0.54 ± 0.24	0.23 ± 0.40	0.64[a] ± 0.23
Total (mg)	53.25 ± 27.37	52.06 ± 46.09	54.79 ± 19.60
Concentrate[1]			
μM	0.42 ± 0.24	0.68 ± 0.40	0.06[a] ± 0.23
Total (mg)	64.41 ± 27.37	96.86 ± 46.09	6.35 ± 19.60

[1]Means are from two animals, all other means from three animals.

[a]Superscript indicates a statistically significant difference due to diet $P<.10$.

Table 8. Intraruminal Tyrosine Administration: Rumen Fluid pH for Control (C) and Treatment (T) Observations

		Time							
Animal		0	2	4	6	8	12	24	48
1									
	C	7.00	6.80	6.23	6.68	6.78	6.63	7.00	7.00
	T	6.57	6.45	6.40	6.61	6.55	6.55	6.80	6.94
2									
	C	6.85	6.85	6.75	6.55	6.65	6.62	6.80	6.80
	T	6.80	6.62	6.66	6.65	6.52	6.67	6.65	6.84
$\bar{X}$									
	C	6.98	6.83[a]	6.49	6.62	6.72[a]	6.63	6.90[b]	6.90
	T	6.69	6.54[a]	6.53	6.63	6.54[a]	6.61	6.73[b]	6.89

[a]Similiar superscripts in columns indicate a difference P<.10

[b]Similiar superscripts in column indicate a difference P<.05

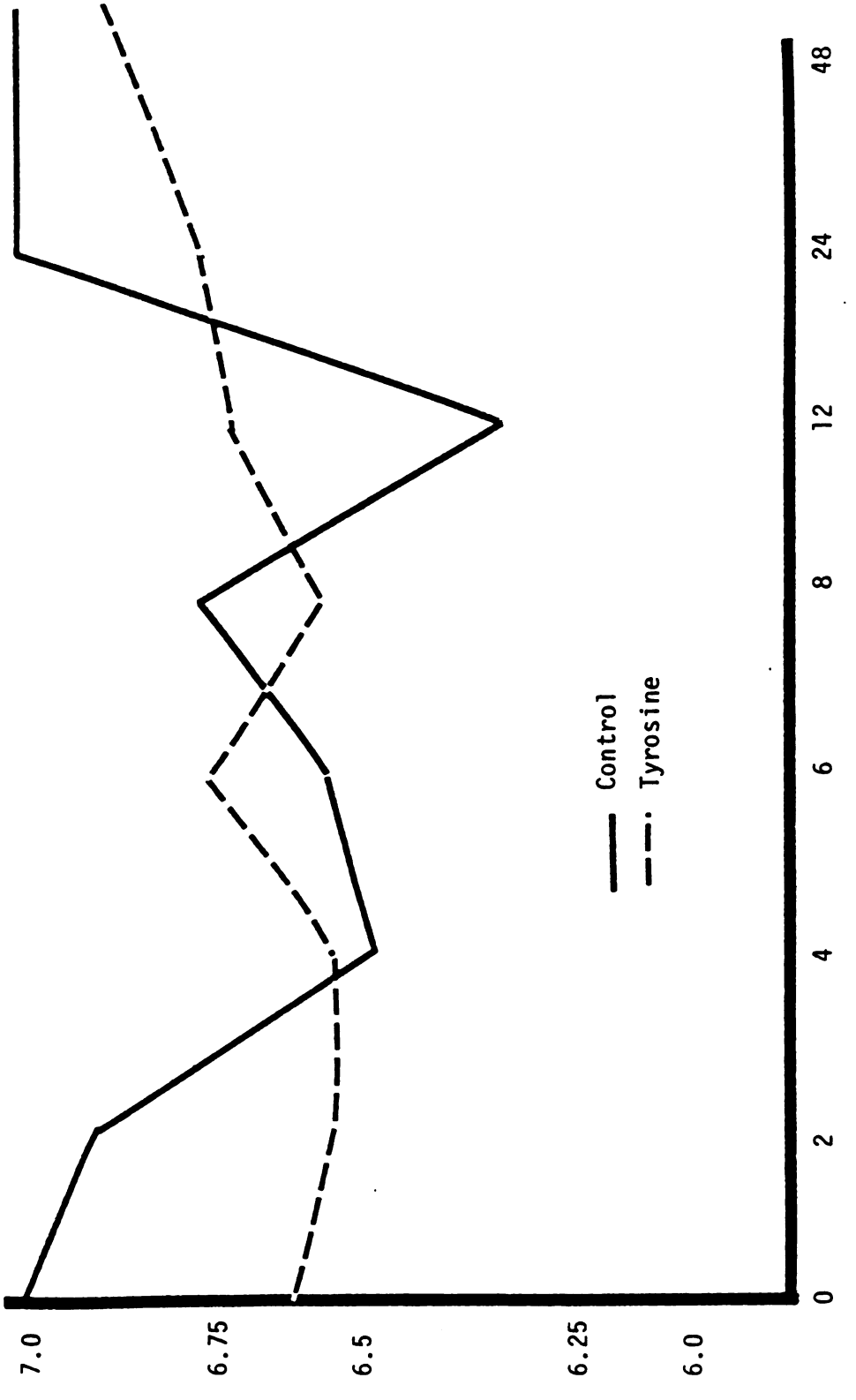


FIGURE 11. Study 2: Intraruminal administration of tyrosine rumen pH.

Rumen Ammonia

Rumen ammonia was found to be significantly increased ($P<.10$) at 0 time (pre-treatment), 4 hours ($P<.005$), 24 hours ($P<.10$) and 48 hours ($P<.01$). Observations at 2, 6, 8 and 12 hours approached significance but were not different at the 90% level of confidence. In all cases the treatment observations were greater than the control observations. The average difference was 5 mg %. Peak ammonia concentrations in the control period occurred at 4 and 12 hours with concentrations reaching 14.76 mg % and 15.94 mg % respectively. Peak concentrations after tyrosine was administered occurred at 4 and 24 hours. These concentrations were 21.95 mg % and 21.30 mg % respectively. Normal diurnal fluctuations in rumen ammonia were evident during the control period and the treatment period.

Volatile Fatty Acids

Volatile fatty acid concentrations were not substantially affected intraruminal tyrosine administration. Acetate concentrations fluctuated for both control and treatment observations; however, no trend was present. Maximum acetate concentrations were seen before feeding for the control period (66.22 molar %) and at 6 hours post tyrosine administration for the treatment observations (68.82 molar %). Propionate concentrations did not differ with treatment except at 2 hours where propionate was significantly depressed ($P<.01$). Butyrate concentrations fluctuated as much as the acetate and propionate concentrations. At 6 hours a significant increase in butyrate was seen ($P<.07$).

Table 9. Intraruminal Tyrosine Administration: Rumen Ammonia Concentrations (mg %) for Control (C) and Treatment (T) Observations.

Animal		Hours 0	2	4	6	8	12	24	48
1									
	C	15.13	14.40	16.36	13.65	15.15	15.63	15.13	14.40
	T	17.20	18.40	23.60	22.00	22.50	20.30	21.60	20.00
2									
	C	12.08	14.48	13.15	12.90	14.03	16.25	12.08	12.08
	T	14.90	23.50	20.30	16.90	17.00	17.40	21.00	17.40
$\bar{X}$									
	C	13.61[a]	14.44	14.76[b]	13.28	14.59	15.94	13.61[a]	13.24[c]
	T	16.05[a]	20.95	21.95[b]	19.45	19.75	18.85	21.30[a]	18.70[c]

[a]Superscripts in columns indicate a significant difference P<.10

[b]Superscripts in columns indicate a significant difference P<.005

[c]Superscripts in columns indicate a significant difference P<.01

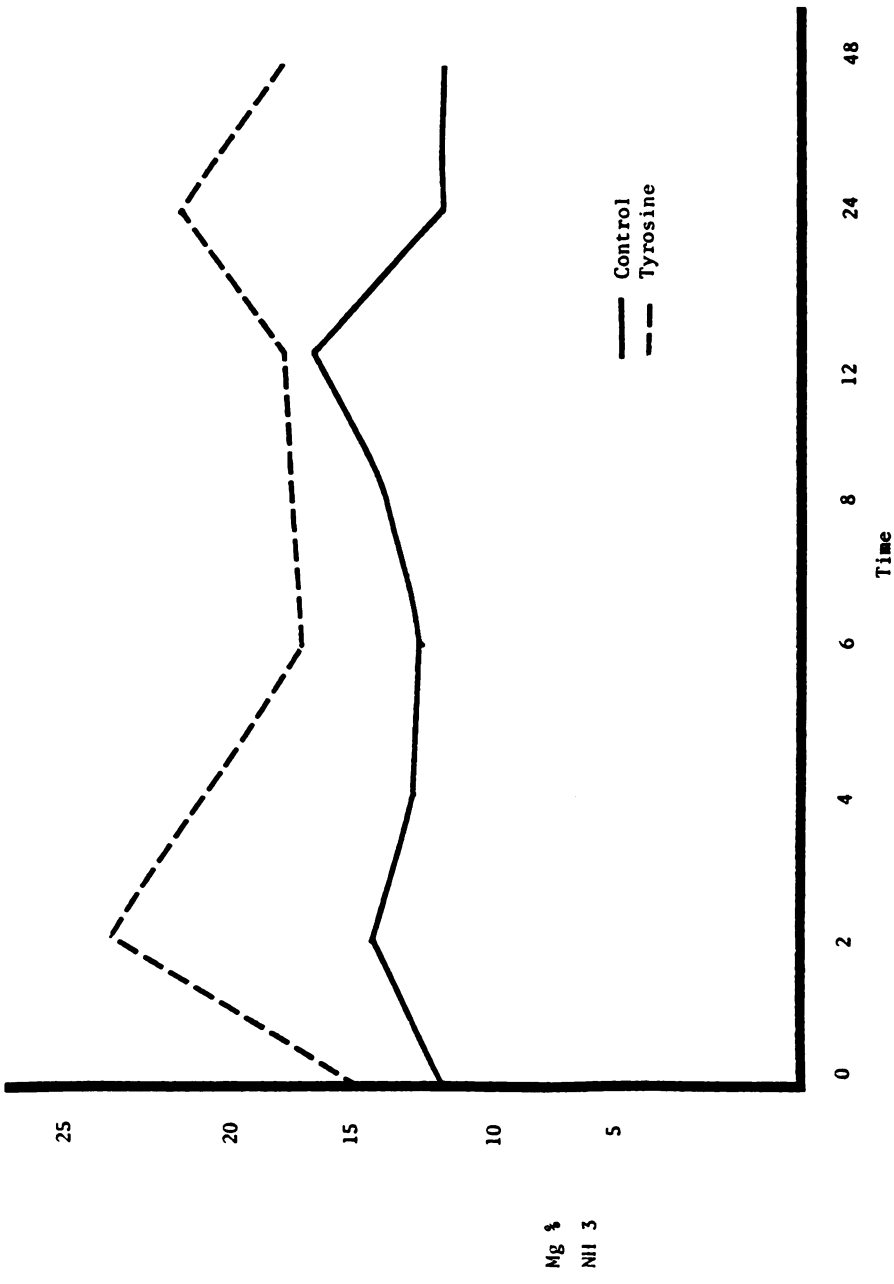


FIGURE 12. Study 2: Intraruminal administration of tyrosine, mean rumen ammonia concentration.

Table 10. Intraruminal Tyrosine Administration: Volatile Fatty Acid Concentration (mMolar) for Control (C) and Treatment (T) Observations

Hour	C	T	C	T	C	T
0	178.69	150.21	38.91	34.38	24.45	17.51
2	200.59	162.55	47.11[a]	34.74[a]	27.59	22.96
4	212.48	185.27	45.66	42.15	29.54	25.19
6	192.97	121.32	43.29	37.73	26.31[b]	23.59[b]
8	187.95	168.83	42.13	36.62	25.69	23.08
12	222.39	187.11	49.97	42.11	30.11	24.92
24	178.69	198.09	139.91	45.86	24.45	26.98
48	178.60	185.65	39.91	39.08	24.45	24.59

[a]Superscripts in rows indicate a significant difference P<.01

[b]Superscripts in rows indicate a significant difference P<.07

Rumen Phenolic Acids

3-Phenylpropionic acid concentration was increased with intraruminal tyrosine administration. The 0 time observation (before treatment) was significantly lower ($P<.025$) than the control observation. Treatment period observations were lower than control observations to 4 hours after which they exceeded control observations. At 8 hours the concentration of 3-phenylpropionic acid was statistically significantly greater ($P<.05$) in the treated animals. Peak 3-phenylpropionic acid concentrations occurred at 6 hours for the control period and 48 hours for the treatment period. There was no defined trend for 3-phenylpropionic acid during the control period with fluctuations occuring in concentration from observation to observation. Treatment concentrations increased to 24 hours where they declined slightly, then doubled to 48 hours. Table 12 illustrates the extreme variation that occurred from animal to animal.

p-Hydroxyphenylacetic acid (PHPAA) concentrations showed the same pattern as the 3-phenylpropionic acid concentrations. At 0 time PHPAA concentrations were significantly lower for the treatment observations than the control observations ($P<.025$). Fluctuations in concentration occurred for both control and treatment observations. At 8 hours PHPAA concentration was significantly increased over control ($P<.05$).

P-cresol concentrations were in all cases greater for the treatment period than for the control period. At 6 hours the difference was statistically significantly different ($P<.05$). Although at first glance there is a great deal of difference between treatment and control, examination of individual animal observations (Table 12) illustrates the wide variation between animals.

Table 11. Intraruminal Tyrosine Administration: Rumen 3-Phenylpropionic Acid (3PPA), 3-Hydroxyphenylacetic Acid (PHPAA) and P-cresol (PC) Concentrations for Control (C) and Treatment (T) Observations (μMolar)

	3PPA		PHPAA		PC	
Time	C	T	C	T	C	T
0	0.50[a]	0.44[a]	0.21[a]	0.10[a]	0.05	0.05
2	0.66	0.61	0.19	0.36	0.03	0.30
4	0.49	0.55	0.06	0.05	0.16	0.96
6	0.67	0.78	0.06	0.12	0.03[b]	0.85[b]
8	0.49[b]	0.82[b]	0.04[b]	0.08[b]	0.04	0.53
12	0.43	0.82	0.05	0.30	0.02	0.12
24	0.50	0.61	0.21	0.21	0.05	0.53
48	0.50	1.21	0.21	0.59	0.05	0.70

[a]Means in rows with similiar superscripts differ P<.025

[b]Means in rows with similiar superscripts differ P<.05

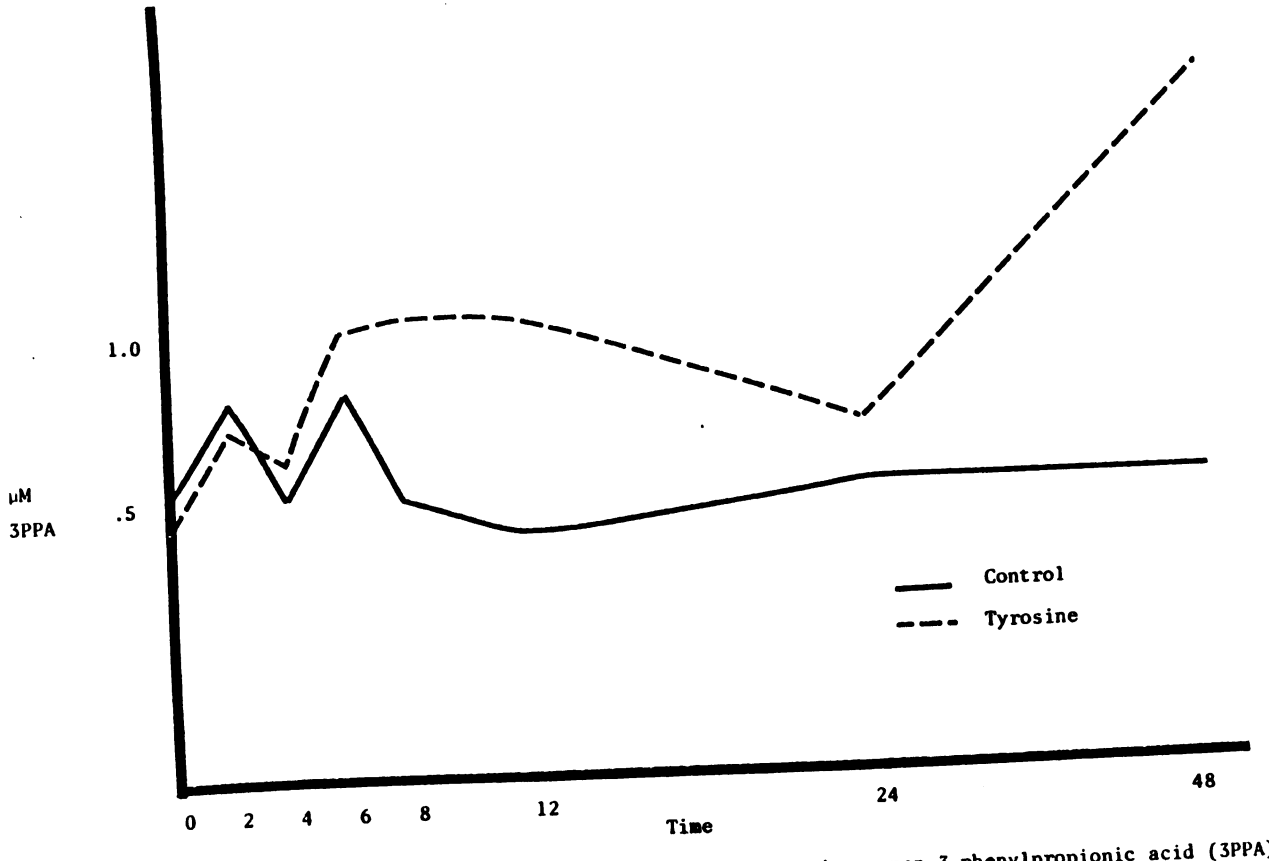


Figure 13. Study 2: Intraruminal tyrosine administration rumen 3-phenylpropionic acid (3PPA) Concentration (µM)

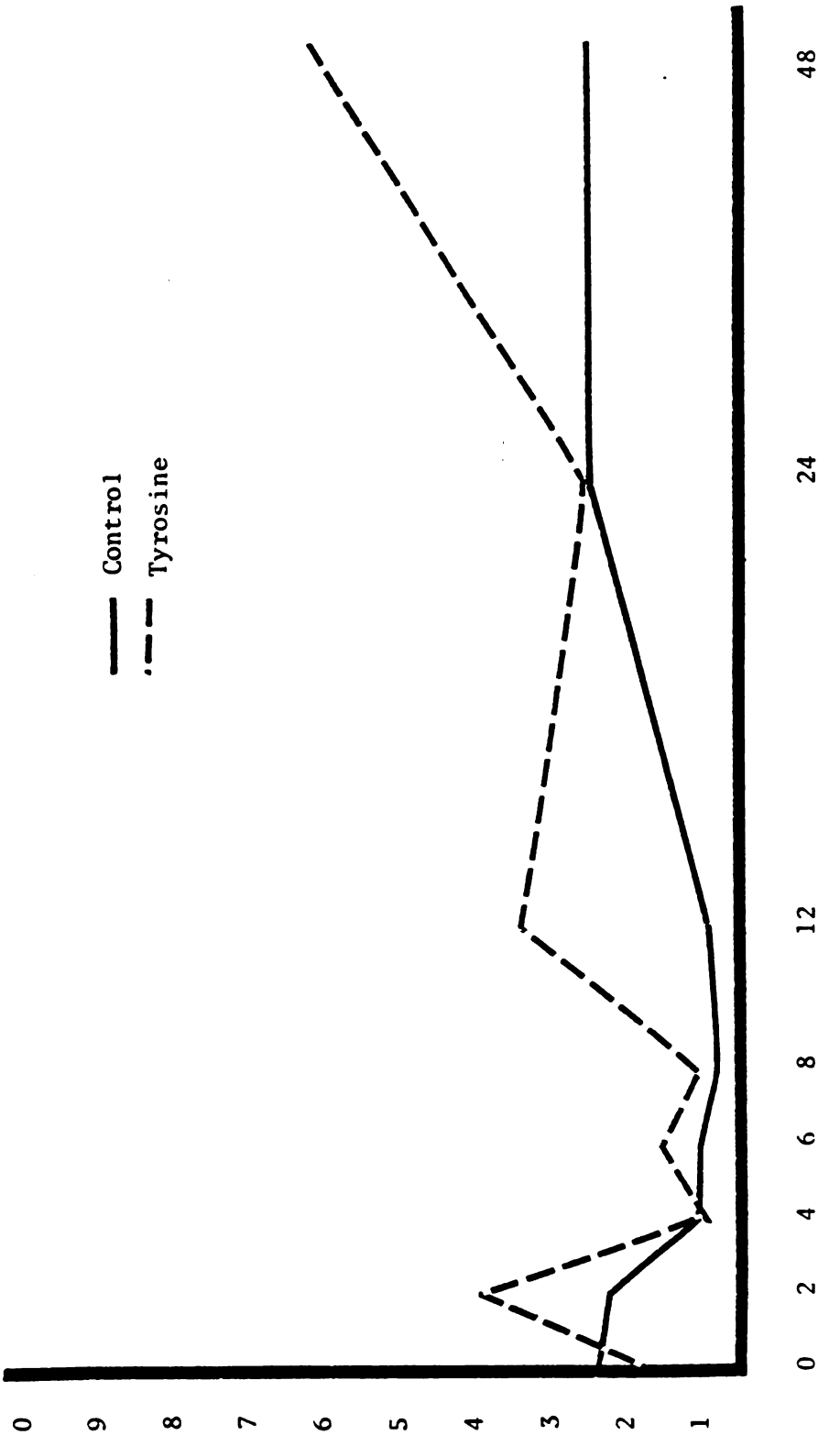


Figure 14. Study 2: Intraruminal tyrosine administration rumen p-hydroxyphenylacetic acid (PHPAA) concentration (µM)

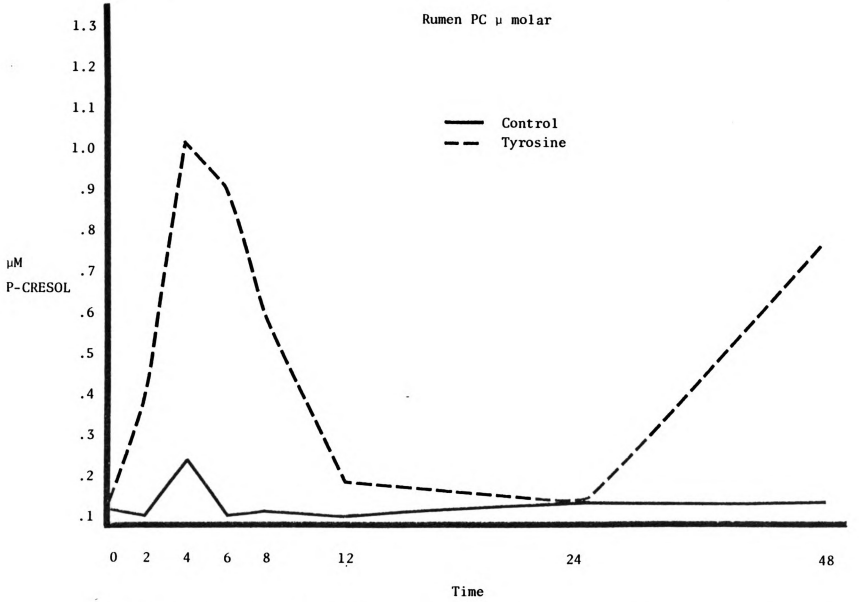


FIGURE 15. Study 2 Intraruminal administration of tyrosine rumen P-cresol concentration (μM)

Table 12. Intraruminal Tyrosine Administration: Individual Animal Observations for Ruminal 3-Phenylpropionic Acid (3PPA) p-Hydroxyphenylacetic Acid (PHPAA) and P-Cresol (PC) Concentrations (μMolar) - Control (C) and Treatment (T) Observations.

Sheep #		3PPA		PHPAA		PC	
1		C	T	C	T	C	T
H	0	.49	.43	.17	.06	.06	.03
O	2	.65	.31	.26	.04	.05	.03
U	4	.50	.24	.08	.03	.30	---
R	6	.78	.75	.04	.11	.05	1.07
S	8	.42	.79	.05	.09	.04	1.08
	12	.48	.58	.05	.62	.03	.25
	24	.49	1.21	.17	.42	.06	1.07
	48	.49	2.03	.17	1.12	.06	1.41
2							
	0	.52	.46	.27	.15	.05	.08
H	2	.67	.92	.13	.69	.03	.58
O	4	.49	.86	.05	.08	.03	1.93
U	6	.58	.82	.11	.14	.03	.64
R	8	.58	.87	.04	.07	.05	---
S	12	.39	1.07	.05	---	.02	---
	24	.52	.03	.27	---	.04	---
	48	.52	.39	.27	.07	.04	---

Blood Phenolic Acid Concentrations

Analysis of whole blood failed to show any of the phenolic compounds at any time for any animal.

Urine Phenolic Acid Concentrations

p-Hydroxyphenylacetic acid was not detected in the urine of these sheep in either of these collection periods. 3-phenylpropionic acid and p-cresol concentrations were significantly different within individual sheep ($P<.001$) but not when testing mean values. Total 3PPA and PC present in the urine was different for each sheep as well as for the mean values. Mean total 3PPA was increased with tyrosine treatment from 11.64 mg to 23.75 mg ($P<.09$). Total p-cresol increased from 357.47 mg to 2696.79 mg ($P<.001$). This statistical significance is due to increased urine output.

Table 13. Intraruminal Tyrosine Administration: Urinary 3-Phenylpropionic Acid (3PPA), p-Hydroxyphenylacetic Acid (PHPAA) and P-cresol (PC) Concentrations - Control (C) and Treatment (T) Observations

	3PPA		PHPAA		PC	
Animal	C	T	C	T	C	T
1						
Concentration (μM)	0.06[a]	0.12[a]	ND	ND	2.21[a]	2.98[a]
Total (mg)	13.95[a]	20.54[a]	ND	ND	318.64[a]	2777.68[a]
2						
Concentration (μM)	0.07[a]	0.08[a]	ND	ND	2.95[a]	3.63[a]
Total (mg)	9.31[a]	26.96[a]	ND	ND	396.28[a]	2615.90[a]
$\bar{X}$						
Concentration (μM)	0.07	0.10	ND	ND	2.58	3.30
Total (mg)	11.63[b]	23.75[b]	ND	ND	357.46[a]	2696.79[a]

[a]Means with similiar superscripts are different P<.001

[b]Means with similiar superscripts are different P<.09

DISCUSSION

The primary degradation products identified in rumen contents by gas-liquid chromatography after tyrosine incubation were p-hydroxyphenylacetic acid and 3-phenylpropionic acid. This is in agreement with previous work by Scott et al. (1964) and Patton and Kesler (1967). Scott and coworkers (1964) have proposed that these compounds are produced through Stickland reactions carried out by rumen bacteria. In the case of 3-phenylpropionic acid formation, tyrosine acts as a hydrogen acceptor in a coupled deamination of a pair of amino acids. The product of this reaction is p-hydroxyphenylpropionic acid (PHPPA). PHPPA then appears to be slowly dehydroxylated to 3-phenylpropionic acid. Scott and coworkers (1964) were able to identify PHPPA in low concentrations early in the tyrosine incubation sequence and demonstrated a decline in PHPPA as 3PPA levels increased. No p-hydroxyphenylpropionic acid was detected in any of the incubations in this study.

Another reaction scheme has been proposed in which tyrosine is initially deaminated to p-hydroxyphenylpyruvic acid. This intermediate compound is then dehydroxylated and reduced in a one step reaction to produce 3-phenylpropionic acid directly. The presence of this reaction mechanism in the rumen would account for the failure to identify p-hydroxyphenylpropionic acid in the incubations carried out in this study.

p-Hydroxyphenylacetic acid is also produced in the rumen via a Stickland reaction (Scott et al., 1964). In this case, however, tyrosine

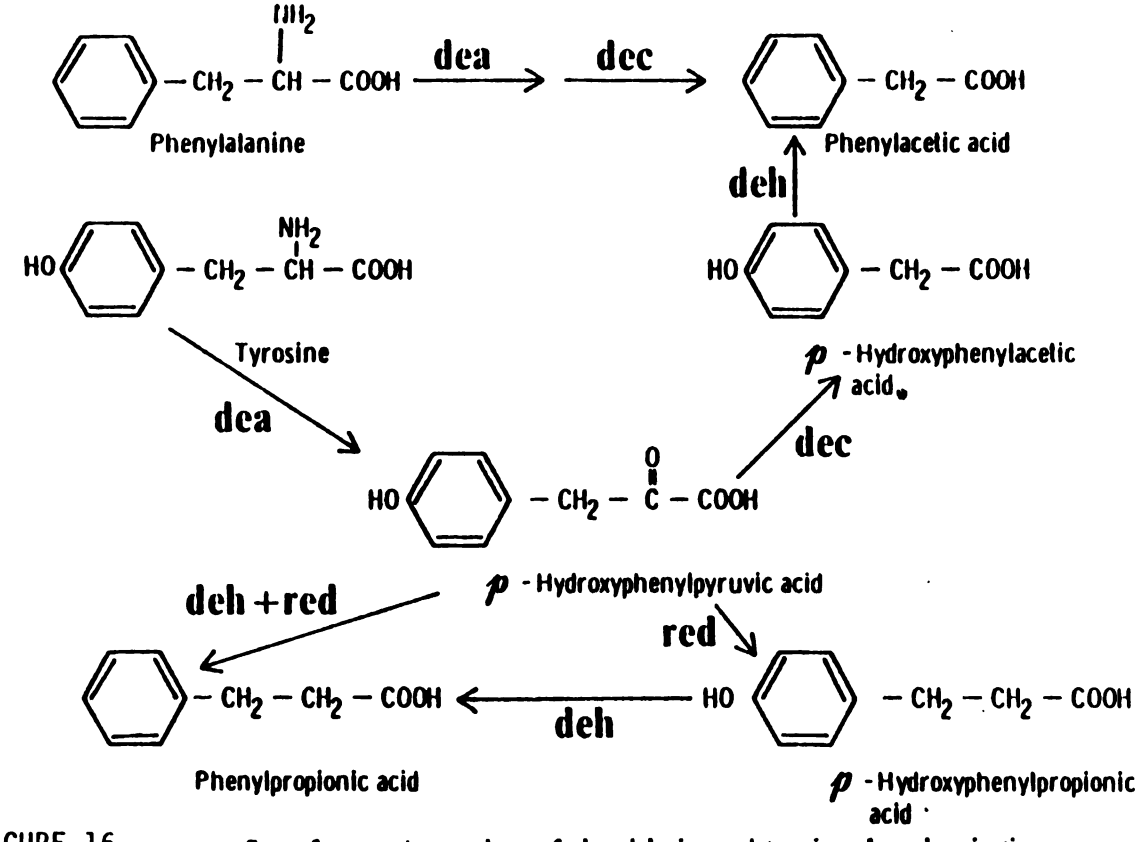


FIGURE 16. Some fermentation products of phenylalanine and tyrosine. dea: deamination; dec: decarboxylation; deh: dehydroxylation; red: reduction. (Prins, 1977).

acts as a hydrogen donor in a coupled deamination-decarboxylation reaction. In other mammalian systems p-hydroxyphenylpyruvic acid is an intermediate product in the production of p-hydroxyphenylacetic acid from tyrosine (Prins, 1977). p-Hydroxyphenylpyruvate has yet to be identified in the rumen. Because of its instability this compound is unlikely to be present under the anaerobic conditions existing in the rumen or in vitro rumen simulations.

P-cresol was also identified by gas-liquid chromatography to be present in rumen incubations. This metabolite arises from the decarboxylation of p-hydroxyphenylacetic acid. The presence of p-cresol in the rumen has been shown previously by Yokoyama and Carlson (1981) and Martin (1982c).

The results of the in vitro tyrosine incubations indicated that there were some differences due to diet. The diet x time interaction present in the statistical analysis would be expected in a time course study in which metabolite concentrations are changing with time of incubation. The diet x observation interaction is important to consider. Day to day variation within animals within diet combined with variation between animals on the same diet may be large enough to mask diet differences. This was apparently the case in this study.

The data do indicate several trends. With the hay diet the reaction sequence that produces p-hydroxyphenylacetic acid seems to be favored over the production of 3-phenylpropionic acid.

There are several possible explanations for the enhanced degradation of tyrosine to PHPAA. First the population of bacteria present in the rumen under a hay feeding regime may have a greater ability or need to utilize and metabolize phenolic acids. The cellulolytic bacteria are

intimately associated with the plant material in the rumen and plant constituents are the primary source of rumen phenolic acids. Recent data from Hungate and Stack (1982) indicate that 3-phenylpropionic acid, possibly derived from lignin degradation, stimulates cellulose digestion by a strain of *Ruminococcus albus*. The data in this study support this observation because the presence of hay in the diet resulted in higher concentrations of the phenolic acids examined.

p-Hydroxyphenylacetic acid is not a terminal metabolite in the rumen. It may be decarboxylated to form p-cresol or resynthesized to tyrosine. If it is dehydroxylated to phenylacetic acid, the phenylacetic acid can be utilized for phenylalanine biosynthesis by rumen bacteria (Kristensen, 1974). These three reactions combine to decrease the concentration of p-hydroxyphenylacetic acid in the rumen. If the enzymes involved in the production of PHPAA are inhibited by end product accumulation, as in a classical feedback regulation mechanism, the utilization of PHPAA in other reaction sequences would permit PHPAA production to continue.

Three-phenylpropionic acid is, on the other hand, a terminal metabolite in the rumen. Under conditions in which the concentration of 3PPA could accumulate the enzymes involved in its synthesis might be inhibited by the presence of excess 3-phenylpropionic acid.

Another explanation lies in the ability of the rumen microbes to carry out decarboxylation and dehydroxylation reactions. Decarboxylations occur frequently in the rumen in response to decreasing rumen pH (Prins, 1977). Dehydroxylation reactions, however, occur much more slowly. Data from Booth and Williams (1963) showed that dehydroxylations occurred at the rate of 5% of the incubated caffeic acid in 18 hours of incubation. If the dehydroxylation reaction to form 3-phenylpropionic acid from

p-hydroxyphenylpropionic acid occurred at this same rate, the 3PPA concentrations present in the rumen incubations of this study are too high to be accounted for by tyrosine metabolism alone. This agrees with the conclusions of Scott and coworkers (1964) and Martin (1978). The most likely source of this extra 3PPA is the phenolic monomers present in forages. The low 3-phenylpropionic acid concentrations seen in both the control and tyrosine treated rumen incubations for those animals fed a concentrate diet would correlate with this hypothesis.

P-cresol concentrations in the rumen incubations (both control and tyrosine treated) increased over time regardless of diet. The greater concentrations seen in those incubations from hay-fed animals reflect the increased production of its precursor, p-hydroxyphenylacetic acid. P-cresol production may be enhanced by the increased availability of the substrate from which the bacteria produces p-cresol. Concentrations of p-cresol may also be enhanced under the conditions of hay feeding.

A limitation of this study is the method employed in straining the rumen contents prior to incubation. By using cheesecloth to separate rumen liquor from feed particles, the cellulolytic bacteria bound to the fiberous material are strained out of the liquor and would be present in lower numbers in the incubations. These bacteria are stimulated by lignin degradation products (Hungate and Stack, 1982) and their presence in the incubations could have resulted in a greater degradation of tyrosine, resulting in higher metabolite concentrations.

Since the incubations of rumen fluid from hay fed sheep resulted in the greatest degradation of tyrosine to p-hydroxyphenylacetic acid as well as increases in 3-phenylpropionic acid and p-cresol, hay was fed

exclusively to the sheep during the second trial.

In vitro trial results need to be carefully interpreted because they do not always simulate the in vivo system under examination. The bacteria removed from the rumen are sensitive to oxidation-reduction potential, temperature changes and fluctuations in pH (Hungate, 1964). Even slight changes in these parameters may alter the in vitro population characteristics and result in incubations that do not reflect the original rumen population sampled. Rates of substrate disappearance and metabolite appearance are also altered because of substrate limitation and product accumulation. In the normal rumen the dilution rate and rate of removal influences the production and concentration of end products. With these considerations in mind a second trial was undertaken to examine tyrosine degradation under normal rumen conditions.

The amount of free tyrosine administred was calculated to be 0.35 g/kg body weight. This level of tyrosine was chosen to introduce the maximum amount of substrate into the rumen while preventing the possibility of an ammonia toxicity. Since tyrosine is 13% nitrogen approximately 3.20 g of nitrogen was given to one animal and 2.99 g given to the other. Assuming the rumen to be 4 liters in capacity and total deamination of all tyrosine administered, rumen ammonia concentrations could reach 80 mg % which is generally considered to be a toxic level.

Intraruminal administration of tyrosine did increase rumen ammonia levels over those seen in the control observations and maintained higher ammonia levels over the 48 hours of observation. This suggested that residual tyrosine was still present in the rumen, and being deaminated up to 48 hours after tyrosine administration. The concentrations of the metabolites measured indicates this as well. Concentrations of p-cresol,

p-hydroxyphenylacetic acid and 3-phenylpropionic acid remained higher than control levels and were still increasing even to 48 hours after tyrosine administration. This is in agreement with work by Rae and Ingalls (1982) in which tyrosine was incubated in nylon bags and rates of disappearance monitored. Due to its low solubility at pH 6, only 38% of the incubated tyrosine disappeared after 24 hours. After 48 hours 73% had disappeared from the bags.

The time sequence observations for the first 24 hours after tyrosine dosing for p-cresol and 3-phenylpropionic acid follow the pattern of bacterial activity normally seen on a hay diet. Just prior to feeding the rumen population is generally in a quiescent state with most of the bacteria present in stationary phase. From 2 hours to approximately 6 hours after feeding the bacterial population is in a stage of high metabolic activity. It is during this time period that peak deaminase activity takes place (Barao, 1983; personal communication). With the depletion of substrate the rumen bacteria return to stationary phase and are again quiescent until the next feeding (Hungate, 1964).

Urinary Phenolic Acids

The total urine phenolic acid concentrations should be interpreted with some concern because the water intake varied markedly under collection conditions. Urine volume also varied greatly within the same animal from day to day and, despite the care taken to prevent drinking water from getting into the collection bucket, there was some water spillage and subsequent urine volume increases (Further modifications were made prior to Trial 2 to prevent this problem).

The values obtained for total urinary 3-phenylpropionic acid and

p-cresol in the first study are lower than previously reported values (Martin, 1975). The decline in p-cresol output seen with the addition of increasing amounts of concentrate in the diet is in agreement with earlier work reported by Martin (1969). In his work the addition of oats and groundnut meal decreased free aromatic acid output in the urine by 53-70% depending on the amount of concentrate included.

Assuming the outflow of digestion from the rumen of sheep fed a hay diet twice a day to be 7 liters per 24 hours (as shown by Hydin) the levels of p-cresol seen in the urine compared to that produced in the rumen indicate that the levels produced in the rumen are not sufficient to account for that found in the urine. This is especially evident when excess tyrosine was introduced intraruminally. The increase in rumen production of p-cresol seen with intraruminal tyrosine administration is far short of that found in the urine. It is likely that lower intestinal fermentation is the source of this additional p-cresol. Ward and Yokoyama (1982) reported the isolation from pig feces of a bacterium that decarboxylates p-hydroxyphenylacetic acid to p-cresol. This strain has been found to be highly active metabolically and is present primarily in the caecum of pigs. Although this bacterium has not been isolated from the caecum of the ruminant it is highly likely that a similiar strain of bacteria would be present in the ruminant lower digestive tract.

Ward and Yokoyama's data indicate that this particular isolate produces p-cresol more rapidly under energy limiting conditions (no glucose) than under energy replete conditions. These observations would suggest a catabolite repression mechanism present under favorable growth conditions. The conditions under which this isolate would produce more

p-cresol are those present in the lower tract under a hay feeding regimen. These are:

1. Lower amounts of dietary and microbial protein available.
2. Increased rate of passage of digesta out of the lower tract.
3. More alkaline pH conditions in the lower tract.
4. Energy limiting conditions (less readily fermentable substrate) (Kaufman et al., 1980).

The presence of readily fermentable substrate in the lower tract, as is found on high concentrate diets, is a condition under which a catabolite repression mechanism would be exhibited by this bacterium. Another possible explanation for the decline in p-cresol on a high concentrate diet revolves around the decrease in pH that occurs in the lower tract as a result of the fermentation of available substrate. The presence of acidic conditions in the lower tract might inhibit the bacteria responsible for p-cresol production.

Urinary p-hydroxyphenylacetic was variable with diet in the first study but was not found to be detected under the conditions of the second study. Urine analysis of the second study gave values more representative of those present in the literature (Martin, 1975).

Table 14 shows the total excretion of 3-phenylpropionic acid, p-cresol and p-hydroxyphenylacetic acid. There are no control observations for urine output of the phenolic acids over 12 hour time spans so no statistical conclusions can be made. Assuming the 24 hour observations taken during the control period to be reflective of the normal amounts of these acids excreted, it is evident that with tyrosine introduced into the digestive tract of the first animal p-cresol excretion

Table 14. Total Urinary 3-Phenylpropionic Acid (3PPA), P-Hydroxyphenylacetic Acid (PHPAA) and p-Cresol (PC) Excretion in Tyrosine Treated Sheep (mg)

	Animal 1			Animal 2		
Hours	3PPA	PHPAA	PC	3PPA	PHPAA	PC
12	9.61	ND	1760.82	15.96	ND	2408.11
24	10.93	ND	1016.86	10.99	ND	207.78
36	1.56	ND	361.25	7.84	ND	281.65
48	6.37	ND	165.34	20.57	ND	497.89

In 12 hours exceeds by 5 times that seen in pretreatment observations for 24 hours. the total p-cresol excretion 24 hours after tyrosine administration is 8 times that seen in a pretreatment 24 hour period of time. After the 24 hour collection was taken p-cresol levels in the urine of the first animal declined to pretreatment levels.

The second animal showed a different excretion pattern altogether. Within the first 12 hours after tyrosine administration p-cresol excretion increased to 6 times that seen normally after 24 hours. After this time p-cresol excretion declined to pretreatment levels.

Excretion of 3-phenylpropionic acid was increased with intraruminal tyrosine administration but not as dramatically as that seen with p-cresol. This increase in urinary 3PPA appears to be more a reflection of increased rumen production than lower intestinal fermentation (Martin, 1982).

The urine excretion data provides the best explanation of what happened in the animal after excess tyrosine was introduced into the digestive tract of the sheep. The majority of the tyrosine passed out of the rumen flowing with the particulate fraction into the lower intestinal tract. Most of this was probably excreted in the feces, although there was a increase in fermentation of tyrosine in the lower intestinal tract to p-cresol which appeared as large increases in the urine of the sheep.

The majority of the tyrosine moved out of the rumen within the first 24 hours again as reflected by urine output. The tyrosine left in the rumen was deaminated, thus maintaining rumen ammonia at levels higher than those seen during the pretreatment period and was metabolized to 3-phenylpropionic acid, p-hydroxyphenylacetic acid and p-cresol.

Metabolism of tyrosine continued to 48 hours post dosing which maintained the levels of these phenolic metabolites above those seen during the pretreatment period.

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