AMINO ACID METABOLISM BY RUMEN MICROORGANISMS

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This is to certify that the

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TRENT R. LEWIS

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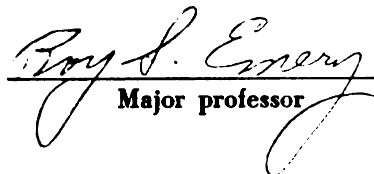

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BY RUMEN MICROORGANISMS

By

Trent R. Lewis

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ABSTRACT

AMINO ACID METABOLISM
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This investigation had a fourfold purpose. Catabolic reactions of amino acids were compared and quantitated in both *in vitro* mediums--rumen liquor and washed suspensions of rumen microorganisms. Each amino acid medium was also examined chromatographically for intermediary products which could have pronounced physiological activity within the host. Certain materials were added to washed cell suspensions in an effort to increase the amino acid dissimilation rate. Lastly, L-arginine, L-lysine and DL-tryptophan were studied *in vivo* to compare *in vitro* and *in vivo* dissimilation patterns and the effect of amino acid administration through the rumen fistula on the plasma amino acids of jugular blood.

Rumen fluid which was to serve as inocula and washed cell sources was collected at three to four hours after feeding. The amino acids were dissolved in each medium to give amino acid concentrations of 10 micromoles per ml. In the *in vivo* studies, each amino acid was added two hours after feeding in quantities to give amino acid concentrations of approximately 20 micromoles per ml.

The post incubation mediums were examined chromatographically and spectrophotometrically for catabolic products. Plasma amino acids from jugular blood were examined qualitatively by paper chromatography zero, one, two and four hours after the administration of the amino acid.

Serine, aspartic acid, glutamic acid, arginine, lysine, cysteine, cystine, threonine and phenylalanine were readily dissimilated when added to both mediums (47 to 100% in rumen liquor; 21 to 99% in washed cells). Tryptophan, histidine, methionine, ornithine, valine, alanine, leucine, isoleucine, delta amino valeric acid, glycine, hydroxyproline and proline were dissimilated at lesser rates (8 to 37% in rumen liquor; 1 to 22% in washed cells). The dissimilation rates were more rapid and complete in rumen fluid studies than in washed cell suspensions. Three or four amino acids incubated together differed from the summation of the ammonia formed from each amino acid only in the cases where proline and alanine were incubated together. The individual usage of 48 hour enriched cultures, pyridoxamine, pyridoxal phosphate, magnesium ions, all potassium buffers, methylene blue or catalase in washed cell incubations failed to significantly promote ammonia production over the control values and were still low as compared to rumen liquor and *in vivo* ammonia production. Ammonia production and amino acid

disappearance, as noted by paper chromatography, were closely correlated. The D- and L- forms of tryptophan and serine were both dissimilated. Arginine yielded ornithine, putrescine and delta amino valeric acid. Lysine yielded cadaverine and delta amino valeric acid whereas ornithine yielded delta amino valeric acid.

Penicillin at 3 I.U./ml. did not inhibit any of the dissimilations whereas 30 I.U. caused a marked inhibition. Tests for amine production from casein hydrolyzate and individual amino acids at pH 4.5, 5.5 and 6.5 were negative, except for cadaverine and putrescine. Arginine produced the highest levels of ammonia in eight hour rumen liquor incubations. The presence of arsenate or fluoride increased ammonia production from serine over the control values. Glutaric acid was not dissimilated _in vitro_ by rumen microorganisms.

The _in vivo_ dissimilations of L-arginine, L-lysine and DL-tryptophan were in good agreement with the _in vitro_ studies. Arginine and lysine both produced delta amino valeric acid. Arginine also yielded ornithine. Indole and skatole were formed from tryptophan. Rumen ammonia levels _in vivo_ paralleled what would have been expected from the _in vitro_ studies. The administration of readily dissimilatable amino acids to the rumen had a generally positive effect on the amino acids from jugular blood

drawn the first four hours following amino acid administration rather than specifically raising the level of the amino acid administered.

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INTRODUCTION

At present, little is known of the ruminal intermediary metabolism of amino acids resulting from protein catabolism. A few reports dealt with volatile fatty acid, ammonia and gas production from the *in vitro* and *in vivo* incubation of individual amino acids, combinations of amino acids and protein preparations. However, information as to those intermediate products formed in amino acid dissimilations and their possible physiological effect upon the host is scanty. Efforts directed towards managing amino acid dissimilation rates and patterns within the rumen offer many practical nutritional applications. Three of the more important applications are the following. The studies on the nature of acute bloat in ruminants indicate that a toxic substance, or substances, is involved in inhibiting the eructation. Pasture usually results in high ruminal ammonia concentrations which in turn affect the utilization of the herbage magnesium. Lysine and methionine supplementation have been found to increase the nutritional value of certain ruminant rations.

Discrepancies have appeared in the literature from studies involving *in vitro* rumen liquor and washed cell suspensions. In addition, there is a complete lack of knowledge regarding the inability of *in vitro* amino acid studies to duplicate the reaction rates noted *in vivo*.

As a result of the foregoing considerations, this investigation had a fourfold purpose. First, catabolic reactions of amino acids were compared and quantitated in both _in vitro_ mediums--rumen liquor and washed cells. Secondly, each amino acid was examined chromatographically for intermediary products which could have pronounced physiological activity within the host. Third, certain materials were added to washed cell suspensions in an effort to increase the amino acid dissimilation rate. And lastly, arginine, tryptophan and lysine were studied _in vivo_ to compare _in vitro_ and _in vivo_ dissimilation patterns.

REVIEW OF LITERATURE

Protein Catabolism

In the past fifteen years there has been considerable interest in the nitrogen metabolism of ruminants and the role of rumen-microorganisms in protein digestion. The nitrogen metabolism of ruminants is unique in that much of the ingested feed protein is broken down by microorganisms present in the rumen prior to entering the major area of amino acid absorption--the small intestines (McDonald and Hall, 1957). A portion of the resultant degradation products are used, in turn, by these same organisms to synthesize microbial protein. Hence the original amino acid composition of the ration may be markedly converted to the amino acid composition of microbial protein, which frequently is nutritionally superior to the ruminant (Williams and Moir, 1951). This marked intervention of the rumen microorganisms in the nitrogen metabolism of the host also permits a partial substitution of nonprotein nitrogen for protein nitrogen in the ration. For instance, urea can effectively replace natural protein in feedstuffs without affecting growth rates and milk yields, provided the protein replacement with urea does not exceed one-third of the total protein of the ration (Briggs, 1947; Owen, 1941).

Studies based on nitrogen balances and nonprotein nitrogen substitutions for protein nitrogen in rations, led many investigators to believe that total protein rather than protein quality was the prime essential in the protein requirements of ruminants. However, it is now realized that irrespective of protein breakdown and synthesis in the rumen, the nature of the ingested material is also of major importance (Ellis et al., 1956).

Sym (1938) was the first to show active proteolysis by suspensions of both rumen bacteria and protozoa, as well as by extracts of acetone powders of these microorganisms. The peptidase activity, however, was weak in his preparations and little free protease was detected in the supernatant rumen liquor. Pearson and Smith (1943c) demonstrated proteolysis by rumen microorganisms in vitro. After the incubation of casein or gelatin with rumen contents, there was a definite evidence of protein degradation whereas blood meal showed no degradation, but rather a net protein synthesis occurred. These results were attributed to a difference in solubilities among the proteins since blood meal unlike gelatin and casein is largely water insoluble. This problem was further examined by McDonald (1948, 1952) who showed that large quantities of ammonia are produced from several different protein sources in the rumen under normal feeding conditions. Ammonia proved to make up the major portion of the nonprotein

nitrogen fraction.

Lewis (1957) stated that numerous factors influence the value of protein to the ruminant other than its digestibility and amino acid composition. Such factors as protein solubility, degree of denaturation, particle size, the previous ration of the animal, other nitrogenous compounds such as urea and amino acids, and the protein level of the ration influence ammonia production in the rumen. McDonald (1954) was one of the first persons to demonstrate quantitatively that a large portion of the feed protein added to the rumen was converted to microbial protein. He found that about 40 per cent of the zein given to a sheep was converted to microbial protein. McDonald and Hall (1957) have shown that about 90 per cent of the casein, which constituted 87 per cent of the nitrogen intake, was digested in the rumen in a relatively short time. These calculations were based on figures obtained by determining protein alkali-labile phosphate; however, it is not necessarily true that the release of inorganic phosphate from casein occurs at the same rate as its degradation. An interesting anomaly is that the biological value of casein, though high for a monogastric animal because of its high digestibility and favorable amino acid composition, is low for a ruminant. Head (1953) compared the nitrogen retention of sheep receiving casein or fish meal as the protein source in a hay and starch ration, and found that raising

the nitrogen intake by raising the casein level of the ration increased the nitrogen balance very little in comparison with an increase after a similar rise in the level of the fish meal supplement. Chalmers and Synge (1954) also showed that lambs grew better on fish meal than on casein. Both groups of workers attribute the low nitrogen retention of casein to a rapid and excessive rumen deamination and an increased urinary nitrogen excretion. In addition, it has been shown that if casein is denatured with formaldehyde so that its solubility is reduced, its nutritive value for the ruminant is increased, presumably by a curtailment of ammonia production in the rumen (Chalmers et al., 1954). McDonald (1952) and Annison et al. (1954) both reported that free ammonia was rapidly formed from casein and ground nut meal, but that flaked maize or its component protein, zein, resulted in low rumen ammonia levels. Fontaine et al. (1944) have suggested that the relative ammonia production in the rumen from standard feed proteins can be estimated by the proportion of their total nitrogen soluble in a molar sodium chloride solution under a standard set of conditions.

In feeding experiments, two main interrelationships have been demonstrated between carbohydrate and protein materials in the rumen. There is a better utilization of proteins in the presence of added carbohydrate (Lewis and McDonald, 1958) and as the protein level is increased,

there is a more rapid attack upon the fibrous components of the ration (Burroughs and Gerlaugh, 1949; Burroughs *et al.*, 1949; Burroughs *et al.*, 1950). The effects of high protein rations on ruminal volatile fatty acids have been investigated in sheep (El-Shazly, 1952a; Annison, 1954) and in lactating dairy cattle (Davis *et al.*, 1957). Increased concentrations of the total volatile fatty acids occurred when high protein rations were fed, in which the relative proportion of acetic acid decreased and of butyric and higher acids increased. Further evidence was provided by *in vitro* studies (El-Shazly, 1952b; Annison, 1956) wherein casein was incubated with washed suspensions of rumen microorganisms. The resultant mixture of volatile fatty acids obtained in both instances was considerably higher in C_4 and C_5 branched-chain acids than was normally found in rumen liquor.

Weller *et al.* (1958) have analyzed the rumen contents of sheep slaughtered at different times after feeding a ration of wheaten hay and found that between 2 and 24 hours after feeding, the microbial nitrogen accounted for 63 to 82 per cent of the total nitrogen; soluble nitrogen accounted for 5 to 10 per cent; the remainder being plant nitrogen. This study along with those previously mentioned (McDonald, 1954; McDonald and Hall, 1957) provide ample evidence that feed protein is converted to microbial protein in the rumen in marked amounts. The question then

arises as to the intermediate steps in this process.

Protein is degraded by the action of the proteolytic enzymes of the rumen microorganisms; peptides and amino acids are produced which, in turn, are attacked by deaminases to give ammonia. The demonstration of peptides and amino acids in the rumen, as intermediates in protein digestion, is of recent origin. Lewis (1955) demonstrated that free amino acids could be detected to a greater extent in concentrated rumen fluid 3 hours after feeding than before feeding, and Annison (1956) showed that detectable quantities of both alpha-amino nitrogen and diffusible peptide-nitrogen were always present, their concentration increasing up to five- or tenfold after feeding. Furthermore, Annison (1956) found about 2 to 3 per cent of the total microbial nitrogen to be due to amino acids either bound on the cellular surface or existing endogenously. These bound amino acids constituted a substantial part of the free amino acids in rumen liquor and were comprised mainly of those amino acids that have been shown to be most readily dissimilated by rumen microorganisms.

Further information on the proteolytic enzymes of ruminal microorganisms was provided by recent *in vitro* studies (Annison, 1956; Warner, 1956). Washed suspensions of rumen bacteria, when used in similar concentrations, caused proteolysis at similar rates irrespective of the

ration of the animal. This phenomenon differs from deamination since El-Shazly (1952b) found that the capacity of rumen bacteria to deaminate amino acids was proportional to the readily attacked protein in the ration. In addition, the proteolytic enzymes in vitro appeared to be relatively resistant to environmental changes and to be unaltered by toluene or acetone. The deaminases were inactivated by these organic solvents; the peptidases were intermediate in their resistance to inactivation by the usual enzyme isolation techniques. About half the proteolytic activity of the microorganisms taken from the rumen of a sheep on a high protein ration was due to the protozoa and about half due to the bacteria. The optimum pH of the bacterial proteolysis was between 6.5 and 7.0. The most active bacterial preparations were obtained 6 to 10 hours after feeding the donor animal. Considerable quantities of amino acids, peptides and ammonia were always produced from casein, ground nut cake, and soya protein, but not from bovine albumin, zein or wheat gluten. At present, no ruminal proteolytic activity has been attributed to enzymes secreted by the ruminant itself. In addition, very little proteolytic activity can be demonstrated in cell free rumen liquor (Warner, 1956).

Further investigation is needed with regard to the kinetic properties and specificity of the proteolytic enzymes, the factors governing the susceptibility of proteins

to attack, the intermediates of proteolysis and the effect of these intermediates upon proteolysis.

Warner (1956) and Blackburn and Hobson (1960a) have measured the proteolytic activity of rumen bacteria and protozoa. The fractions were obtained by differential centrifugation of rumen contents from sheep. After washing, each fraction was added to incubation flasks containing buffered casein under toluene. The fractions--whole fluid, protozoa, large bacteria, small bacteria--were naturally somewhat mixed, but each contained a preponderance of the designated organisms. These results showed that proteolytic activity appears to be distributed over the whole range of rumen organisms. Little activity occurred in the cell-free supernatant. The demonstration of proteolytic activity of rumen protozoa is difficult since it is almost impossible to get a preparation of protozoa completely free from bacteria. Attempts to free protozoa of bacteria by washing resulted in a rapid death of the protozoa and a loss of enzymatic activity. Excellent indirect evidence for protozoan proteolysis was obtained by two means. In a number of experiments using stained protein particles some oligotric protozoa were observed to ingest and digest these particles. Secondly, experiments employing simple freezing and thawing resulted in a considerable amount of active protease from a suspension of rumen protozoa contaminated with a few bacteria, but very

little protease was obtained from a similar suspension of rumen bacteria only.

The proteolytic enzymes of washed suspensions of rumen bacteria were stimulated by cysteine (Warner, 1956; Blackburn and Hobson, 1960), resembling in this respect the anaerobic clostridia (Weil *et al*., 1939). Other reducing agents such as ascorbic acid or sodium sulphide were without effect as was magnesium ions; ferrous ions stimulated proteolysis for one group but was inactive for the other. These proteolytic enzymes appeared to be relatively unaffected by oxygen.

All these results suggest that a number of different rumen organisms play a part in proteolysis. The results of attempts to isolate proteolytic bacteria have not, however, confirmed this. A few microscopic observations on the possible proteolytic bacteria in rumen contents have been made. Van der Wrath (1948) and later Warner (1956) noted increased concentrations of gram-positive coccobacilli, bacilli and large cocci in association with casein suspended in the rumen. Masson (1950), observing particles of casein in the general mass of rumen contents, found that they were surrounded by streptococci, and Gall *et al*. (1951) noted masses of gram-positive cocci in the rumen of a sheep getting casein as the sole protein. Burroughs *et al*. (1950) also noted chains or clumps of cocci in the rumens of cattle getting two rations high in

casein.

Appleby (1955) studied three sheep on two different rations with the express object of isolating proteolytic bacteria and found that in spite of using an anaerobic technique nearly all the organisms were facultative anaerobes, the isolated organisms belonging to the genera Bacillus, Micrococcus, Clostriduim and Flavobacterium. The most abundant proteolytic species being Bacillus types, with Bacillus licheniformis predominating. The medium used was made solid with agar and was initially cloudy because of the casein present. After growth, colonies of organisms with extracellular proteolytic properties could be recognized by the clear zones they produced in their vicinity. Bacillus spores were present in large numbers on the food of the animal and were shown to germinate in the rumen. It appears as though most of the isolates may have been derived from the environment or feed of these sheep. Hunt and Moore (1958) isolated a Flavobacterium sp. from the rumen which behaved similar to the proteinases of some aerobic organisms studied by Weil et al. (1939) but which was inhibited by cysteine. The presence of numbers of facultatively anaerobic proteolytic bacteria may, of course, help to explain the comparative stability of rumen proteinases to air.

Byrant and Small (1956) found Butyrivibrio fibrisolvens, a common rumen inhabitant, to be proteolytic and

this type of organism may play an important role in rumen proteolysis. Gutierrez (1953) isolated in pure culture proteolytic gram-positive bacteria, and Byrant and Burkey (1953a, b) isolated proteolytic gram-positive and weakly proteolytic gram-negative bacteria from the rumen. Only one out of eleven strains of Selenomonas ruminatum tested by Byrant (1956) was proteolytic when tested with casein.

Amino Acid Metabolism

The literature on amino acid metabolism is voluminous and provides ample evidence of the striking advances which have been made since the isolation of the first amino acid one hundred and fifty-five years ago. Asparagine and cystine, the first two amino acids to be identified were discovered in 1806 and 1810, respectively (Vanquelin and Robiquet, 1806; Wollaston, 1810). Although the existence of 80 amino acids has been demonstrated, there are only 22 amino acids which may be said to occur frequently in protein hydrolyzates.

Knowledge concerning the intermediary metabolism of the amino acids has developed from a variety of experimental procedures and observations. Pure nutritional studies have provided important evidence leading, in the long run, to the identification of metabolic reactions. The use of mutant strains of microorganisms has proved to be an excellent tool in the study of metabolic pathways,

either in the biosynthesis or dissimilation of amino acids. Mutants blocked at various stages of biosynthesis and dissimilation accumulate intermediates, which may often be detected by their growth effects on other mutant or non-mutant strains. Human mutations, alcaptonuric and phenylpyruvic oligophrenic patients, have also been studied, and as in the case with microorganisms, have led to a better knowledge of normal metabolic pathways. Considerable information has resulted from studies with perfusion mechanisms, tissue slices, tissue homogenates and extracts, and purified enzymes. Isotopes have been widely used in studies of intermediary metabolism and this technique has provided both initial clues and final proof of the occurrence of many biochemical transformations.

There is little doubt that species differences occur in the metabolism of amino acids--for example, the absence of biochemical pathways in mammals when compared to microorganisms, or between microorganisms themselves. On the other hand, many pathways are essentially the same among widely different species. As a result, microbes may be used to study animal mechanisms and vice versa. Likewise, the reactions leading to the synthesis and degradation of amino acids are frequently, but not always different.

Since the rumen is an anaerobic or facultatively anerobic environment, the major portion of this discussion

will be limited to amino acid pathways demonstrated by rumen preparations and to pathways demonstrated in strict anaerobes or facultative anaerobes. Detailed investigations of amino acid metabolism by rumen microorganisms are of a relatively recent origin. In 1952, El Shazly (1952b), working with whole strained rumen fluid and washed cell suspensions, reported that the main reaction products obtained from the incubation of rumen bacteria with casein hydrolyzate were ammonia, carbon dioxide and volatile fatty acids. Analysis of the volatile fatty acids, by gas-liquid partition chromatography, revealed that straight and branched chain C_2 to C_5 fatty acids were present. When the concentrations of the fatty acids produced by incubation of rumen liquor with casein hydrolyzate were compared with the concentrations normally present in rumen fluid, an increase in the branched chain C_4 and C_5 fatty acids was observed. Paper chromatographic analysis of the reaction mixtures indicated a general decrease in the concentration of all the amino acids, with a complete disappearance of phenylalanine, tyrosine and proline. A new spot consistently appeared on the chromatograms and was subsequently identified as delta aminovaleric acid. On incubation of rumen liquor with proline alone, there was no obvious disappearance of proline, nor was any delta aminovaleric acid formed. But, upon incubation of proline and alanine with rumen liquor, both decreased markedly in

concentration and delta aminovaleric acid was produced. Alanine, incubated alone with rumen liquor, simply showed a decrease in concentration. On the basis of these results, El Shazly concluded that delta aminovaleric acid was formed by a Stickland reaction between alanine and proline. He further postulated that the amino acids valine, leucine and isoleucine act as hydrogen donors, while proline serves as a hydrogen acceptor in the fermentations with casein hydrolysate. Thus, valine, leucine, and isoleucine would be oxidized to the branched chain C_4 and C_5 volatile fatty acids, and proline reduced to delta aminovaleric acid. He also pointed out that other reducible substances may function in the place of proline if carbohydrate is being fermented simultaneously.

A comparison of washed cell suspensions versus rumen liquor indicated very similar relative rates of degradation, however, the deaminating power of washed cell suspensions was closely related to the diet of the animal. In the washed cell studies, the rough equivalence of moles of ammonia, carbon dioxide and volatile fatty acids produced to moles of amino acid destroyed was striking.

Sirotnak _et al_. (1953) using washed cell suspensions, reported that decarboxylation and deamination occurred only with aspartic acid, glutamic acid, serine, arginine, cysteine and cystine. Arginine and cystine were incompletely utilized after 72 hours dissimilation while

the other four amino acids were nearly completely utilized. In each case, volatile fatty acids were produced and hydrogen sulfide gas was produced from cystine and cysteine. A further observation was a significant enhancement of both decarboxylation and deamination in the presence of maltose.

In 1955, Lewis (1955) examined further the deamination of individual amino acids by washed cell suspensions. In this case, significant deamination occurred only in the presence of L-aspartic acid and L-cysteine, although a small production of ammonia occurred in the presence of L-alanine, L-glutamic acid, L-serine and L-threonine. In studies using mixtures of amino acids, it was clear from the results obtained that there was no definite group or number of amino acids necessary to bring about rapid deamination. Since the rate of attack upon single amino acids *in vivo* was considerably more rapid than when washed cells were used, Lewis felt that the method of preparation of the suspension in some way rendered inactive the enzymes responsible for the deamination. Thicker suspensions were used, other redox agents were tested, greater care was taken to maintain anaerobiosis and the effect of adding a portion of ammonia-free Seitz-filtered rumen contents was investigated. Ammonia production from most of the amino acids when tested individually, however, still was comparatively slow.

Labeled casein dissimilation was studied with washed cells by Otagaki _et al_. (1955). Unfortunately, 83% of the labeled carbon appeared in glutamic and aspartic acid, and no appreciable quantities of isotope appeared in the so-called essential amino acids. Fatty acids accounted for 21%, carbon dioxide for 7%, and microbial protein for less than 0.5% of the recovered activity, respectively. Studies with glutamate $-1-C^{14}$ and leucine $-3-C^{14}$ were also undertaken. Nearly 1/5 of the total activity of the former was recovered as carbon dioxide; an unexplainably high C^{14} content (12%) was recovered in the volatile fatty acid fraction, since carbon atom one would be expected to be lost preliminary to any alteration of the five carbon skelton. Studies with $C^{14}O_2$ indicated the activity in the volatile fatty acid fraction was not due to carbon dioxide fixation. About fifty per cent of the administered leucine isotope was recovered in the volatile acid fraction, ten per cent in the cellular material and almost none in the carbon dioxide fraction, indicating the main end product of leucine degradation is volatile fatty acids. Lacoste _et al_. (1958) reported the marked conversion of phenylalanine to phenylacetic acid by enriched cultures of rumen microorganisms.

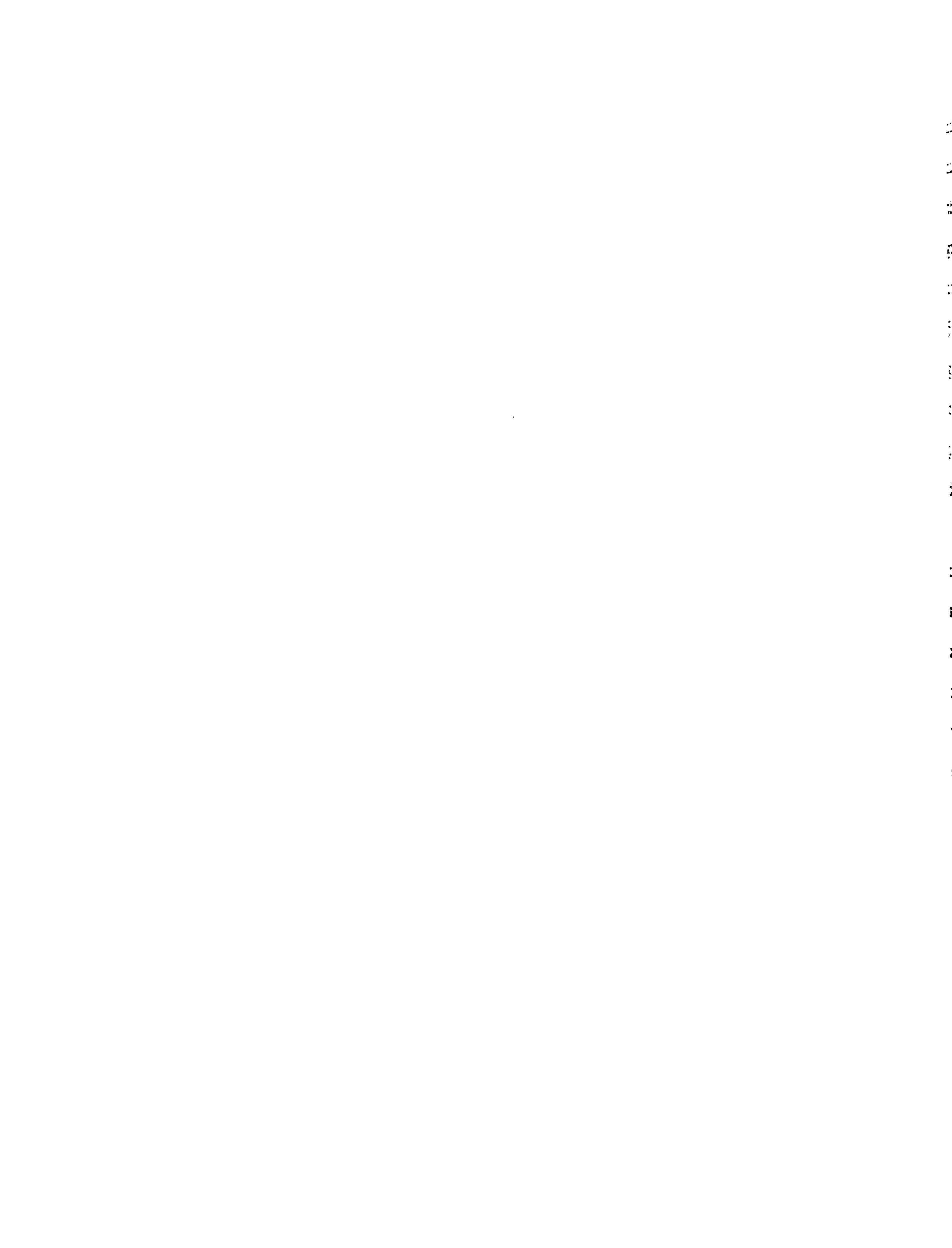
The identification of the amino acids valine, proline, leucine, and isoleucine as the components from autolyzed yeast, casein hydrolyzate and alfalfa extract which

increased cellulose digestion in vitro (Bentley et al., 1954; MacLeod and Murray, 1956) lead Dehority et al. (1957, 1958) to study their metabolism in order to elucidate their function in this process. Valine and proline were found to have an additive effect on cellulose digestion, leucine or isoleucine could replace valine, but not proline, indicating that the action of valine, leucine, and isoleucine were similar. This led to the idea of a two component system, consisting of a branched-chain component from valine, leucine, or isoleucine and a straight-chain component from proline. A subsequent study by Dehority et al. (1958) presented evidence that valine is oxidatively deaminated and decarboxylated by rumen microorganisms in vitro to isobutyric acid, and that proline undergoes reductive ring cleavage and deamination at the delta position to form valeric acid. In addition, the proposed intermediates in leucine and isoleucine oxidative deamination and decarboxylation metabolism were found to possess cellulolytic activity for rumen microorganisms in vitro, thus supporting these proposed metabolic pathways.

Both indole and skatole have been reported in rumen contents but little has been done about the mechanism of their production. Spisni and Cappa (1954) found the indole content of the rumen to be variable and stated the indole could either be of plant origin or arise by bacterial action on the tryptophan of the ration. Cappa (1956)

investigated the indole content of the rumen in order to explain the presence of indican in bovine urine. He found in the rumen liquor of 15 animals indole levels ranging from 0.09 to 3.0 (av. 0.73) mg./l. Conochie (1953) has reported indole and skatole in the milk of ruminants feeding on Lepiduim sp. Nearly saturated solutions of indole (0.1%) and skatole (0.025%) are highly toxic to rumen protozoa, particularly the Isotrichia, Dasytrichia and Ophryoxcolex (Eadie and Oxford, 1954).

Investigations by Dougherty et al. (1949) revealed the presence of toxic compounds in the rumen liquor of sheep suffering from acute indigestion. Intravenous injections of this rumen liquor into an anesthetized dog or sheep induced a pronounced and prolonged drop in blood pressure along with decreased rumen motility in the sheep. A subsequent study by Dain et al. (1955) identified histamine and tyramine as the toxic constituents in the rumen ingesta of experimentally over-fed sheep. Fatal histamine levels exceeding 70 mcg. per ml. were obtained. The histamine content increased as the rumen acidity became lower than pH5 and reached its highest level below pH4.5, normal sheep ingesta was essentially free of histamine. Dougherty (1942) has likewise reported histamine in the rumen liquor of three steers which had died of bloat. Rodwell (1953) isolated eight species of Lactobacilli from the rumen of an over-fed sheep which were able to decarboxylate



histidine and form histamine. Shinozaki (1957) identified histamine and methylamine in the rumen liquor of most of the sample taken from animals fed ladino clover or pasture grazed. On rare occasions putrescine and ethylamine were identified. Histamine concentrations were from 2.9 to 5.6 mcg. per ml. on an average; histamine was produced in greater amounts on pasture than on hay rations. High concentrations of histamine infused into a rumen pouch appeared not to be absorbed; this was also true for high dosages of histamine administered via the rumen fistula.

Washed suspensions of the LC microorganism, a gram-negative coccus isolated from the rumen of a sheep, fermented L-serine, L-threonine, and L-cysteine with the formation of ammonia, hydrogen, carbon dioxide and volatile fatty acids (Lewis and Elsden, 1955). Acetone powders of this coccus deaminated both serine and threonine but not cysteine indicating that in this organism, cysteine desulphurase is distinct from the enzyme which deaminates serine and threonine. Walker (1958) has found in the LC organism that one enzyme appears to attack both serine and threonine.

Perhaps, the dissimilation of aspartic acid by rumen microorganisms has been more thoroughly studied than for any other one amino acid. Two groups of workers (Sirotnak _et_ _al_., 1954; Van Den Hande _et_ _al_., 1959) have attempted to elucidate completely the degradative route

or routes of aspartic acid. Three major points of discrepancy appear between the two groups. Sirotnak (1954) excluded fumarate as an intermediate in the dissimilation of aspartate because fumarate was only decarboxylated and no increase in volatile fatty acids occurred. However, under the experimental conditions of Van Den Hande *et al.* (1959) fumarate gave the same end products as aspartic acid; thus it was concluded by these authors that fumarate is an intermediate in the fermentation of aspartic acid. Sirotnak's data indicated to him that malate and oxaloacetate were not intermediates, but that volatile fatty acids arose only from the decarboxylation of succinate to propionic acid and the interconversion of propionate to acetate and butyrate. Whereas Sirotnak found ethyloxaloacetate not to be fermented, Van Den Hande found that oxaloacetate gave the same end products as malate. Van Den Hande believed the main pathway of aspartic acid metabolism to be via fumarate to succinate to propionate, and did not account for acetate and butyrate from propionate but rather by another minor pathway from fumarate to malate to oxalacetate to pyruvate and then to acetate and butyrate.

At the present, limited information is available on the dissimilation of amino acids in the rumen *per se*. Hueter (1958) comparing *in vitro* and *in vivo* techniques, reported deamination of DL aspartic acid, DL lysine and

mixture of DL alanine and glycine. Five individual amino acids, beta-alanine, DL aspartic acid, L-glutamic acid, glycine and L-lysine were fed to a steer by Looper _et_ _al_. (1959). Beta-alanine, DL aspartic acid and L-glutamic acid were all readily deaminated whereas glycine and L-lysine failed to raise ammonia levels over that of the control. At present, amino acids appear to be poorly absorbed from the rumen (Tsuda, 1956b; Annison, 1956).

In reality, little is known about the deaminating bacteria in the rumen. In fact, only a few of the proteolytic organisms isolated from the rumen produce ammonia (Appleby, 1955). Byrant _et_ _al_. (1958) have reported that some strains of _Bacteroides_ _ruminicola_ produced ammonia. The deaminating ability of the "LC" organism has been mentioned previously (Lewis and Elsden, 1955); however, this organism occurs in greater numbers in young than in older animals (Hobson _et_ _al_., 1958). Dohner and Carlson (1954) reported an interesting case of symbiosis between two strains of _Escherichia_ _coli_. Pure cultures failed to ferment lysine, but the two together did so, to give ammonia and butyric and acetic acids. Most of the rumen organisms that exhibit deaminating power are gram-negative (Hobson, 1959); Phear and Ruebner (1956) studying intestinal organisms from man found that ammonia production by gram-negative bacteria was greater than by gram-positive ones. A number of rumen bacteria have been shown to grow

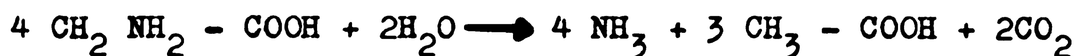
on one or more amino acids as sole nitrogen sources, but some rumen bacteria need growth factors, which appear to be peptides and which are found in casein hydrolysate, e.g., Bacteroides ruminicola (Byrant et al., 1958).

The following is a brief resume of amino acid metabolism by strict and facultative anaerobes in pure cultures; however, this resume is meant only to define specific biochemical reactions and is not a complete review on the subject. Each amino acid will be discussed individually.

Glycine. Brasch (1909a, b) reported in cultures of Clostridium lentoputrescens a reductive deamination of glycine by molecular hydrogen yielding acetic acid and ammonia. Cellular suspensions of Cl. tetanomorphum (Wood and Clifton, 1937), Cl. sporogenes (Stickland, 1935b; Woods, 1934; Hoogerheide and Kocholaty, 1938), Cl. histolyticum (Bessey and King, 1934), Cl. welchii (Woods, 1942), Cl. tetani, Cl. botulinum A and B (Clifton, 1942), Cl. bifermentans and Cl. sordelli (Prevot et al., 1947), Cl. acetobutylicum, Cl. butylicum, Cl. flabelliferum, Cl. saccharobutylicum, Cl. codophilum and Bacillus teras (Nisman et al., 1948a) do not deaminate glycine. The same negative results have been reported for several other anaerobes (Nisman and Thouvenot, 1948).

Another anaerobe whose washed suspensions have been reported to deaminate glycine is Diplococcus glycinophilus

(Cardon, 1942). This organism has been studied in detail by Cardon and Barker (1946, 1947) who showed that the only compound attacked of any appreciable nature by this bacterium was glycine. Serine and pyruvate were poorly attacked in the presence of glycine. The balance sheets of the dissimilation of glycine by washed suspensions of this bacterium corresponded to the following equation:

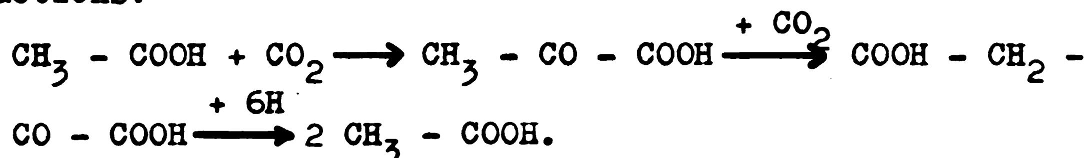


$$4\ CH_2\ NH_2 - COOH + 2H_2O \longrightarrow 4\ NH_3 + 3\ CH_3 - COOH + 2CO_2$$

These same authors (1947) reported that only dipeptides in which the carboxyl group of glycine was free were dissimilated. The only compound containing two adjoining peptides which was degraded was hippurylglycine. Under these conditions hydrogen is a product of the degradation of glycine.

The experiments carried out by Barker, Volcani and Cardon (1948) with C^{14} revealed that about 75% of the methyl and 54% of the carboxyl group of acetic acid arose from the methylene carbon of glycine. These observations indicated that one of the principal reactions of this fermentation is the condensation of two molecules of glycine or one of its derivatives through the methylene groups. The two terminal carbons of the resulting compound, which may be a C_4 dicarboxylic acid, were converted mainly to carbon dioxide, the two central atoms being oxidized to acetic acid. In addition, at least 6% of the methyl carbon and 38% of the carboxyl carbon of the acetic acid arose

from carbon dioxide. Glycine then was not directly converted to acetic acid. The fact that acetic acid was not metabolized eliminated the possibility that the fixation of carbon dioxide observed involved the following series of reactions:



$$CH_3 - COOH + CO_2 \longrightarrow CH_3 - CO - COOH \xrightarrow{+\ CO_2} COOH - CH_2 -$$
$$CO - COOH \xrightarrow{+\ 6H} 2\ CH_3 - COOH.$$

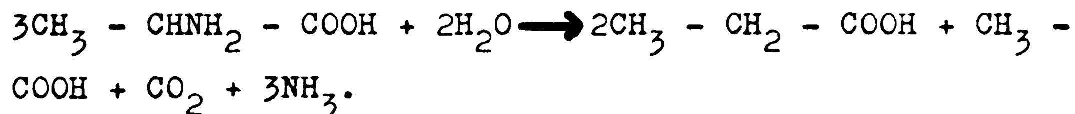
Such a mechanism would have produced an effective redistribution of labeled carbon in the acetate molecule.

A strain of *Achromobacter* oxidized glycine aerobically to ammonia and hydrogen peroxide (Paretsky and Werkman, 1950); presumably the pathway was similar to that which occurs in mammalian tissues and involved glyoxylate and formate as intermediates (Campbell, 1955). Stadtman (1958) has presented evidence that a quinone operates in the overall process of glycine reduction to acetic acid and ammonia by *Clostridium sticklandii*; this same quinone does not appear to operate in proline reduction to delta aminovaleric acid.

Alanine. Brasch (1909a, b) reported for alanine results analagous to those obtained for glycine with *Cl. lentoputrescens*, i.e., alanine was reduced to propionic acid and ammonia. Negative results for the dissimilation of alanine by suspensions of various anaerobic organisms have been reported by numerous investigators (Woods and

Clifton, 1937; Stickland, 1935a; Bessey and King, 1934; Woods and Trim, 1942; Clifton, 1942; Prevot et al., 1947; Nisman et al., 1948a; Nisman and Thouvenot, 1948).

Cardon (1942) isolated the organism, Clostridium propionicum which has been studied in detail by Cardon and Barker (1946, 1947). Of the several compounds assayed, only DL-alanine, DL-serine, DL-threonine, pyruvate, DL-lactate, acrylate and L-cysteine were fermented by this bacterium. The results of the analysis of the products from the degradation of alanine by cultures of this organism in a medium where the only organic constituents were alanine and yeast autolysate corresponded to the following equation:



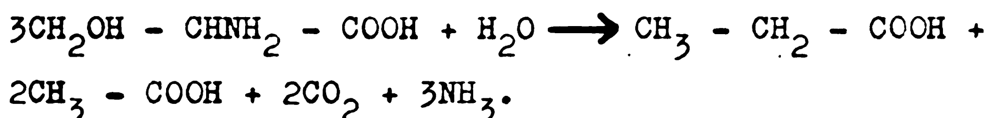
$$3CH_3 - CHNH_2 - COOH + 2H_2O \rightarrow 2CH_3 - CH_2 - COOH + CH_3 - COOH + CO_2 + 3NH_3.$$

The dissimilation of alanine appears to be mainly a propionate fermentation.

Serine. Numerous anaerobic and facultative anaerobic organisms attacking this amino acid have been described. Cultures of Cl. lentoputrescens deaminate serine yielding propionic and formic acids, ammonia and other products which were not identified (Brasch, 1909a, b). Washed suspensions of Cl. tetanomorphum (Woods and Clifton, 1937), Cl. propionicum (Cardon, 1942; Cardon and Barker, 1946, 1947), Cl. botulinum (Clifton, 1939), Cl. tetani (Clifton,

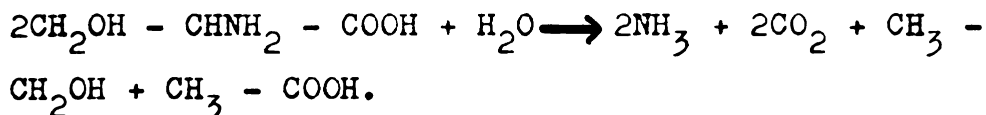
1942; Pickett, 1941), *Cl. bifermentans* and *Cl. sordelli* (Prevot *et al.*, 1947), *Cl. perfringens* (Woods and Trim, 1942; Chargaff and Sprinson, 1943a, b) deaminated this amino acid. Chargaff and Sprinson (1943a, b), utilizing *Cl. perfringens*, demonstrated the formation of pyruvic acid from serine in the presence of arsenite. Furthermore, they demonstrated that the alcoholic hydroxyl group of serine must be free for the dissimilation to occur, DL-O-ethylserine, L-phosphoserine and phosphatidyl serine were not attacked. These facts suggested to these investigators that the mechanism of this reaction involved an initial dehydration followed by a hydrolysis of the resulting imino acid. A D-serine dehydrase was obtained from *Escherichia coli* which was activated by pyridoxal phosphate (Metzler and Snell, 1952a, b). On the other hand, an L-specific serine deaminase prepared from *E. coli* was not activated by pyridoxal phosphate but was activated by glutathione and adenylic acid (Wood and Gunsalas, 1949).

Cardon and Barker (1947), employing *Cl. propionicum*, noted the following catabolic scheme for serine:



$$3CH_2OH - CHNH_2 - COOH + H_2O \longrightarrow CH_3 - CH_2 - COOH + 2CH_3 - COOH + 2CO_2 + 3NH_3.$$

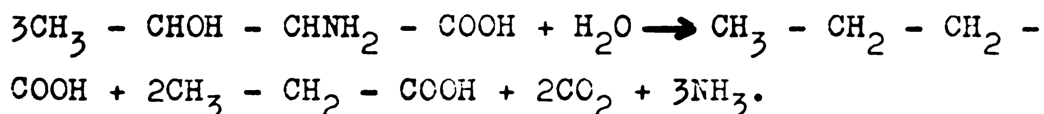
Clifton (1940b), employing washed suspensions of *Cl. botulinum* type A, suggested that the degradation of serine by this organism corresponded to the following equation:



$$2CH_2OH - CHNH_2 - COOH + H_2O \longrightarrow 2NH_3 + 2CO_2 + CH_3 - CH_2OH + CH_3 - COOH.$$

Clifton (1942) studying the degradation of serine by *Cl. tetani* discovered, in addition to acetic and butyric acids, a significant quantity of alcohol. Cohen *et al*. (1948) reported the formation of equimolar quantities of acetic and butyric acid from serine with washed suspensions of *Cl. saccharobutyricum*, corresponding to the results of Clifton with *Cl. tetani*. The interconversion of glycine and serine is of considerable significance for microorganisms and is believed to be folic acid dependent (Lascelles and Woods, 1950; Lascelles *et al*., 1954; Holland and Meinke, 1949).

Threonine. Woods and Trim (1942), employing suspensions of *Cl. perfringens*, reported a deamination of threonine and the formation of hydrogen, carbon dioxide and ammonia. Chargraff and Sprinson (1943a), employing the same organism, isolated alpha-ketobutyric acid which resulted from the oxidative deamination of threonine. Cardon and Barker (1947) suggested the following equation for the propionic fermentation of threonine by suspensions of *Cl. propionicum*:



$$3CH_3 - CHOH - CHNH_2 - COOH + H_2O \longrightarrow CH_3 - CH_2 - CH_2 - COOH + 2CH_3 - CH_2 - COOH + 2CO_2 + 3NH_3.$$

Cohen *et al*. (1948) reported the production of 2 parts acetic and 1 part propionic acid by *Cl. saccharo-*

butyricum. Pickett (1941), Cl. tetani, and Nisman and Thouvenot (1948), several species of Cl. sporogenes, reported the deamination of threonine but did not analyze the volatile acids produced. Barker and Wiken (1948) studied in detail the mechanism of the formation of butyric acid in the degradation of threonine by suspensions of Cl. propionicum. They tried to resolve if butyric acid was formed by the condensation of two molecules of acetic acid or one of its derivatives, as is the case for much of the butyric acid resulting from clostridial fermentation. To accomplish this, threonine was incubated with washed suspensions of Cl. propionicum in the presence of acetate labeled with C^{14} on both carbons. After chromatographic separation of the mixture of acids by the method of Elsden (1940), almost all of the radioactivity was recovered in the acetic acid fraction, thereby, excluding the latter as an intermediate in the formation of butyric acid from threonine.

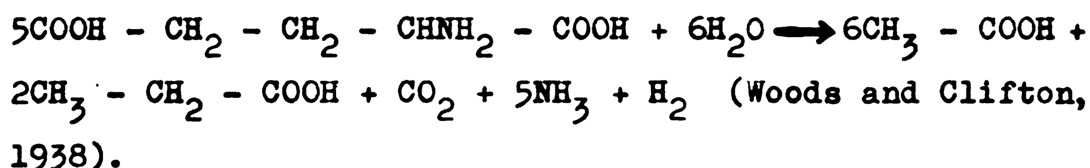
Aspartic acid. Brasch (1909a, b) observed that cultures of Cl. lentoputrescens dissimilated aspartic acid to propionic and succinic acids. Cohen et al. (1948) found washed suspensions of Cl. saccharobutylicum fermented aspartic acid producing acetic and butyric acids in the proportion of B/A equal to 1/1. Cohen-Bazire and Cohen (1949) having noted that this same bacterium fermented

oxaloacetate and yielded the same volatile end products, postulated that the degradation of aspartic acid involved first a deamination, then a decarboxylation of oxaloacetic acid ensued which was followed by the dissimilation of the resultant pyruvic acid. Such a postulation was supported by a series of experiments in which clostridia fermented the intermediate compounds of the metabolic scheme (Cohen and Cohen-Bazire, 1948, 1949a, b). The degradation of aspartate has been reported by Woods and Clifton (1937), employing _Cl. tetanamorphum_, and by Clifton (1942) and Pickett (1941), employing _Cl. tetani_. In these reports the volatile end products were not determined.

Clostridium welchii decarboxylates the beta-carboxyl group of L-aspartic acid to form L-alanine rather than the alpha-carboxyl group (Meister _et al._, 1951a, b). Small quantities of alpha-keto acids as well as pyridoxal phosphate activate this enzyme. Alpha-keto acids apparently stimulate the formation of pyridoxal phosphate by transamination of the added alpha-keto acids with pyridoxamine phosphate present in the enzyme preparation. This reaction is not a decarboxylation of oxalacetate, which could result from a transamination between the alpha keto acid and aspartic acid, since decarboxylation in the presence of labeled pyruvic acid results in non-labeled alpha-alanine. Crawford (1958) reported an aspartic decarboxylase in _Nocardia globerula_ which was inactive toward all the

other amino acids tested. Another pathway of aspartic acid metabolism is carried out by the microbial enzyme aspartase which reversibly catalyzes the conversion of aspartate to fumarate and ammonia (Woolf, 1929; Erkama and Virtanen, 1951).

Glutamic acid. A detailed paper on the degradation of glutamic acid by suspensions of *Cl. tetanomorphum* led to the following proposed overall reaction:



$$5COOH - CH_2 - CH_2 - CHNH_2 - COOH + 6H_2O \longrightarrow 6CH_3 - COOH + 2CH_3 - CH_2 - COOH + CO_2 + 5NH_3 + H_2$$

(Woods and Clifton, 1938).

Barker (1937), employing a clostridium which was later identified as *Cl. cochlearium* (Barker, 1939), noted the same end products and the same quantitative relationships. Comparable results were obtained by Clifton (1942) with *Cl. tetani*, B/A = 1/2, and by Cohen *et al*. (1948) with *Cl. saccharobutylicum*, B/A = 1/2. The latter authors identified alpha-keto glutaric acid as the first product of the reaction. They also verified that *Cl. saccharobutylicum* degraded alpha-keto glutaric acid, forming the same acids in the same proportions. While the initial deamination of glutamic acid was rather rapid, the subsequent attack of alpha-ketoglutarate was relatively slow. Krebs (1948) reported a glutamic decarboxylase in *Cl. welchii*.

Valine. The strict anaerobes studied to date are very poor dissimilators of valine as the sole substrate; the negative results obtained may be found in the literature previously cited for negative results with glycine and alanine. Valine has been shown to be decarboxylated by Proteus vulgaris (Ekladius et al., 1957). Further on, it will be found that valine may be deaminated by the Stickland reaction whereby volatile acids are formed.

Leucine and Isoleucine. Schmidt, Peterson, and Fred (1924) reported the formation of l-leucic acid, $(CH_3)_2 - CH - CH_2 - CHOH - COOH$, by a hydrolytic deamination of leucine in cultures of Cl. acetobutylicum. Other anaerobic organisms have shown little activity towards either leucine or isoleucine; however, both leucine and isoleucine undergo the Stickland reaction. Leucine and isoleucine have been reported to be decarboxylated by Proteus vulgaris (Ekladius et al., 1957).

Phenylalanine. The strict anaerobes appear to be poor dissimilators of phenylalanine. Several facultative anaerobes have been reported which deaminate phenylalanine to phenylpyruvic acid (Henricksen, 1950).

Tyrosine. Brasch (1909a, b) reported that cultures of Cl. lentoputrescens reduced tyrosine to p-hydroxyphenylpropionic acid. Janke (1930) and Rhein (1921) noted the

formation of p-cresol with Cl. cresologenes, a species whose cultures unfortunately are no longer in collections. Rhein (1921) observed the formation of phenol by Cl. tetani and Cl. pseudotetanicum. Prevot and Saissac (1947) obtained phenol and p-cresol from tyrosine with Inflabilis teras and Cl. corallinum, respectively.

Histidine. Woods and Clifton (1937) reported the deamination of histidine by Cl. tetanomorphum. Pickett (1941) showed that suspensions of Cl. tetani deaminated histidine with an opening of the imidazole ring, the volatile acids formed being butyric and acetic acids in the respective proportions of 0.31 to 2.11 moles per mole of histidine fermented. Cohen et al. (1948) observed an analogous phenomenon with suspensions of Cl. saccharobutylicum when they obtained a formation of butyric and acetic acid in the proportion of B/A = 1/2. They proposed that the opening of the imidazole was due to a histidase analagous to that discovered by Edlbacher and Kraus (1930) in tissues and implied an intermediary formation of glutamic acid. Histidine is fermented by Micrococcus aerogenes to ammonia, carbon dioxide, acetate, butyrate, lactate, and traces of formate, with urocanate as an intermediate product (Whiteley, 1957). Glutamate was also an intermediate in this case.

Proline and hydroxyproline by themselves are quite refractive to degradation by suspensions of anaerobic bacteria but are attacked in the presence of hydrogen donating amino acids, e.g., alanine, valine, leucine or isoleucine (Stickland, 1935a).

Tryptophan itself is not deaminated by the strict anaerobes studied to date. Hopkins and Cole (1903) demonstrated that indole and skatole were formed from tryptophan by E. coli. The mechanism of the over-all reaction of indole formation was established by Woods et al. (1947) and was confirmed by Davis and Happold (1949). Two alternate pathways for the complete oxidation of tryptophan have been reported for microorganisms (Happold, 1950). Both of these pathways apparently are initiated by the peroxidase-oxidase system and formylase which leads to the formation of kynurenine from tryptophan. The presence of kynureninase leads to the aromatic pathway and the formation of anthranilic acid and catechol whereas the quinoline pathway depends on the presence of a transaminase which induces the formation of kynurenic and xanthurenic acid.

Cystine and cysteine. Several microorganisms have been reported which cause a nonoxidative desulfhydration with either a simultaneous or subsequent deamination of cysteine (Fromageot, 1951). Woods and Clifton (1937) reported the deamination of cystine and cysteine by suspen-

sions of Cl. tetanomorphum.

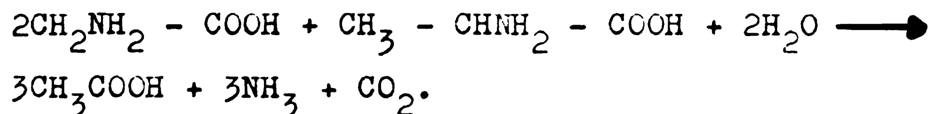
Methionine. Woods and Clifton (1937) and Pickett (1941) have reported the deamination of methionine by Cl. tetanomorphum and Cl. tetani. Nisman and Thouvenot (1948), employing Cl. sporogenes, isolated alpha-keto-gamma methiobutyric acid from methionine in the presence of sodium arsenite; mercaptan formation was also noted. The anaerobic degradation in certain strains of Pseudomonas produced ammonia, alpha-ketobutyric acid and methyl mercaptan (Kallio and Larson, 1955).

Lysine was decarboxylated to cadaverine by Bacillus cadaveris and E. coli (Gale, 1940) whereas none of the anaerobes studied were found to deaminate lysine (Cohen, 1949).

Arginine and ornithine. These amino acids have been deaminated by a number of anaerobic species; Woods (1936) reported Cl. sporogenes reduced ornithine to delta-aminovaleric acid (47%) and that arginine also gave rise to delta-aminovaleric acid. Stadtman (1954), working with an amino acid-fermenting Clostridium, observed that 6 per cent of ornithine - 2 - C^{14} was reduced to delta-aminovaleric acid and 1 per cent was converted to proline. Arginine and ornithine are decarboxylated to agmatine and putrescine, respectively, by E. coli (Gale, 1940) and a strain of Lactobacillus (Rodwell, 1953).

The Stickland Reaction

Stickland (1935a) reasoning from the earlier experiences of Knight and Fildes (Stickland, 1935b) on the nutrition of Cl. sporogenes, discovered that the energy necessary for the growth of this bacterium resulted from a reaction between amino acids. Stickland placed amino acids and Cl. sporogenes in Thunberg tubes with methylene blue or brilliant cresol blue as oxidation-reduction indicators. He found that alanine, valine, leucine and pyruvate were good donators of hydrogen. Utilizing the leucoderivative of benzyl violet, Stickland observed that the following amino acids were hydrogen acceptors: glycine, proline and hydroxyproline. Then he placed both a hydrogen donating amino acid and a hydrogen accepting amino acid along with washed bacterial suspensions into Thunberg tubes and measured the deamination. By this means, Stickland demonstrated that two amino acids were deaminated whereas each one individually was not. The results of Stickland (1935) for alanine and glycine may be represented by the following equation:



$$2CH_2NH_2 - COOH + CH_3 - CHNH_2 - COOH + 2H_2O \longrightarrow 3CH_3COOH + 3NH_3 + CO_2.$$

The importance of such a reaction to strict anaerobes, which do not utilize oxygen as a hydrogen acceptor, is readily apparent. When proline served as the hydrogen acceptor, Stickland (1935a) observed that the reduction of

proline was made without deamination, giving rise to delta aminovaleric acid. Neuberg (1911) and Ackerman (1907, 1908, 1909) had previously discovered delta aminovaleric acid in mixed cultures of putrefactive bacteria.

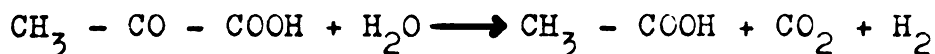
Stickland (1935a) proposed that the oxidation of alanine occurred as follows: (1)

$$CH_3 - CHNH_2 - COOH + 2H_2O \longrightarrow CH_3COOH + CO_2 + NH_3 + H_2$$

Initially alanine would be converted to pyruvic acid: (2)

$$CH_3 - CHNH_2 - COOH + H_2O \longrightarrow CH_3 - CO - COOH + NH_3 + H_2$$

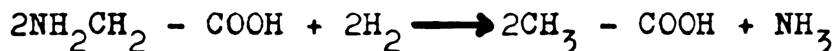
Pyruvic dehydrogenase would then produce acetic acid as follows: (3)



$$CH_3 - CO - COOH + H_2O \longrightarrow CH_3 - COOH + CO_2 + H_2$$

Reaction (1) then represents the sum of reactions (2) and (3).

The reduction of glycine utilizes the $2H_2$ formed during the oxidation of alanine and is brought about by deamination: (4)



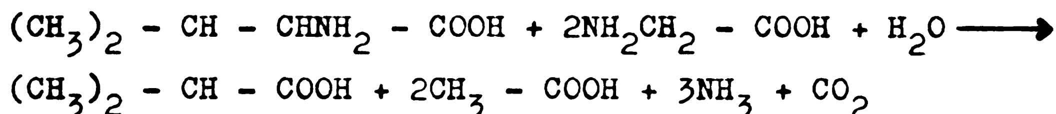
$$2NH_2CH_2 - COOH + 2H_2 \longrightarrow 2CH_3 - COOH + NH_3$$

The Stickland reaction then is represented by the sum of reactions (1) and (4). In the presence of 10^{-3} molar sodium arsenite the Stickland reaction is totally inhibited, however, Nisman and Vinet (1949b) have demonstrated that in the presence of this inhibitor and methylene blue as the hydrogen acceptor, the oxidation of the hydrogen donating substrate is carried out normally, whereas the oxidation by glycine of the leucophenosafranine is inhibited.

Therefore, two distinct enzymes appear to be operative in the coupled deamination.

Woods (1934) demonstrated that ornithine and arginine are also hydrogen acceptors. Their reduction gave rise to delta aminovaleric acid. Valine and leucine were shown by Stickland (1935a) to be good hydrogen donators; Hoogerheide and Kocholaty (1938) added isoleucine. These authors, however, did not conduct experiments comparable to that which Stickland (1935b) made for alanine. With this in mind, Cohen-Bazire *et al*. (1948a, b) performed experiments designed to better elucidate the dissimilative patterns of valine, leucine and isoleucine in the Stickland reaction. Prevot and Zimmes (1946) had established that *Cl*. *valerianicum* and *Cl*. *caproicum* produced respectively an acetic-valeric and acetic-caproic fermentation when they were grown on meat broth and 10% glycogen. Cohen-Bazire *et al*. (1948a, b), however, observed the same fermentations in a non-glucose medium and surmised that valeric and caproic acids were not catabolites arising from the degradation of glucose. Only acetic acid was formed when glucose or pyruvate were substrates. These authors then concluded that isobutyric, isovaleric and valeric acids were arising from the branched chain amino acids valine, leucine and isoleucine. Washed suspensions of *Cl*. *caproicum* and *Cl*. *valerianicum* did not deaminate these amino acids when they were used as individual substrates,

but deamination did occur in the presence of hydrogen accepting amino acids, either glycine or proline. The balance sheet of Cohen-Bazire (1948a, b) permitted the following equation to be written for valine:



$$(CH_3)_2 - CH - CHNH_2 - COOH + 2NH_2CH_2 - COOH + H_2O \longrightarrow$$
$$(CH_3)_2 - CH - COOH + 2CH_3 - COOH + 3NH_3 + CO_2$$

The exactness of the proposed catabolic equations was verified by the ammonia levels, the measured volatile acidity, the analysis of the nature and proportion of the acids formed and the manometric measure of liberated carbon dioxide. Proline proved to be a better hydrogen donator than glycine for these analyses patterns of valine, leucine and isoleucine in the Stickland reaction. Prevot and Zimmes (1946) had established that *Cl. valerianicum* and *Cl. caproicum* produced respectively an acetic-valeric and acetic-caproic fermentation when they were grown on meat broth and 10% glycogen. Cohen-Bazire *et al.* (1948a, b), however, observed the same fermentations in a non-glucose medium and surmised that valeric and caproic acids were not catabolites arising from the degradation of glucose. Only acetic acid was formed when glucose or pyruvate were substrates. These authors then concluded that isobutyric, isovaleric and valeric acids were arising from the branched chain amino acids valine, leucine and isoleucine. Washed suspensions of *Cl. caproicum* and *Cl. valerianicum* did not deaminate these amino acids when they were used as

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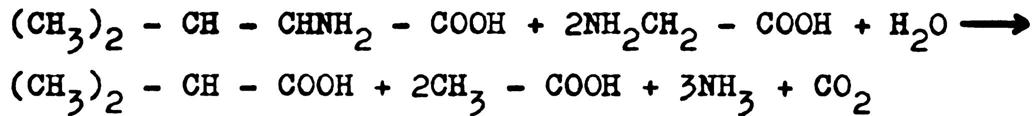

$$(CH_3)_2 - CH - CHNH_2 - COOH + 2NH_2CH_2 - COOH + H_2O \longrightarrow$$
$$(CH_3)_2 - CH - COOH + 2CH_3 - COOH + 3NH_3 + CO_2$$

Proline proved to be a better hydrogen donator than glycine for these analyses due to the formation of non-volatile delta aminovaleric acid. Optically active valeric acid was not definitely established as the volatile acid arising from isoleucine due to inherent errors in the analytical technique employed, but the acid obtained proved not to be optically active caproic acid which could have arisen by a reductive deamination of isoleucine.

Earlier, Neuberg and Karczag (1909) had described reductive deamination by mixed cultures of bacteria whereby isovaleric acid arose from valine and optically active caproic acid from isoleucine. Optically active caproic acid has been reported in tobacco leaf sap (Sabety and Panouse, 1947) and in petroleum (Quebedaux _et al_., 1943). To date, however, there has been no evidence in the literature of reductive deamination of valine, leucine and isoleucine by anaerobic bacterial suspensions (Cohen-Bazire _et al_., 1948a, b). Wagner _et al_. (1925) observed a marked accumulation of ammonia and volatile acids in cultures of _Cl. botulinum_ grown on peptone broth. The volatile acids were

a mixture of acetic, butyric and valeric acids. Clifton (1940a) reported that _Cl. botulinum_ was capable of performing the Stickland reaction and thought that the valeric acid fraction of Wegner _et al._ (1925) could have arisen from the deamination of delta aminovaleric acid by certain strains of _Cl. botulinum_. At present, it is known that the valeric acid fractions reported by earlier workers was probably a mixture of optically active valeric acid, isovaleric and isobutyric acids and these acids originated from branched amino acids via the Stickland reaction.

In addition to _Cl. sporogenes_, _Cl. botulinum_, _Cl. valerianicum_ and _Cl. caproicum_, Nisman _et al._ (1948a, b) have extended the Stickland reaction to the following species: _Cl. histolyticum_, _Cl. flabelliferum_, _Cl. saprotoxicum_, _Cl. sordelli_, _Cl. bifermentans_, _Cl. butyricum_, _Cl. acetobutylicum_ and _Inflabilis indolicus_. _Cl. saccharobutylicum_, _Cl. tetani_, _Cl. iodophilum_, _Cl. tetanomorphum_, _Cl. perfringens_ and _I. teras_ were incapable of performing the Stickland regardless of the pair of amino acids studied; this was also true for the following facultative anaerobes: _Staphylococcus aureus_, _Proteus vulgaris_, _Klebsiella pneumonia_ and _Escherichia coli_. It would appear then that the enzymes of the Stickland reaction are limited in general to the proteolytic species of the _Clostridiacea_. Species giving the Stickland reaction produce a mixture of acetic, C_4 and C_5 acids whereas other

species have an acetic-butyric fermentation. The latter acids therefore arise from the degradation of glucose and the deamination of those amino acids which give principally acetic and butyric acids; this group constitutes the majority of the Clostridiaceae.

Raynaud and Macheboeuf (1946) reported that the Stickland reaction carried out by Cl. sporogenes is inhibited in the presence of glucose. Cohen-Bazire et al. (1948a, b) and Saissac et al. (1948) reported this same phenomenon for other species. Nisman and Thouvenot (1948) demonstrated that Cl. aerofoetidum, Cl. carnofoetidum, Cl. mitelmani and Cl. goni possessed the enzymes of the Stickland reaction. Nisman et al. (1948b) reported that in this reaction glucose, pyruvate, acetaldehyde and ethanol, but not lactate and succinate, could replace the hydrogen donating amino acids; glycine, for example, was just as well deaminated in the presence of these compounds as in the presence of alanine (Prevot and Zimmes, 1946).

It has been demonstrated recently that the strict anaerobes possess enzymatic systems capable of utilizing oxygen as a hydrogen acceptor (Rosenberg and Nisman, 1949). Under aerobic conditions, Nisman and Vinet (1949) observed that amino acids of the hydrogen donor group were converted mainly to the corresponding fatty acids; however, small quantities of the corresponding alpha-keto acids were formed. These authors found that, under aerobic conditions,

the amino acids of the hydrogen acceptor group and oxygen competed for the hydrogen liberated from the amino acid donor. There was evidence that diphosphopyridine nucleotide was involved in the carrier system (Nisman, 1954; Mamelok and Quastel, 1953); it was demonstrated that diphosphopyridine nucleotide might be reduced by alanine and that the reduced coenzyme could undergo reoxidation by proline or glycine.

Non-Protein Nitrogen

The knowledge of the ability of rumen bacteria to utilize non-protein nitrogen for their own protein needs dates back to the nineteenth century. For it was in 1891 that Zuntz (1891) proposed the utilization of amides and ammonium salts as precursors of ruminal bacterial protein and the subsequent digestion and assimilation of this protein by the host. However, it was the impetus of the scarcity of nitrogenous concentrates in Germany prior to and during World War I and the development of processes for the synthesis of non-protein nitrogen compounds that led German scientists to be very active in research projects dealing with the usage of urea and other non-protein nitrogen materials as protein substitutes in ruminant rations. From these studies and the many subsequent investigations which followed, certain conclusions have been reached regarding those conditions suitable for the most

efficient utilization of urea nitrogen. Pearson and Smith (1943c) and Mills et al. (1944) have shown that the degree of protein synthesis is related to the amount and type of carbohydrate present in the ration, starch being the most efficient. In vitro studies by Wegner et al. (1940) demonstrated the importance of the level of protein in the ration on the utilization of urea nitrogen. These workers found that urea utilization was lessened when the total protein concentration of the rumen ingesta exceeds 12 per cent. Later work by Johnson et al. (1942) and Hamilton et al. (1948) showed that the nutrient value of rations for growing lambs was diminished when less than 16 per cent of the total nitrogen is in the form of preformed protein. Loosli and Harris (1945) reported in lambs both higher absolute amounts of nitrogen and higher percentages of absorbed nitrogen on 10 per cent protein rations containing urea plus methionine than on urea alone.

In 1944, Johnson et al. (1944) defaunated the rumen of sheep by feeding copper sulfate and showed that it was the bacteria and not the protozoa in the rumen which were responsible for protein synthesis from urea. Defaunating the rumen with copper sulfate did not reduce the ability of sheep to utilize urea. Smith and Baker (1944) likewise demonstrated that protein synthesis from urea was not reduced in the absence of the rumen protozoa. These authors further stated that the chief contributors to protein

synthesis are the small rods, cocci and vibrios of the microiodophilic population. Mann et al. (1954) and Mackay and Oxford (1954) succeeded in isolating gram-positive micrococci, and a gram-negative rod from the rumen which were urease-positive and facultatively anaerobic. In addition, Gibbons and Doetsch (1959) have isolated from the rumen a gram-positive, nonmotile, asporogenous, pleomorphic rod resembling Lactobacillus bifidus which hydrolyzed urea and was obligately anaerobic. The urease in rumen fluid resembles in activity that from soya and jack beans in that changes occur in its activity with changes in temperature and pH and its behavior in the presence of inhibitors as quinone and sodium fluoride is typical of enzymes of the urease type (Pearson and Smith, 1943). These authors found the inhibition of urease by quinone could be prevented by thiol compounds such as hydrogen sulfide and cysteine.

Ruminants appear to differ from nonruminants in blood urea metabolism, a phenomenon which is largely attributable to the rumen microflora. In the ruminant, urea formed by the liver moves via the blood or saliva into the rumen where it is utilized for protein synthesis. The subsequent digestion and absorption of protein in the small intestines constitutes a mechanism whereby protein nitrogen can be continually regenerated. Of course, the regeneration

rate would be governed by the urinary urea excretion rate. Here again the ruminant shows a uniqueness since Schmidt-Nielsen *et al*. (1957, 1958) has demonstrated that the mechanism of urea excretion in ruminants differs from that observed in other mammals. This conclusion is based on their findings that camels and sheep, when placed on a low protein ration, decreased the fraction of filtered urea appearing in the urine to very low values. It was found in the camel that low protein intake caused the urea clearance to decrease to values of only 1 to 2% while the urea filtered in the glomeruli rose to 40% when a normal ration was fed. The authors also observed in both the camel and sheep that the kidneys continue to conserve urea for some time after nitrogen intake is increased following a prolonged period of low nitrogen intake. This regulation was found to be independent of the glomerular filtration rate, plasma urea concentration and osmotic load and therefore the regulation appears to be on the tubular level (Schmidt-Nielson *et al*. 1958). These authors further stated that their data was consistent with the previously suggested hypothesis "that the excretion of urea in the mammalian kidney is brought about through a regulated active transport of urea, accentuated by a counter-current multiplier system represented by Henle's loop and vasa recta." Indeed, the urinary urea excretion rate appeared to be governed by the nitrogen intake and growth rate.

When known quantities of urea were injected intravenously into protein depleted sheep fed a low nitrogen, high carbohydrate ration, only about 52% of the injected urea could be recovered in the urine (Houpt, 1959). However, the utilization of injected urea decreased to a mean of 22 per cent when dietary carbohydrate was withheld during the urea injection studies. In experiments in which the isolated rumen of anesthetized sheep was emptied and replaced with warm physiological saline, Houpt found the total urea -N transfer to the rumen to be 5.2 mmoles urea -N/hr. This value was from 40 to 67% lower than the rates found in the urea injection experiments (7.8 - 13.0 mmole/hr.) and was attributed by the author to the experimental conditions. The 7.8 to 13 millimoles/hr. reported utilized by Houpt in the rumen agrees well with the 8.6 mmol. urea -N/hr./4 liter rumen reported by Pearson and Smith (1943c). In the rumen saline experiment on sheep, 5.5 to 16 times as much urea passed directly from the blood to the rumen as moved with saliva. McDonald (1948) has estimated that the salivary urea nitrogen in the sheep contributes at least 0.5 g. nitrogen to the rumen daily which is somewhat higher than the value of 0.28-0.36 g. given by McDougall (1948). Somers (1958) also found 0.29 g. urea nitrogen in the daily salivary secretion of sheep. Unfortunately, accurate data is not available on the urea nitrogen contributed to the bovine rumen, but calculations based on urea concentrations

of 10-13 mg. % and an average salivary secretion of 85-90 liters per day would be 8.5 to 11.7 g. per day (Phillipson and Mangan, 1959).

Another interesting facet of urea metabolism in ruminants was studied by Tsuda (1956a, b) employing a Pavlov pouch in a goat's rumen. In attempting to elucidate whether urea is absorbed directly from the rumen, Tsuda placed three concentrations of urea solutions 0.2, 1 and 5 per cent into this miniature rumen. Only at the 5 per cent level did appreciable absorption take place; and since when urea is fed to ruminants, the concentration in the rumen fluid is generally below 0.5 per cent, it would appear that direct absorption of urea through the rumen wall does not occur under practical feeding conditions.

In summary, urea transfer appears to be a one way mechanism from the peripheral blood system or saliva to the rumen which occurs in both normal and stressing conditions. The unique urea conservation mechanism operative in the renal tubules of the ruminant serves to continually regenerate nitrogen so essential to the rumen microbial flora during periods of low nitrogen intake.

Ammonia is a key intermediate of microbial nitrogen metabolism in the rumen. Rumen ammonia is derived from several sources: (1) a breakdown of feed protein, (2) a deamination of free amino acids in feedstuffs, (3) a proteolysis of the rumen microorganism, (4) a hydrolysis of

feed, salivary and ruminal urea, (5) a breakdown of ammoniated feedstuffs and (6) a reduction of feed nitrates.

In contrast to the relatively high level of ammonia in the ruminal and portal veins of the ruminant, the concentration of ammonia in the peripheral blood, other body fluids, and the tissues of ruminants is very low (McDonald, 1948). McDonald (1948) calculated that ammonia absorption from the sheep's rumen could amount to about 4 to 5 g. ammonia nitrogen per day when ruminal ammonia levels averaged 23 mg. per cent. Normally, the ammonia concentration of rumen contents approximates 8 to 40 mg. per cent and reaches a maximum at about 2 to 3 hours after feeding (Head, 1959). Lewis, et al. (1957) demonstrated a close correlation between changes in rumen-ammonia levels and the levels of ammonia in the portal blood. No regulatory mechanism for the adsorption of ammonia appeared to exist; ammonia transfer seemed to be one of simple diffusion.

Excessive levels of ammonia may appear in the peripheral circulation whenever the urea synthesizing capacity of the liver is exceeded and in such a case, is deleterious to the animal. Toxicity studies have revealed that the rumen ammonia concentration at which ammonia appeared in the peripheral blood was not constant. Head and Rook (1955) observed peripheral blood ammonia at rumen ammonia concentrations as low as 30 to 40 m. moles per liter, while Annison et al. (1957) reported rumen ammonia concentrations

as high as 85 m. moles per liter without noticing any toxic effects. A level of 1 to 2 mg. per cent of ammonia in the peripheral blood of ruminants produces toxic symptoms (Repp *et al.*, 1955; Lewis *et al.*, 1957; Dinning *et al.*, 1949).

Pearson and Smith (1943a) and Smith *et al.* (1956) observed a marked stratification in the ammonia concentration of rumen ingesta. In both instances, a higher concentration of ammonia occurred in samples taken from the top ingesta than in samples from the bottom. Reis and Reid (1958) reported that the optimum pH for ammonia production from casein in the rumen varied between 6.0 and 6.7 on different rations; ammonia production fell rapidly on the acid side on the optimum pH and less rapidly on the alkaline side.

Ammonia is a key intermediate in the dynamic state of mammalian protein metabolism. Schoenheimer and his associates found that when N^{15} (as ammonia or amino acids) was given to rats, the isotope subsequently appeared in almost all of the amino acids (Foster *et al.*, 1939; Rittenberg *et al.*, 1939; Schoenheimer *et al.*, 1939a, b). In general, examination of the body revealed that the amino acid originally fed exhibited the highest concentration of isotope followed in order of isotope concentration by glutamic and aspartic acids. When N^{15} aspartic acid was fed, the isolated glutamic acid had the greatest concentra-

tion of isotope.

Two amino acids, lysine and threonine, appear to occupy a special metabolic position in mammals in that they do not appreciably incorporate administered N^{15} from ammonia or other amino acids. When lysine labeled with deuterium and N^{15} was fed to rats, N^{15} was found in other amino acids, but the lysine incorporated into the tissues had a deuterium to N^{15} ratio which was almost the same as that of the administered amino acid (Weissmann and Schoenheimer, 1941; Ratner _et al._, 1943). Similar findings were also noted with threonine (Elliott and Neuberger, 1950).

Lewis (1951a) has demonstrated the conversion of nitrate to ammonia in the rumen and postulated that nitrite and hydroxylamine were intermediates in this reduction. An accumulation of nitrite in the rumen may occur under conditions whereby large amounts of nitrates are present in the ration. Nitrite then may be absorbed with toxic effects due to a methemoglobinemia. Lewis (1951b) has also shown, using washed suspensions of rumen bacteria, that hydrogen was a very active donor for the reduction of nitrate, nitrite and hydroxylamine. Formate, succinate, lactate, citrate, glucose, malate and mannitol were also hydrogen donors for nitrate reduction, but less active than hydrogen itself.

Protein Anabolism

Since the rumen is a dynamic system, the factors affecting the quantity of protein synthesized in the rumen are numerous and complex. Most of these factors were discussed previously and included the level of protein in the ration, the nature of the ration protein, other constituents in the ration, etc. Knowledge about the extent of this protein synthesis and processes involved are essential for the understanding of the contribution of this phenomenon to the nutrition of the ruminant. This section then will include the magnitude of protein synthesis within the rumen and a few specific examples of the anabolic processes.

It has recently been reported (Gray *et al.*, 1958) that in sheep fed on wheaten hay the amount of nitrogen reaching the abomasum and passing on to the duodenum was equivalent to almost 100 per cent of the nitrogen ingested; in other words there was no overall loss of nitrogen from the rumen by absorption. Since subsequent studies (Weller *et al.*, 1958), employing diaminopimelic acid for the assay, revealed that throughout the entire day 61 to 82 per cent of the nitrogen in the rumen was present as microbial nitrogen, it would appear that this range represented the extent of the conversion of plant nitrogen to microbial nitrogen.

Pearson and Smith (1943c) and McNaught and Smith (1947) estimated the extent of microbial protein production in the mature cow to be 100 to 500 grams per day. Duncan *et al*. (1952) found 19 to 190 per cent more protein in the rumen six hours after feeding a purified ration containing urea as the sole source of nitrogen. Agrawala *et al*. (1953) demonstrated a 33 to 109 g. increase in true protein in the rumens of six month old calves fed a purified ration and a 252 g. increase on a natural ration.

Lysine synthesis by rumen bacteria has been demonstrated by McNaught (1951) and Edwards and Darroch (1956). McNaught demonstrated a 12 per cent increase in the lysine content of *in vitro* fermentation flasks whereas Edwards and Darroch demonstrated the ruminal synthesis of lysine by feeding a ration devoid of lysine to lactating goats and demonstrating lysine containing proteins in the milk. These latter authors also calculated that the ruminal synthesis of protein in these goats amounted to 40 g. per day. Allison *et al*. (1959) incubating *Ruminococcus flavefaciens* in the presence of isovalerate - 1 - C^{14} recovered radioactive leucine from protein hydrolyzates of this organism. Block *et al*. (1951) and Emery *et al*. (1957a) demonstrated the incorporation of radioactive inorganic sulfate into cystine and methionine by rumen bacteria. The latter authors found that the formation of cystine was about twice as great during the three-hour incubation

period as the formation of methionine. When examining the response of single strains of bacteria, Emery *et al.* (1957b) noted that only five out of ten strains studied utilized significant amounts of inorganic sulfate in the synthesis of organic sulfur compounds.

EXPERIMENTAL PROCEDURE

In Vitro Studies

The donor animals for these studies were mature cows, weighing from 1050 to 1200 pounds, fitted with the screw cap, plastic fistula plugs described by Hentschl *et al.* (1954). These animals were fed rations of 6 to 10 pounds of alfalfa hay and 10 to 14 pounds of a concentrate mixture, composed of 77.5% ground shelled corn, 20% soybean oil meal, 1% calcium carbonate, 1% dicalcium phosphate and 0.5% trace mineralized salt. The concentrate mixture also contained 5000 international units of vitamin A and 450 international units of vitamin D per pound. The crude protein content was 15.8%. All the cows received 50 grams of trace mineralized salt daily. The experimental rations were fed once daily at 7 a.m. for at least three weeks before the investigations were initiated. The animals had free access to water.

General methods. Samples of rumen fluid which were to serve as inocula were collected at 3 to 4 hours after feeding. The rumen ingesta was strained through a double layer of cheese cloth to remove all extraneous solid material. In those studies which are designated as rumen liquor incubations the material described above served as the incubating medium. The amino acids were dissolved in

200 ml. of rumen liquor in amounts equivalent to 0.01M solutions. The washed suspensions of rumen bacteria were prepared in the following manner. The cheese cloth strained material was centrifuged in an International centrifuge size 2 model V for 5 minutes at 250 x G to remove the large feed particles and protozoa. The resulting supernatant fluid was then subjected to a force of 27,600 x G for 10 minutes in a Serval centrifuge type SS-1. The supernatant was discarded and the bacterial residue was suspended in 25 ml. of 0.1M phosphate buffer at pH 6.5, previously boiled and cooled, to which was added 0.02% (w/v) $Na_2S \cdot 9H_2O$. This material was again centrifuged at 27,600 x G for 10 minutes; the supernatant was discarded and the bacterial residue resuspended in the above buffer. Three parts of the bacterial suspension (dry weight, 4 to 7 mg./ml) were then added to ten parts of the buffer containing the amino acid or amino acids. Quantities of amino acids equivalent to 0.01M solutions in 13, 130, and 260 ml. total volumes were dissolved in the buffer solutions just prior to the addition of the bacterial suspension.

Each amino acid was incubated alone and in combination with two and three other amino acids in rumen liquor and washed cell preparations. Whenever tryptophan was studied, the amino acid was solubilized in 5 ml. of 1N Na OH prior to the addition of the rumen liquor or phosphate

buffer. Whenever aspartic or glutamic acid was studied, the amino acid was neutralized to pH 6.9 with sodium carbonate prior to incorporation in the incubation medium.

Anaerobic conditions were obtained by gassing for five minutes with carbon dioxide freed from oxygen by bubbling the gas through a chromous acid solution. The rumen liquor incubations were conducted for 8 or 24 hours at 39° C., whereas the washed cell suspensions were incubated at 39° C. for 8, 24 or 48 hours.

Samples for amino acid chromatographic analysis were obtained by adding 0.5 ml. of the amino acid medium to 2 ml. of absolute alcohol just prior to incubation (zero hour) and at the termination of the experiment. Samples for ammonia and volatile and nonvolatile acids were taken just prior to incubation (zero hour) and at the termination of the experiment. These samples were preserved by adding 1 ml. of 50% (v/v) sulfuric acid to 50 ml. of sample. This volume of acid lowered the pH to 1.5 to 2.0 and yielded a final sample in which most of the suspended solids were precipitated. Samples arising from incubation studies with tryptophan were preserved by adding 1 cc. of a saturated solution of mercuric chloride to 39 cc. of the incubating medium. These samples were stored at 6° C. until analyzed which was always less than a month.

The Keeney column (1955) was used for the determination of volatile fatty acids. Ammonia was determined by

aeration or steam distillation from an alkaline sample. The ammonia was collected in a 2% solution of boric acid and titrated to the mixed Kjeldahl indicator (100 mg. methyl red + 30 mg. methylene blue) endpoint.

Glutaric acid analysis. Paper chromatography was used as a semiquantitative measure of glutaric acid dissimilation. A series of glutaric levels of 5, 10, 15, 20 and 25 mcM./ml. were added to rumen fluid and washed cell endogenous preparations to serve as standards. Twenty-five micromoles of glutaric acid per milliliter previously neutralized with 1N NaOH was used in the duplicated 24-hour rumen fluid and washed cell dissimilation studies. At the end of 24 hours, 15 ml. were taken from each of the dissimilation and standard solution flasks and steam distilled under acid conditions to remove the volatile acids. The samples were then evaporated on a hot plate to a volume of 5 ml. The 5 ml. was extracted successively with three 10 ml. portions of peroxide-free ethyl ether. The ethyl ether was evaporated on a hot plate and concentrated to a final volume of 3 ml. One hundred microliters of the ethyl ether extract was chromatographed onto Whatman no. 1 filter paper. Descending chromatography was employed using ethyl alcohol, water and ammonium hydroxide (80:10:10). The paper was air dried and sprayed with a 0.04% solution of bromophenol blue in 95% ethyl alcohol adjusted to a definite blue color (pH 6.7 as determined with a glass elec-

trode) with dilute sodium hydroxide (Buch *et al.*, 1952). Another spray reagent employed with a mixture of 2 g. glucose, 2 cc. aniline, 20 cc. water, 20 cc. ethanol, and 60 cc. butanol (Bastie, 1957). The sheets were placed in an oven at 115° C. for 10 minutes. The acid spots appeared dark brown on a pale yellow background. Concentration differences of 5 mcM./ml. were readily detected.

Amino acid analysis. The amino acids and their dissimilation products were identified by unidimensional descending paper chromatography. A number of the well-known amino acid developing solvents were used for this purpose: (1) butanol, acetic acid, water (4:1:1); (2) pyridine, acetic acid, water (50:35:15); (3) phenol, water (80:20); (4) ethyl alcohol, butanol, pyridine, water (60:10:5:25); (5) isobutyric acid, water (80:20). Leucine and isoleucine were separated with tertiary amyl alcohol, propanol, and water (4:1:1). Cadaverine and putrescine were separated with phenol, water (80:20) in a 0.3% ammonia atmosphere with 100 mg. of sodium cyanide in 4-6 ml. of water at the bottom of the chamber.

Thirty microliters of the sample was spotted on Whatman no. 1 paper. The chromatograms were air dried over night. The separated amino acids were visible as fluorescent spots under ultraviolet light. The chromatograms were sprayed with 1% ninhydrin in 95% ethanol and developed in the dark at room temperature.

Since Rf values may undergo fluctuations, the unknown ninhydrin-reactive compounds were identified by comparing the suspected known, the unknown and a mixture of the suspected known and unknown compounds on the same chromatogram. This technique compensates for any deviation of the Rf value in the unknown caused by extraneous material. Repeatable results, by this technique, in three to four different solvents constituted identification of the unknown.

Analysis of tryptophan derivatives. Indole and skatole were identified by means of paper chromatography using isopropyl alcohol, 28% ammonia, water (10:1:1). The chromatograms were air dried and sprayed with Ehrlich's reagent. This reagent is of particular value in that it reveals both the indole structure and compounds containing free amino groups. In addition, the colors obtained with different compounds containing the indole structure vary widely and characteristically, as do the time required for these colors to appear. Indole and skatole were likewise identified using the ultraviolet regions of the spectrum of a Beckman DK-2 ratio recording spectrophotometer. Indole was isolated by extracting the tryptophan incubation medium with three times its volume of peroxide-free ethyl ether. The incubation medium was extracted three times successively with its own volume of ethyl ether. The ethyl

ether was dried over anhydrous sodium sulfate and evaporated off in a rotary vacuum flash evaporator into 2 ml. of distilled water. Concentrations were chosen so as to obtain a maximum absorbancy for the sample of 0.50-0.85 units, the absorbancy being controlled by dilutions or reduction of the cell path from 1.0 cm. to 0.1 cm. Indole and skatole were determined quantitatively using a modification of the p-dimethylaminobenzaldehyde method of Meyers (1950). In this case, the chloroform was evaporated off at 57° C. in a current of air rather than siphoned off.

Amine analysis. Samples for amine analysis were divided into two portions. The first portion was made alkaline by adding 4% by volume of 10% sodium hydroxide. This sample was then extracted three times successively with its own volume of peroxide-free ethyl ether. The ethyl ether was evaporated off in a rotary vacuum evaporator into one ml. of 2N HCl. Such a procedure should have resulted in a 30 to 100 fold concentration, depending upon the original volume of the sample selected. The second portion was chromatographed directly.

In Vivo Studies

The animals used in these studies were the same mature fistulated cows used in the *in vitro* studies and were receiving 12 lb. of alfalfa hay and 8 lb. of the previously described concentrate mixture. The experimental ration

was fed once daily at 7 a.m. for at least three weeks before the investigations were initiated. The animals had free access to water.

In the *in vivo* amino acid dissimilation studies the endogenous, L-lysine and DL-tryptophan studies were performed using the same animal whereas the L-arginine study was performed on another animal. The two animals, however, were of similar body size and were fed identical rations. For purposes of calculating the amount of each amino acid to be administered via the fistula, it was assumed that each animal weighed 1200 lb., the rumen contents constituted 14% of the total body weight and the rumen contents were comprised of 85% water, by weight. Using these figures, the rumen would contain 65 liters of aqueous phase. Equivalent amounts of DL-tryptophan, L-arginine . HCl, or L-lysine . HCl were dissolved in a liter of water so that the 65 liters of aqueous phase in the rumen contained the equivalent of a 0.02M solution of amino acid. The level of amino acid amounted to 265.5, 274, and 237 grams of DL-tryptophan, L-arginine . HCl and L-lysine . HCl, respectively. The DL-tryptophan was solubilized with 800 cc. of 1N NaOH and then made up to one liter with water prior to fistular administration.

Each amino acid solution was administered via the fistula two hours after feeding. The amino acid solution was thoroughly mixed with the rumen ingesta by stirring

with the arm and fist for five minutes. A portion of the rumen ingesta was then strained through a double layer of cheese cloth to remove the larger feed particles. This rumen liquor served as the zero hour sample. Rumen liquor samples were taken for paper chromatography and ammonia analyses at 0, 1, 2, 3, 4, 6, 8, 10, 13 or 14, 23 or 24 hours after the administration of each amino acid. Samples for amino acid chromatographic analysis were obtained by adding 0.5 ml. of rumen fluid to two ml. of absolute alcohol. Rumen fluid samples in the L-lysine and L-arginine studies were preserved by adding one ml. of 50% (v/v) sulfuric acid to 50 ml. of sample whereas in the tryptophan studies the rumen samples were preserved by adding one ml. of a saturated solution of mercuric chloride to 39 ml. of rumen liquor.

Ammonia was determined by the permutite method described by Hawk et al. (1954) with modifications. Three ml. of rumen fluid were added to a 100 ml. volumetric flask containing two grams of amberlite IR-120. The mixture was allowed to stand several minutes before the rumen fluid was decanted from the flask and the resin washed with distilled water. Two ml. of a 10% sodium hydroxide solution were added and the flask was allowed to stand again. After the addition of 75 ml. of distilled water to the mixture, two drops of Gum Ghatti were added followed by 10 ml. of Nessler's reagent. Five minutes was allowed for color

development before diluting to 100 ml. The optical density of the sample was then determined at 490 millimicrons in a Beckman B Spectrophotometer. A standard curve was prepared in a similar manner using aqueous ammonium sulfate solutions. Indole and skatole were determined quantitatively using a modification of the p-dimethylaminobenzaldehyde method of Meyers (1950).

Blood samples were obtained by jugular venepuncture 0, 1, 2 and 4 hours after the fistular administration of the amino acid solution. For plasma samples, approximately 40 to 45 ml. of blood were drawn into tubes in which the anticoagulant was one ml. of a 10% potassium oxalate solution evaporated to dryness. Fifty ml. of ethyl alcohol were added to five ml. of plasma. After standing for 15 minutes the tubes were centrifuged for 5 minutes at 250 x G to remove the plasma protein. The supernatant was decanted and evaporated to dryness at below 50° C. under vacuum.

The ninhydrin reactive components of the bovine plasma were purified and separated into three fractions by employing the technique of Thompson _et al_. (1959). The water-soluble residue was taken up in five ml. of water and transferred to a seven cm. column (30 x 1 cm.) of Dowex 50-X4 in the ammonium form. Two 3 ml. washings were likewise transferred to the above resin. The column of Dowex 50-X4 in ammonium form retained the basic amino acids and

amines. The eluate was allowed to drip onto a seven cm. column (30 x 1 cm.) of Dowex 50-X4 in the hydrogen form which retained the neutral and acidic amino acids.

The basic amino acids--arginine, lysine, and histidine--were eluted with 80 ml. of 2N ammonium hydroxide. The eluate was dried down under carbon dioxide free conditions at moderate temperatures ($< 50°$ C.). The column was washed free of excess NH_4OH with 40 ml. of deionized water, the ammonium ion was removed from the resin with 50 ml. of 0.50 ± 0.02N HCl and the eluate was discarded. The strongly basic amines were eluted with 50 ml. of 6N HCl.

The column of acid resin was treated with small portions of 2N NH_4OH until the effluent was just basic (8-10 ml.) and then washed with 40 ml. of deionized water. The effluent, containing the neutral and acidic amino acids, was dried in vacuum at below 50° C.

The three fractions were taken up individually in 5 ml. of 50% alcohol-50% water. One and a half milliliters of this mixture was used in the paper chromatography studies.

Whatman No. 3 mm. paper (16½" x 22½") was used for one or two dimensional descending chromatography. The basic amino acids and amines were chromatographed unidimensionally employing pyridine/acetic acid/water (50/35/15), phenol/water (80/20) in a 1% ammonia atmosphere, or ethanol/diethylamine (77/1). The chromatograms were air dried and

sprayed with a 1% solution of ninhydrin in 95% ethanol. The neutral and acidic amino acids were separated employing two dimensional descending chromatography. Phenol/water (80/20) in a 1% ammonia atmosphere was used as the first solvent and n-butanol/acetic acid/water (62/12/26) as the second. The second system contained 0.1% ninhydrin (w/v). The chromatograms were air dried and allowed to develop overnight in the dark.

RESULTS

In Vitro Studies

The first phase of this project was concerned with the relative rates of dissimilation of amino acids added individually to the two *in vitro* mediums--cheese cloth-strained rumen fluid and washed suspensions of rumen microorganisms. The extent of deamination of each amino acid in cheese cloth-strained rumen liquor are presented in Table 1 and in washed suspensions of rumen microorganisms in Table 2. The amino acids are arranged in the tables in decreasing order of deamination and can be roughly grouped into three separate classes based on their activity. L- or DL-serine, L-cysteine, L-aspartic acid, L-threonine, and L-arginine were attacked most completely, followed by L-glutamic acid, L-phenylalanine, L-lysine, L-cystine and DL-lysine forming an intermediate group, and a third group in which deamination was much less pronounced was DL-tryptophan, delta amino valeric acid, L-methionine, L-alanine, L-valine, L-isoleucine, L-leucine, L-ornithine, L-histidine, glycine, L-proline and L-hydroxyproline. Ammonia production from arginine revealed only a 57 to 80% dissimilation whereas paper chromatography on the same samples revealed arginine to be completely dissimilated within 24 hours. Apparent quantities of ornithine, however,

Table 1. Ammonia production from individual amino acids with cheese cloth-strained rumen fluid. (24 hr. incubation; 0.01M soln. of amino acid. The ammonia values represent the total ammonia minus the control value)

	Ammonia N (mg/100 ml)		
Sample	Theor. Yield of Amino N	Actual Yield[1]	% dissimilation[2]
L-Serine	14.01	13.98	100
DL-Serine	14.01	14.06	100
L-Cysteine	14.01	13.42	96
L-Aspartic Acid	14.01	13.32	95
L-Threonine	14.01	11.66	83
L-Arginine HCl	56.04	44.91	80
L-Phenylalanine	14.01	10.52	75
L-Glutamic Acid	14.01	8.90	64
L-Lysine HCl	27.99	16.02	57
L-Cystine	28.02	13.04	47
DL-Lysine HCl	27.99	11.34	41
DL-Tryptophan	14.01	5.14	37
L-Histidine HCl	14.01	4.50	32
L-Ornithine	28.02	8.04	29
L-Valine	14.01	4.01	29
L-Alanine	14.01	3.89	28
L-Leucine	14.01	3.42	24
L-Isoleucine	14.01	3.10	22
Delta AVA[3]	14.01	2.90	21
Glycine	14.01	1.35	10
L-Hydroxyproline	14.01	1.26	9
L-Proline	14.01	1.18	8

[1]Mean of three values.

[2]Range ± 10 per cent.

[3]Delta amino valeric acid.

Table 2. Ammonia production from individual amino acids with washed suspensions of rumen microorganisms. (24 hr. incubation, 0.01M soln. of amino acid. The ammonia values represent the total ammonia minus the control values)

Sample	Ammonia N (mg/100 ml)		
	Theor. Yield of Amino N	Actual Yield[1]	% dissimilation[2]
L-Serine	14.01	13.80	99
DL-Serine	14.01	13.90	99
L-Aspartic Acid	14.01	12.10	86
L-Cysteine	14.01	11.86	85
L-Threonine	14.01	9.62	69
L-Glutamic Acid	14.01	7.92	57
L-Arginine HCl	56.04	31.82	57
L-Lysine HCl	27.99	10.40	37
L-Cystine	28.02	9.62	34
Delta AVA[3]	14.01	3.02	22
L-Phenylalanine	14.01	2.98	21
L-Methionine	14.01	2.50	18
D-Tryptophan	14.01	2.32	17
DL-Tryptophan	14.01	2.06	15
L-Histidine HCl	14.01	2.03	14
L-Ornithine HCl	28.02	3.92	14
L-Alanine	14.01	1.98	14
L-Valine	14.01	1.54	11
L-Leucine	14.01	1.48	11
L-Isoleucine	14.01	1.39	10
Glycine	14.01	0.32	2
L-Proline	14.01	0.24	2
L-Hydroxyproline	14.01	0.16	1

[1]Mean of three values.

[2]Range ± 10 per cent.

[3]Delta amino valeric acid.

still persisted. Serine, L-cysteine and L-aspartic acid were markedly dissimilated, 80-100%, in 24 hours regardless of the medium used. The other dissimilatable amino acids were present in variable quantities at the end of 24 hour incubations. The amino acids catabolized in rumen fluid were likewise dissimilated by washed cell suspensions and in approximately the same sequence of magnitude. The dissimilation rates, however, are more rapid and complete in the rumen fluid studies than in the washed cell suspensions. The one exception was delta amino valeric acid which was not dissimilated at a distinguishable faster rate in rumen fluid.

When three or four amino acids were incubated together with washed suspensions of rumen microorganisms, the only phenomenon which differed from amino acids incubated alone was the marked increase in the dissimilation of proline and alanine incubated together. The actual yields of ammonia from a mixture of three or four amino acids can be compared to the sum of ammonia production from each amino acid in Tables 3 and 4. In this trial the amount of each amino acid added was the same, 10 mcM./ml., resulting in a final amino acid concentration of 30 to 40 mcM./ml. These results demonstrated that the most readily dissimilated amino acids were responsible for the major portion of the ammonia arising from mixtures of three or four amino acids. Ammonia production and visible

Table 3. Ammonia production from amino acid mixtures and individual amino acids with washed suspensions of rumen microorganisms. (24 hr. incubation, 30 mcM./ml., 10 mcM./ml. of each amino acid. Ammonia values represent the total ammonia minus the control value.)

Sample	Ammonia N (mg/100 ml)			
	Theor. Yield of Amino N	Actual Yield	% Dissimilation	Individual Yield
L-Lysine HCl				15.30
L-Aspartic Acid	56.01	42.18	75	11.70
DL-Serine				13.85
				40.85
L-Leucine				1.20
L-Methionine	42.03	4.88	12	2.20
L-Phenylalanine				2.95
				6.35
Glycine				0.16
L-Aspartic Acid	42.03	12.90	31	13.10
L-Proline				0.00
				13.26
L-Arginine				20.80
DL-Tryptophan	84.06	22.84	27	3.40
L-Valine				1.10
				25.30
Glycine				0.00
L-Alanine	42.03	3.70	9	2.60
L-Isoleucine				1.56
				4.16

Table 4. Ammonia production from amino acid mixtures and individual amino acids with washed suspensions of rumen microorganisms. (24 hour incubation, 40 mcM./ml., 10 mu M/ml. of each amino acid. Ammonia values represent the total ammonia minus the control value.)

Sample	Ammonia N (mg/100 ml)			
	Theor. Yield of Amino N	Actual Yield	% Dissimilation	Individual Yield
L-Aspartic acid				12.40
L-Threonine	56.04	24.90	44	8.70
L-Isoleucine				1.72
L-Norvaline				1.48
				24.30
L-Glutamic				7.60
L-Proline	56.04	16.88	30	0.40
L-Phenylalamine				3.24
L-Alanine				2.10
				13.34
L-Histidine				1.84
DL-Tryptophan	112.08	32.68	29	2.42
L-Lysine HCl				10.40
L-Arginine HCl				20.90
				35.56
L-Leucine				1.70
L-Methionine	56.04	5.10	9	2.10
L-Histidine				1.84
L-Proline				0.16
				5.80

disappearance of the amino acids as noted by paper chromatography were closely correlated.

A third phase of this study dealt with the metabolism of the optical isomers of six amino acids. The amino acids employed were serine, tryptophan, aspartic acid, lysine, threonine and phenylalanine. The results of this experiment are found in Table 5. Ammonia production from both the L form and either the D or DL form was determined. The ammonia levels corresponded well with the amount of amino acid dissimilated as noted by paper chromatography. These results indicate that both isomers of certain amino acids, such as serine and tryptophan, are dissimilated equally as well whereas only the L isomers of certain amino acids, such as aspartic acid, lysine, threonine and phenylalanine are readily dissimilated and the D enantiomorph are either catabolized slowly or not at all.

Since the peak of ammonia production usually occurs in the rumen at one to three hours after feeding, six amino acids were dissimilated <u>in vitro</u> using rumen liquor as the incubating medium in order to elucidate the relative magnitude of ammonia production <u>in vivo</u> from individual amino acids. An eight hour incubation period was employed for two reasons. The first was the fact that since only one amino acid served as an ammonia source, an incubation period of greater than one to three hours would be necessary to resolve apparent differences. The second

Table 5. Ammonia production resulting from the metabolism of optical isomers of six amino acids. (24 hour incubation, 10 mcM./ml. of amino acid).

Amino Acid	Isomer	Medium	Ammonia N (mg/100 ml) Theor. Yield	Actual Yield	% Dis-similation
Serine	L	rumen liquor	14.01	13.98	100
	DL	rumen liquor	14.01	14.06	100
Serine	L	washed cells	14.01	13.80	99
	DL	washed cells	14.01	13.90	99
Tryptophan	DL	washed cells	14.01	2.06	15
	D	washed cells	14.01	2.32	17
Aspartic Acid	L	rumen liquor	14.01	13.32	95
	DL	rumen liquor	14.01	8.40	53
Lysine	L	rumen liquor	27.99	16.02	57
	DL	rumen liquor	27.99	11.34	41
Threonine	L	rumen liquor	14.01	11.66	83
	DL	rumen liquor	14.01	5.82	42
Phenylalanine	L	rumen liquor	14.01	10.52	75
	DL	rumen liquor	14.01	6.19	44

was that the incubation period must be short enough to prevent substrate depletion from entering into the results. The results of this experiment are found in Table 6. In eight hours arginine contributed approximately five times as much total ammonial nitrogen as did any one of the other five amino acids studied.

Table 6. Ammonia production from six amino acids in eight hour rumen fluid incubations (10 mcM./ml of amino acid).

Amino Acid	Ammonia N (mg/100 ml) Theor. Yield	Actual Yield	% Dissimilation
L-Arginine	56.04	24.14	43
L-Aspartic Acid	14.01	5.16	37
L-Serine	14.01	4.52	32
L-Cysteine	14.01	4.39	31
L-Lysine	14.01	2.56	18
DL-Tryptophan	14.01	1.82	13

The final phase of this study dealt with various attempts to increase the magnitude of deamination in washed suspensions of rumen microorganisms. Modifications of the usual washed cell suspensions are given below and the results of each are summarized in Table 7.

Experiment 1. Use of Enriched Cultures. Eight individual amino acids, 30 mcM./ml., were incubated for 48 hours. The rumen microorganisms were centrifuged out and resuspended in a medium containing the same amino acid (10 mcM./ml.) with which they had been previously incubated. Experiment 1 reveals that such enriched cultures are very poor dissimilators of amino acids as compared to Experiment 0 in which conventional washed cell suspensions were employed. A slide was prepared from each of the 72 hr.

Table 7. Attempts to increase ammonia production from amino acids incubated with washed suspensions of rumen microorganisms (10 mcM./ml. of amino acid; 24 hr. incubations at 39° C.; all the results, given in mg. ammonical N/100 ml., have been corrected for the control values).

Substrate	Experiment							
	0	1	2	3	4	5	6	7
Control	1.64	0.63	1.61	1.72	0.84	0.98	2.12	1.22
L-Arginine	25.82	1.62	23.14	25.54	24.62	23.35	28.54	30.16
L-Lysine	9.60	0.23	7.88	8.86	8.54	8.38	4.46	8.74
Delta AVA[1]	3.02	0.06	2.76	2.84	3.08	2.96	1.48	3.36
L-Aspartic Acid	12.10	1.12	12.68	11.98	10.22	10.78	10.64	9.84
DL-Tryptophan	3.06	0.29	3.50	3.68	3.36	3.42	1.48	3.64
L-Glutamic Acid	7.60	0.52	6.90	7.20	7.46	7.90	7.80	8.10
L-Phenylalanine	3.40	0.20	2.86	2.94	3.18	3.46	3.84	3.72
L-Ornithine	4.06	0.40	3.48	4.12	3.72	4.28	2.22	3.46

[1] Delta amino valeric acid.

incubation flasks and compared with a slide made of the original microbial suspensions. All the slides were gram stained. The preincubation slide revealed a variety of morphological forms of gram-positive and gram-negative microorganisms whereas the 72 hr. incubation slides revealed only gram-negative individual cocci.

Experiment 2. Pyridoxamine. In order to reduce the ammonia content of endogenous washed cell suspensions, it is necessary to wash the microorganisms several times. This procedure, however, invariably results in a considerable reduction of activity. Such a phenomenon indicated a loss of cofactors essential in the deamination of amino acids. Experiment 2 was designed to study the effect of added pyridoxamine on the magnitude of deamination of eight amino acids. Pyridoxamine was added in a quantity that resulted in a final concentration of 2.5×10^{-5} M. The results of experiment 2 failed to reveal any consistent effect of added pyridoxamine on amino acid dissimilation by washed suspensions of rumen microorganisms.

Experiment 3. Pyridoxal Phosphate. Experiment 3 was similar to experiment 2 except that the coenzyme pyridoxal phosphate was substituted for its vitamin analogue pyridoxamine. The pyridoxal phosphate was added in quantities equivalent to a final concentration of 2.5×10^{-6} M. The results of experiment 3 also were of a negative

nature, failing to reveal any significant effect of added pyridoxal phosphate on amino acid dissimilation by washed suspensions of rumen microorganisms.

Experiment 4. Magnesium ion. Since pyridoxamine or pyridoxal phosphate apparently were not the sole essential cofactor lost, experiment 4 was performed to ascertain if an exogenous source of divalent cations would increase the magnitude of ammonia production from amino acids. Magnesium ion in the form of magnesium sulfate was added to the incubation medium, giving a final concentration of magnesium ions of 3×10^{-4} M. The results of the addition of magnesium ions to washed suspensions of rumen microorganisms were negative.

Experiment 5. Potassium ion. In experiment 5 an all potassium buffer was compared with the control buffer (approximately equal amounts of potassium and sodium ions) due to the fact that cellular and extracellular potassium levels influence amino acid uptake by Ehrlich ascites tumor cells (Riggs et al., 1958). The all potassium buffer had a potassium ion concentration of 0.097 M whereas the control buffer had a potassium ion concentration of 0.066 M and a sodium ion concentration of 0.063 M. In order to maintain these cationic ratios in the control buffer, aspartic and glutamic acids were neutralized with equivalent amounts of sodium and potassium bicarbonate whereas potas-

sium carbonate alone was employed in the experimental flasks. This procedure increased the cationic strength but did not alter the molar ratios of sodium and potassium. The ammonia production in each of the two buffer systems was essentially the same, indicating that such an alteration in the extracellular potassium level had no apparent influence on amino acid dissimilations by washed cell suspensions of rumen microorganisms.

Experiment 6. Methylene blue addition. Washed cell suspensions of rumen microorganisms are highly reductive systems and the possibility existed that amino acid dissimilation rates are slow due to a failure to reoxidize the reduced forms of coenzymes I and II and flavinadeninedinucleotide. Methylene blue chloride was added to each incubation flask to give a final concentration of 0.1% methylene blue. The results were not consistent but a fifty per cent reduction in ammonia production did result from the addition of methylene blue to L-lysine, L-ornithine, DL-tryptophan and delta amino valeric acid incubation flasks.

Experiment 7. Catalase addition. One ml. of technical grade catalase solution[1] was added to the amino acid incubation in an attempt to offset the possible formation of toxic hydrogen peroxide in the dissimilation studies.

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[1]Nutritional Biochemicals Corporation, Cleveland 28, Ohio.

This catalase had a potency of 50 units per ml. which decomposed 75 times its weight of H_2O_2. The ammonia production of the catalase-added flasks and the control flasks were essentially the same, demonstrating that catalase addition to washed cell suspensions of rumen microorganisms was of no benefit in the catabolism of amino acids.

Ammonia production and amino acid disappearance as noted by paper chromatography were highly correlated in experiments 1 through 7.

Experiment 8. Metabolic Poisons. In an analagous study, an attempt was made to increase the dissimilation rate of serine and thereby produce catabolic end products that would be present in greater concentrations. An eight hour incubation period was employed with L-serine, 10 mcM/ml. Two metabolic poisons, sodium fluoride and sodium arsenate which block key steps in the glycolytic cycle (Fruton and Simmons, 1959), were used as 0.017% and 0.029 M solutions, respectively. The ammonia levels were 4.32, 5.97 and 6.58 mg. of ammonical nitrogen per 100 ml. for the endogenous, sodium arsenate and sodium fluoride incubation mediums, respectively. Such results would indicate that arsenate and fluoride in the above concentrations inhibited the endogenous metabolism of rumen microorganisms.

One ml. of 0.2% dinitrophenylhydrazine in 2N HCl had also been added to each flask in an attempt to isolate the pyruvic acid produced in serine dissimilation. The

hydrazone was not found in any of the three cases. It would appear that pyruvate is a very labile substrate in rumen microbial metabolism and as such is very difficult to isolate in the hydrazone form. Another excellent possibility would be that the dinitrophenylhydrazine was extracellular only whereas the pyruvic acid existed only intracellularly.

Experiment 9. Penicillin. The final phase of this study dealt with the effect of penicillin on amino acid dissimilation by washed cell preparations. Three International Units (I.U.) of penicillin per ml. had no effect on amino acid dissimilations, whereas a penicillin concentration of 30 I.U. per ml. markedly inhibited amino acid dissimilations, Table 8.

Intermediate Products in Amino Acid Dissimilations

The second major aspect of this study deals with the intermediary products formed in amino acid dissimilations by rumen microorganisms. This was accomplished by chromatographic and spectrophotometric examination of the amino acid fermentation mediums prior to and after incubation.

Paper chromatographic analyses of fermentation mixtures, to which L-arginine, L-ornithine and L-lysine had been added, alone and in various combinations, revealed that these three amino acids decreased in concentration as the fermentation proceeded. All three of these amino acids

Table 8. The effect of two levels of penicillin, 3 I.U. and 30 I.U./ml., on amino acid dissimilation by washed suspensions of rumen microorganisms (10 mcM./ml. of amino acid; 24 hr. incubations at 39° C.; all the results, given in mg. ammonical N/100 ml., have been corrected for the control value).

Substrate	Control	3 I.U.	30 I.U.
None	0.88	1.04	0.18
L-Arginine	25.36	27.64	0.46
L-Lysine	9.06	9.36	0.04
Delta AVA[1]	3.10	2.56	0.00
L-Aspartic Acid	10.82	10.16	0.16
DL-Tryptophan	3.12	3.18	0.00
L-Glutamic Acid	7.42	8.06	0.12
L-Phenylalanine	3.66	2.26	0.00
L-Ornithine	3.88	3.72	0.18

[1]Delta amino valeric acid.

gave rise to further ninhydrin reactive products when incubated in either rumen fluid or washed cell suspensions. One of these spots proved to be delta amino valeric acid. In the identification of delta amino valeric acid, it was noted that the new spot corresponded in Rf value to those obtained with a standard of this amino acid. Addition of known delta amino valeric acid to the unknown mixture did not result in the appearance of any new spots when tested in several solvent systems. This was true using both one- and two-dimensional paper chromatography.

The formation of delta amino valeric acid from L-lysine, L-ornithine and L-arginine was much easier to demonstrate in rumen fluid than in washed cell incubations. The appearance of this amino acid de novo was demonstrated in rumen fluid in all ten experiments employing lysine, ornithine and arginine whereas its presence in washed cell suspensions was demonstrated only in those incubations which indicated a good dissimilation of the original amino acid and after the incubation mediums were concentrated twenty fold.

An attempt was made to ascertain the metabolic products formed in the dissimilation of delta amino valeric acid. However, delta amino valeric acid dissimilation was slow in both rumen fluid and washed cell suspensions. Although Rothstein and Miller (1955) have shown that delta amino valeric acid is converted largely to glutaric acid in the intact rat, paper chromatographic analyses of post incubation samples of arginine, lysine, ornithine and delta amino valeric acid failed to reveal the presence of glutaric acid.

An experiment was performed to determine if glutaric acid would be dissimilated by rumen microorganisms. Incubations of 25 mcM./ml. of glutaric acid were carried out in rumen fluid and washed cell suspensions. No increase in volatile fatty acids was detected in this one trial, Table 9. A series of glutaric acid standards of 5, 10, 15,

Table 9. The post-incubation concentrations (mcM./ml.) and molar percentages of volatile fatty acids from glutaric acid *in vitro* studies.

Sample	Higher	M%	Valeric	M%	Butyric	M%	Propionic	M%	Acetic	M%	Total Acids
R.F. End.[1]	0.81	0.52	6.10	3.92	16.11	10.35	28.08	18.05	104.51	67.16	155.61
R.F.+G.A.#1[2]	0.81	0.55	4.95	3.37	15.65	10.66	27.28	18.59	98.07	66.82	146.76
R.F.+G.A.#2	0.58	0.40	5.18	3.60	15.65	10.87	27.28	18.94	95.30	66.19	143.99
Washed Cell End.[3]	0.35	2.74	0.23	1.80	1.38	10.79	3.40	26.59	7.43	58.09	12.10
W.C.+G.A.#1[4]	0.23	1.90	0.58	4.79	1.44	11.90	3.00	24.79	6.85	56.61	13.94
W.C.+G.A.#2	0.29	2.08	0.92	6.60	2.13	15.28	3.11	22.31	7.49	53.73	12.79
W.C. End.[5]	0.82	1.31	1.08	1.72	4.94	7.86	15.65	24.90	40.36	64.21	57.14
W.C.+G.A.#2[5]	0.64	1.00	2.28	3.58	5.51	8.66	13.37	20.99	41.88	65.77	57.88

[1]Rumen fluid endogenous (without added substrate).
[2]Rumen fluid plus 25 mcM./ml. of glutaric acid.
[3]Washed cell endogenous (without added substrate).
[4]Washed cell plus 25 mcM./ml. of glutaric acid.
[5]Five-fold concentration.

20, and 25 mcM./ml. were processed and spotted chromatographically. A comparison of the post-incubation mediums failed to reveal a dissimilation of glutaric acid by rumen microorganisms.

In addition to delta amino valeric acid, three other ninhydrin-reactive products arose from amino acid dissimilations by rumen microorganisms. Arginine was dissimilated to ornithine in all ten rumen fluid and washed cell incubations. Moreover, putrescine was found to be produced in 50 per cent of the fermentations (5 out of 10) and not to be produced in the other 50 per cent. Lysine also gave rise to cadaverine in 50 per cent of the fermentations (5 out of 10) but did not in the other 50 per cent. Putrescine and cadaverine when produced arose from the same inocula and were likewise absent in other sets of similar inocula. In addition, whenever these two amines were produced in rumen fluid incubations, they were likewise produced by washed cell suspensions from this same rumen liquor. The converse of this was also true--an absence in one medium was followed by an absence in the other medium. The inocula which produced putrescine and cadaverine were obtained from two fistulated cows whereas the inocula which failed to produce these two amines came from three different fistulated animals. These results would indicate that inocula differences do occur in cows on similar rations and are reflected in subsequent dissimilation

studies.

Ornithine, putrescine and cadaverine were identified by the same means as were used with delta amino valeric acid. The addition of known ornithine, putrescine and cadaverine to the unknown lysine and arginine dissimilation mixtures did not result in the appearance of any new spots when tested in several solvent systems. This was true using both one- and two-dimensional paper chromatography.

Figure 1 is a photograph of a rumen fluid incubation study. Reading from left to right strips 1 and 2 are DL-lysine, before and after incubation, strips 3 and 4 are L-lysine, before and after incubation, and strips 5 and 6 are DL-tryptophan, before and after incubation. Strip 7 is a post-incubation sample of arginine from a previous study. Strip 8 is a chromatogram of the rumen fluid inoculum prior to incubation.

The lysine strips demonstrate the much greater dissimilation of the L-lysine versus the DL-lysine (10 mcM./ml. in each case) and the formation of cadaverine in both cases, being more pronounced from L-lysine. Delta amino valeric acid was produced in both cases, being slightly more apparent from L-lysine; but concentrations were too low to be apparent on the chromatograms. The arginine strip shows the dissimilation of arginine to ornithine; small quantities of delta amino valeric acid are present

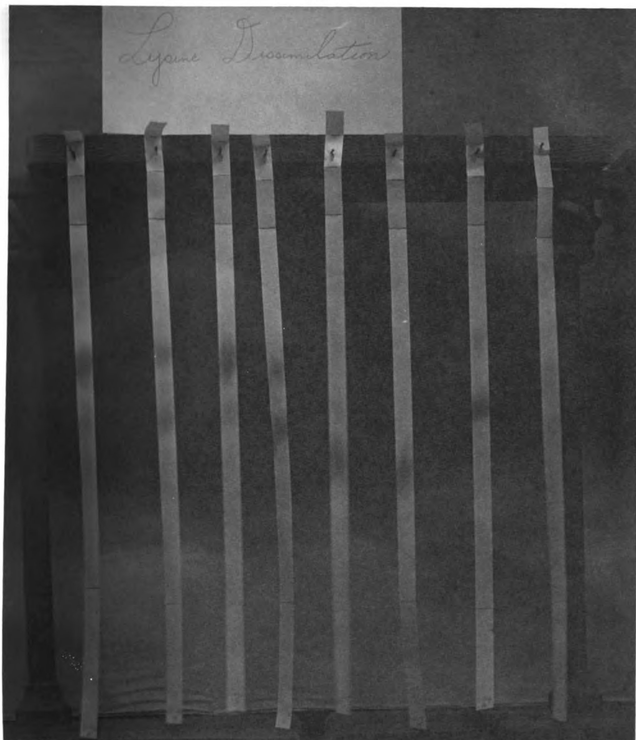


Figure 1. <u>In vitro</u> rumen fluid incubation studies with L-lysine, DL=lysine, DL-tryptophan and L-arginine.

but are not visible. The diminution in spot intensity on chromatogram 6 when compared to chromatogram 5 demonstrates a partial dissimilation of DL-tryptophan. The presence of ninhydrin-reactive spots on strips 5 and 6 establish the presence of endogenous amino acids or amines in the rumen fluid. All these endogenous compounds were dissimilated in the 24 hr. incubations to a concentration where they were no longer detectable by the methods employed. The disappearance of the endogenous ninhydrin-reactive spots is demonstrated in strip 6.

Casein hydrolysate and 18 amino acids were incubated in both rumen fluid and washed cell suspensions at pH 6.5. All the incubation mediums were tested for amines and only putrescine and cadaverine were found. Since most of the bacterial decarboxylases are formed in large quantities only when the organisms are grown in an acid medium, pH 2.2 to 5.5 depending on the organism (Gale, 1946), a single study was initiated wherein histidine, lysine, phenylalanine, tryptophan and arginine were incubated at pH 4.5, 5.5 and 6.5. Phenylalanine, histidine, tryptophan and arginine did not form amines at any of the three pH's used. Lysine did not form amines at pH 4.5 and 6.5 but a marked amount of cadaverine was produced in the rumen liquor buffered at pH 5.5. Paper chromatography established that approximately 40 to 50 per cent of the lysine had been converted to cadaverine at pH 5.5 whereas only nine per cent

of the lysine was completely deaminated (Table 10). The paper chromatographic procedure used indicated that 100 per cent of the arginine was converted to ornithine when incubated at pH 6.5 but only 80 per cent was converted at pH 5.5. No putrescine was produced at either pH. Since Sirotnak et al. (1953) reported that the presence of maltose in the incubation medium significantly increased the production of ammonia and carbon dioxide from aspartic acid, a trial was carried out in which arginine and lysine (10 mcM./ml.) were incubated with 0.2% maltose in unbuffered rumen liquor. In this study the final pH of the mediums were 5.6 for the lysine flask and 5.8 for the arginine flasks; cadaverine was produced whereas putrescine was not. These results from the three pH studies and the maltose addition study indicate that lysine decarboxylase activity in rumen fluid is much greater at a more acid pH than is ornithine decarboxylase.

Table 10 shows the per cent dissimilation, as determined by ammonia production, of each of the five amino acids at pH 4.5, 5.5 and 6.5. Ammonia production was markedly reduced at pH 4.5. This pH appeared to almost completely inhibit rumen microbial activity and also to destroy the colloidal stability of the rumen liquor inocula.

In the process of the ethyl ether extraction of post incubation samples and the subsequent evaporation of the ethyl ether into 2N HCl, it was noted that tryptophan

Table 10. Ammonia production from amino acids at three pH's in 24 hr. rumen fluid incubations (10 mcM./ml. of amino acid).

Sample	pH	Ammonia N (mg./100 ml.) Theor. Yield	Actual Yield	% Dis-similation
L-Phenyalanine	4.5	14.01	0.00	0.00
	5.5	14.01	11.02	78.65
	6.5	14.01	10.62	75.80
DL-Tryptophan	4.5	14.01	1.87	1.33
	5.5	14.01	7.96	56.81
	6.5	14.01	9.27	66.16
L-Lysine	4.5	27.99	0.34	1.22
	5.5	27.99	2.56	9.14
	6.5	27.99	6.19	21.74
L-Histidine	4.5	14.01	0.34	2.42
	5.5	14.01	2.11	15.06
	6.5	14.01	5.03	35.90
L-Arginine	4.5	56.04	1.24	2.21
	5.5	56.04	33.16	59.20
	6.5	56.04	37.44	66.80

dissimilation samples turned red in the acid solution. The red color did not appear, however, when distilled water replaced the acid solution. Several attempts were made to

isolate the intermediary product but these attempts failed due to the low concentration present and the marked solubility of this compound in the various solvent systems tested. Paper chromatograms examined under ultra violet light and sprayed with Ehrlich's reagent demonstrated the presence of two compounds, indole and skatole, in rumen fluid and indole alone in washed cell suspensions. Indole gave a carmine color with Ehrlich's reagent both immediately after spraying and 24 hours later, whereas skatole was carmine initially and turned blue 24 hours later. Indole and skatole were clearly separated and appeared as two separate spots under ultraviolet light using a solvent mixture of 10 parts isopropyl alcohol, 1 part ammonium hydroxide (28% ammonia) and 1 part water.

Two samples of rumen fluid and two samples of washed cell suspensions all of which had been incubated with DL-tryptophan were extracted with ethyl ether. The ethyl ether was evaporated off into 4 ml. of distilled water. The ultraviolet spectrum of these samples and indole and skatole standards are presented in Figures 2 and 3. Only one spectrum of the washed cell suspension and the rumen fluid sample are presented in Figures 2 and 3, respectively, since the duplicates were essentially the same.

A quantitative estimation of the amount of indole and skatole formed from the <u>in vitro</u> fermentation of tryptophan was performed using both rumen fluid and washed cell

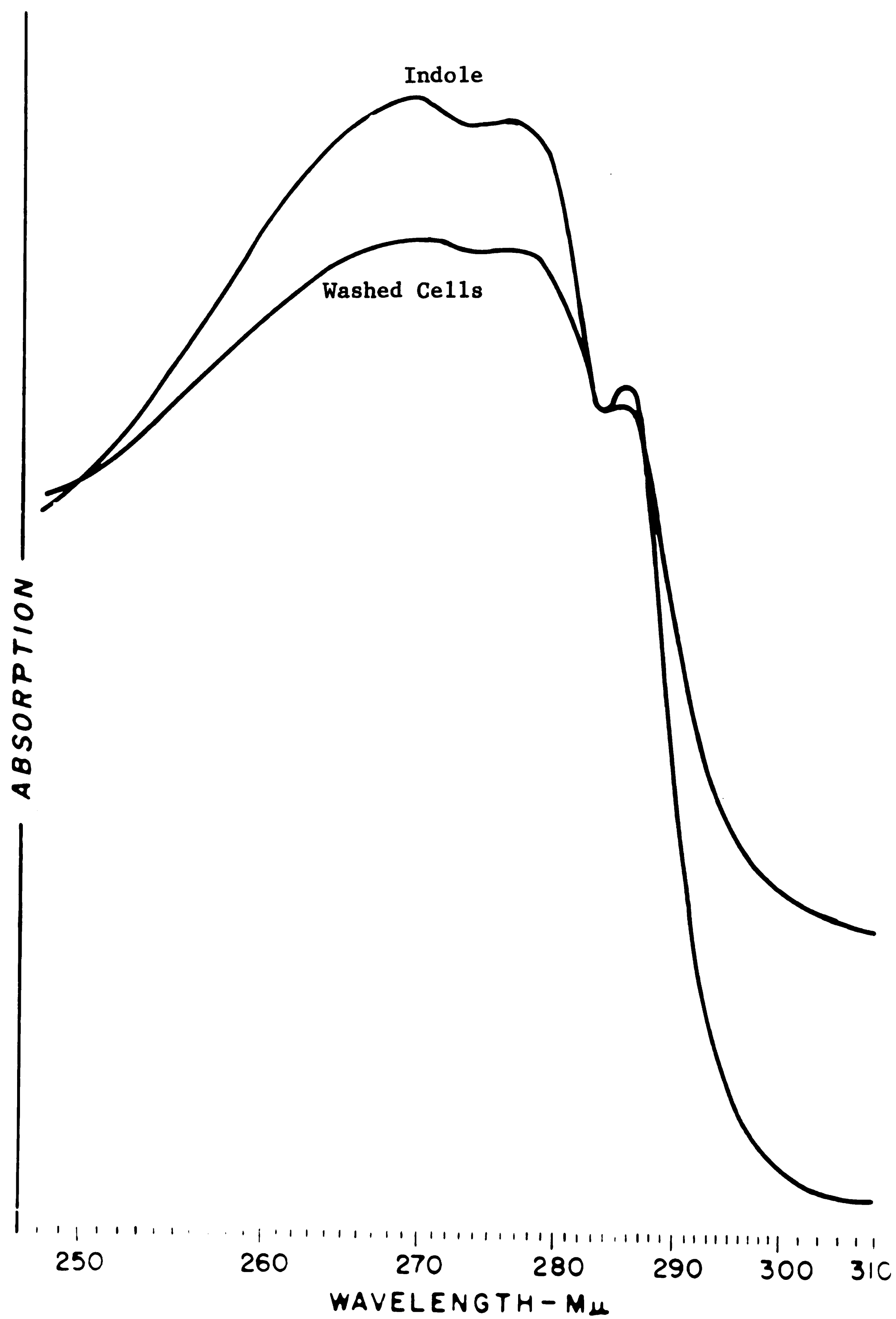


Figure 2. Ultraviolet spectra of the tryptophan fermentation product from washed cell suspensions and an indole standard.

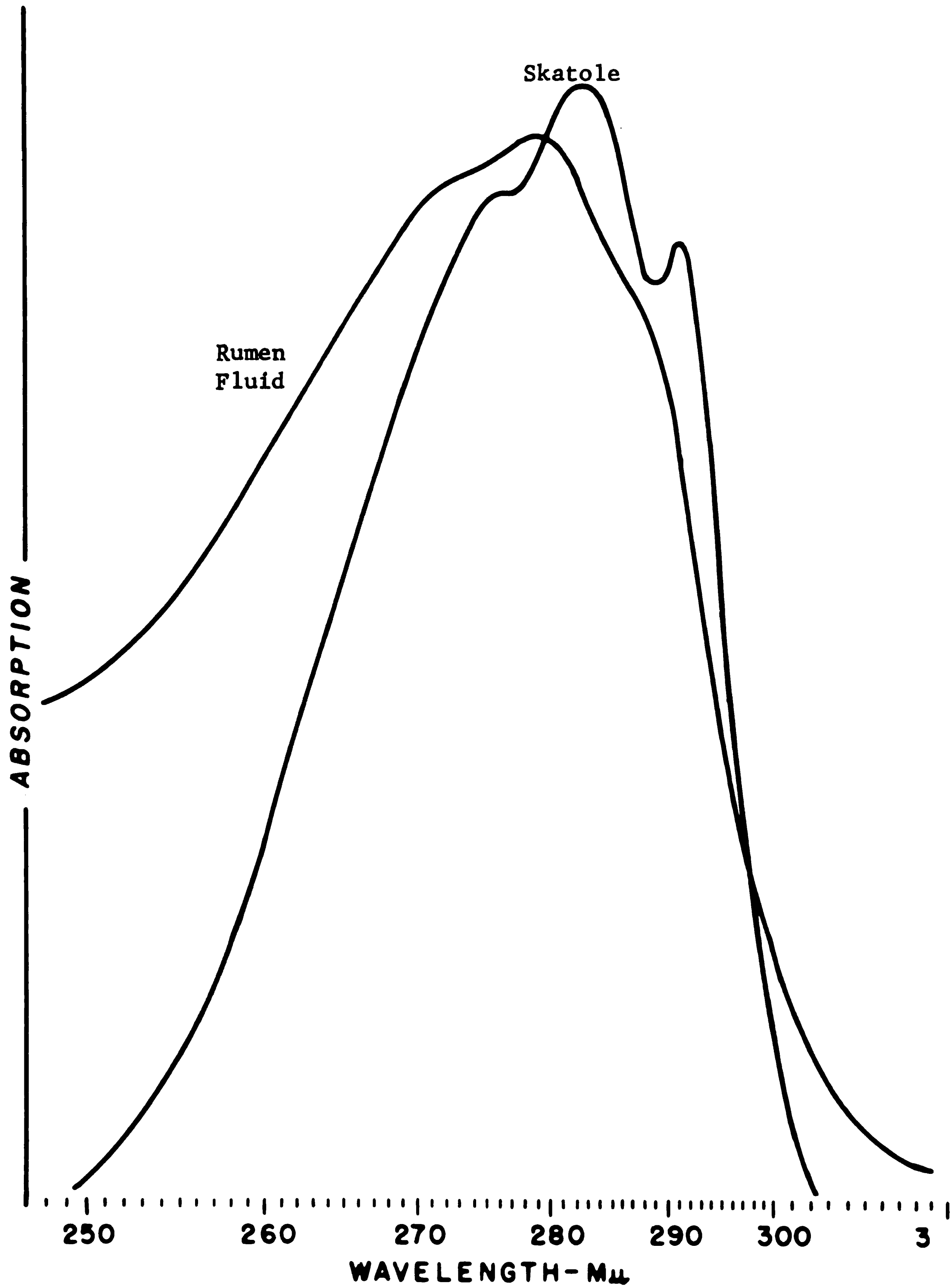


Figure 3. Ultraviolet spectra of the tryptophan fermentation product from rumen fluid and a skatole standard.

suspensions, with and without the addition of maltose. Indole and skatole were estimated by the method of Meyers (1950) and the results are shown in Table 11. The production of indole and skatole was much greater in rumen fluid than in washed cell suspensions. Indole formation could be increased in washed cell suspensions by increasing the incubation period from 24 hr. to 48 hr. Maltose addition to rumen had little effect on indole and skatole formation whereas maltose addition resulted in the production of small quantities of skatole in washed cell suspensions.

An estimation of the extent of formation of volatile fatty acids from certain amino acids was performed using rumen fluid with and without maltose. Rumen fluid was incubated with either lysine, ornithine, arginine, delta amino valeric acid or tryptophan as the sole substrate and with each amino acid plus 0.2% maltose as substrates. The volatile fatty acids formed in rumen fluid alone are presented in Table 12 and in rumen fluid plus maltose in Table 13. Results in rumen fluid alone were variable; lysine and ornithine both formed small amounts of fatty acids, whereas delta amino valeric acid, tryptophan and arginine all decreased the volatile fatty acid concentrations to levels lower than the endogenous values. Results in rumen fluid plus maltose demonstrated that DL-tryptophan, L-arginine and L-lysine were dissimilated to volatile fatty acids, largely acetic, whereas DL-lysine was not. Delta

Table 11. Indole and skatole production in rumen fluid and washed cell dissimilation studies (10 mcM./ml. of tryptophan; 24 or 48 hr. dissimilation studies).

Rumen Fluid	Incubation Time	Indole	Skatole
		mcg./ml. of indole and skatole	
Blank, zero hour	24 hr.	0.7	0.0
Endogenous	24 hr.	1.0	0.0
Tryptophan	24 hr.	12.8	6.0
Tryptophan	24 hr.	19.2	7.8
Tryptophan + maltose (0.2%)	24 hr.	17.6	6.9
Tryptophan + maltose (0.2%)	24 hr.	14.6	5.7

Washed Cells	Incubation Time	Indole	Skatole
Blank, zero hour	24 hr.	0.0	0.0
Endogenous	24 hr.	0.0	0.0
Tryptophan	24 hr.	1.0	0.0
Tryptophan	24 hr.	0.8	0.0
Tryptophan	48 hr.	1.8	0.0
Tryptophan	48 hr.	1.6	0.0
Tryptophan + maltose (0.2%)	24 hr.	0.8	0.5
Tryptophan + maltose (0.2%)	24 hr.	1.0	0.2

Table 12. The concentrations (mcM./ml.) and molar percentages of volatile fatty acids from *in vitro* studies of amino acid dissimilations in rumen fluid.

Sample	Higher	M%	Valeric	M%	Butyric	M%	Propionic	M%	Acetic	M%	Total Acids
Inoculum	0.22	0.18	2.25	1.88	18.43	15.40	21.47	17.94	73.33	64.60	119.70
Endogenous	0.45	0.36	2.36	1.38	19.56	15.56	23.72	18.87	79.58	63.62	125.67
L-lysine	0.34	0.26	2.81	2.16	20.46	15.72	23.94	18.39	82.61	63.47	130.16
L-ornithine	0.45	0.34	2.47	1.85	23.27	17.46	23.49	17.62	83.63	62.73	133.61
DAVA[1]	0.67	0.54	2.47	2.00	20.46	16.56	22.70	18.38	77.22	62.52	123.52
L-arginine	0.56	0.56	2.70	2.70	17.87	18.01	16.07	16.19	62.04	62.52	99.24
DL-tryptophan	0.22	0.18	1.69	1.38	20.46	16.71	22.48	18.36	77.56	63.36	122.41

[1]Delta amino valeric acid.

Table 13. The concentrations (mcM./ml.) and molar percentages of volatile fatty acids from *in vitro* studies of amino acid dissimilations in rumen fluid + 0.2% maltose.

Sample	Higher	M%	Valeric	M%	Butyric	M%	Propionic	M%	Acetic	M%	Total Acids
Inoculum	0.59	0.48	2.13	1.73	16.21	13.17	29.34	23.85	74.77	60.77	123.04
Endogenous	0.71	0.35	3.19	1.56	41.52	20.35	40.93	20.06	117.71	57.68	204.06
DL-Tryptophan	0.83	0.39	3.79	1.80	41.40	19.69	41.17	19.58	123.03	58.52	210.22
L-Arginine	0.59	0.28	4.14	1.97	41.88	19.90	40.46	19.22	123.39	58.63	210.46
L-Lysine	0.59	0.29	3.67	1.78	42.23	20.49	40.46	19.63	119.13	57.81	206.08
DL-Lysine	0.83	0.41	3.43	1.68	41.52	20.39	40.34	19.81	117.47	57.70	203.59
DAVA[1]	0.71	0.35	3.31	1.65	41.29	20.64	41.76	20.87	112.98	56.48	200.05

[1]Delta amino valeric acid.

amino valeric acid again lowered the total volatile fatty acid concentration to a level below that found in the endogenous fermentation. The major effect was to lower the acetic acid concentrations, both with and without maltose, with little effect on the other individual fatty acids. Since the addition of an amino acid produced only a three to five per cent increase in the total volatile acids and this was distributed into five fractions, this analysis proved to be of limited value.

An estimation of the extent of formation of volatile fatty acids from lysine, ornithine, delta amino valeric, tryptophan, aspartic acid, arginine and serine was performed using washed cell suspensions of rumen microorganisms. In order to have a sufficient acid concentration to perform the volatile fatty acid analyses, the incubation mediums were concentrated five fold. With the exception of serine, aspartic acid and arginine, the volatile fatty acid concentrations in the amino acid dissimilation flasks closely approximated the levels in the endogenous flasks. Differences between the endogenous and experimental values are presented in Table 14. Serine and aspartic acid formed primarily acetic and propionic acids, respectively, whereas arginine yielded a much more uniform mixture of volatile fatty acids.

Table 14. The concentrations (mcM./ml.) and molar percentages of volatile fatty acids from <u>in vitro</u> studies of amino acid dissimilations in washed cell suspensions. Values represent a five-fold concentration minus the endogenous values.

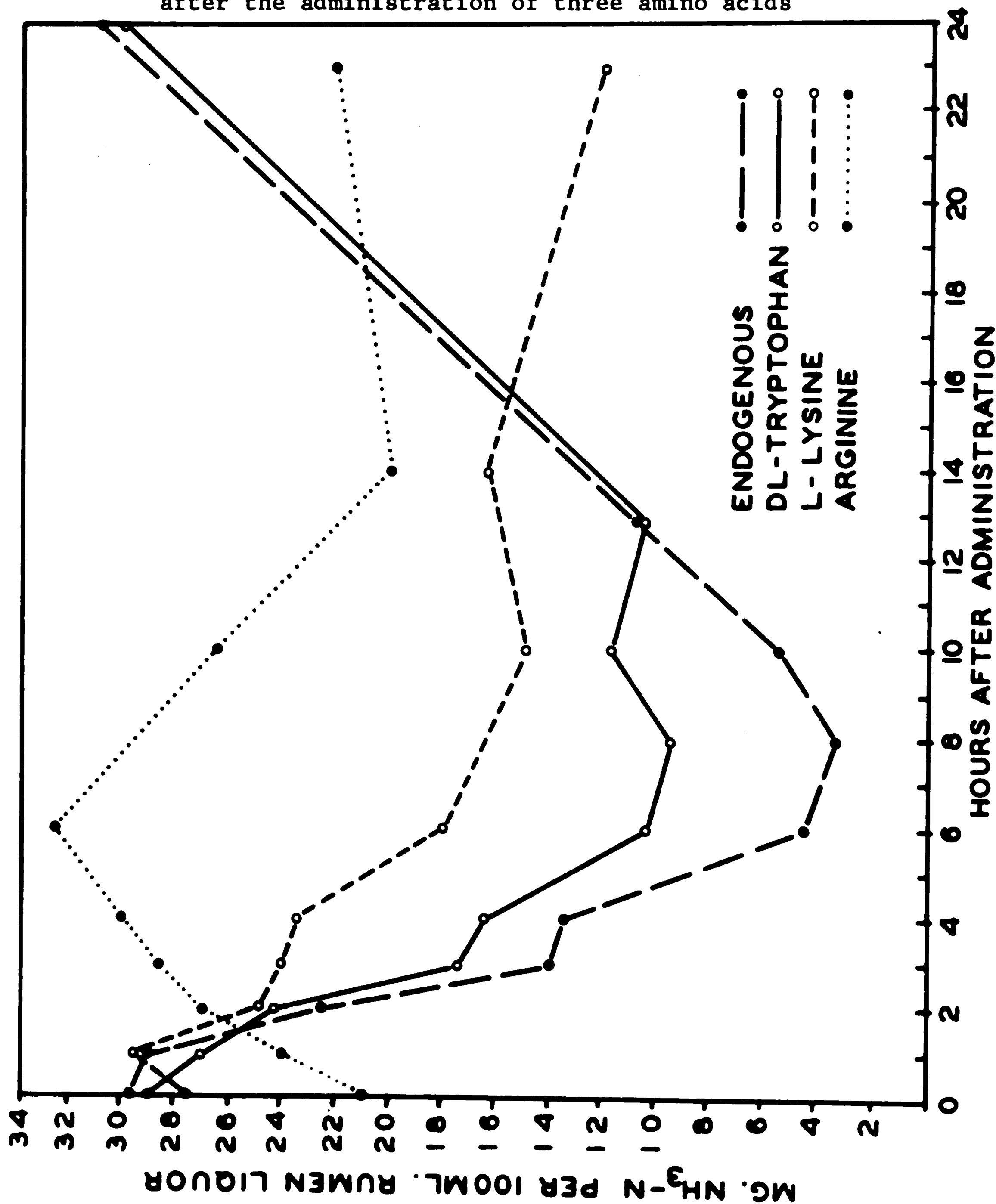
Sample	Higher	M%	Valeric	M%	Butyric	M%	Propionic	M%	Acetic	M%	Total Acids
DL-Serine	0.00	0.00	0.00	0.00	6.14	14.00	3.17	7.20	34.60	78.90	43.91
L-Aspartic Acid	0.00	0.00	0.00	0.00	4.32	8.86	33.98	69.69	10.46	21.45	48.76
L-Arginine	0.90	2.47	4.80	13.19	6.75	18.54	10.20	28.02	13.75	37.77	36.40

In Vivo Studies

The final phase of this thesis is concerned with the *in vivo* rumen microbial dissimilations of L-arginine, L-lysine and DL-tryptophan and a comparison of the extent to which the *in vitro* dissimilations were duplicated *in vivo*. A second aspect of this study was a qualitative examination of the amino acids and amines of jugular vein blood plasma after administering these three amino acids into the rumen through a fistula.

The results of these three trials are summarized in Figure 4. Arginine had the most pronounced effect upon rumen ammonia concentrations. Rumen ammonia levels continued to rise for at least the first six hours after administration of arginine but had decreased by ten hours. L-lysine had little effect upon rumen ammonia levels the first three hours after administration, but thereafter L-lysine tended to keep rumen ammonia concentrations at much higher levels than the endogenous ration. The ammonia levels after lysine administration, however, were still considerably below those from arginine supplementation. The addition of DL-tryptophan had little effect upon rumen ammonia concentration until about six hours after administration. Even then these levels were much lower than those noted for L-arginine and L-lysine. By the fourteenth hour the tryptophan supplemented and endogenous rumen levels were the same.

Figure 4. The levels of ammonia nitrogen in rumen liquor after the administration of three amino acids



The *in vivo* studies with L-arginine, L-lysine and DL-tryptophan substantiate the *in vitro* studies with these three amino acids. In both mediums, L-arginine markedly raised the ammonia concentration in the rumen fluid over the endogenous levels. L-lysine and DL-tryptophan likewise were dissimilated at similar rates *in vivo* and *in vitro*. L-lysine was one of the intermediate amino acids in dissimilation rate *in vivo* whereas DL-tryptophan was only slowly dissimilated *in vivo*. These results were similar to those obtained *in vitro*; each amino acid falling, *in vivo* and *in vitro*, into one of three general classes with regard to their magnitude of dissimilation.

At the same time samples were being prepared for ammonia analyses, rumen liquor samples were likewise prepared for paper chromatographic analyses to indicate and identify the degradation products of the amino acids. Arginine (arg.) glutamic acid (g.a.), alanine (al.) and delta amino valeric acid (d.a.v.a.) were present in the rumen liquor immediately after the addition of L-arginine to the rumen. The ruminal concentrations of these amino acids changed as a function of time in the following manner:

1 hr. arg. decreased; ornithine (orn.) was formed; g.a., al., d.a.v.a. increased.
2 hr. arg. decreased; g.a., al., orn., d.a.v.a. increased.
3, 4, 6 hr. arg., al., g.a. decreased; orn., d.a.v.a. increased.
10 hr. arg., al., g.a., orn. decreased; d.a.v.a. increased.
14, 23 hr. only trace amounts of all five amino acids present.

Equal amounts of arginine and ornithine were present on the chromatogram of the sample taken at the sixth hour.

Lysine (ly.), glutamic acid (g.a.), alanine (al.) and delta amino valeric acid (d.a.v.a.) were present in the rumen liquor immediately after the addition of L-lysine to the rumen. The ruminal concentration of these amino acids changed as a function of time in the following manner:

1 hr. ly. decreased; al., g.a., d.a.v.a. increased.
2 hr. ly. decreased; d.a.v.a. increased; al., g.a. remained the same.
3 hr. ly., al. decreased; d.a.v.a. increased; g.a. remained the same.
4, 6, 10 hr. ly., al. decreased; d.a.v.a., g.a. remained the same.
14, 23 hr. ly., al. trace; d.a.v.a., g.a. remained the same.

The lysine concentration decreased to about 50 to 60 per cent of the original level on the chromatogram of the sample taken at the sixth hour.

Alanine and glutamic acid were present in low concentrations in both studies; the largest increases being one hour after lysine administration and two hours after arginine addition. Cadaverine and putrescine were not present in detectable concentrations in either of these *in vivo* dissimilation studies. The chromatograms of the rumen fluid from *in vivo* dissimilations of L-arginine and L-lysine are found in Figures 5 and 6, respectively.

The chromatograms of the *in vivo* dissimilation of DL-tryptophan failed to reveal the formation of new ninhydrin-reactive products. Glutamic acid and alanine were

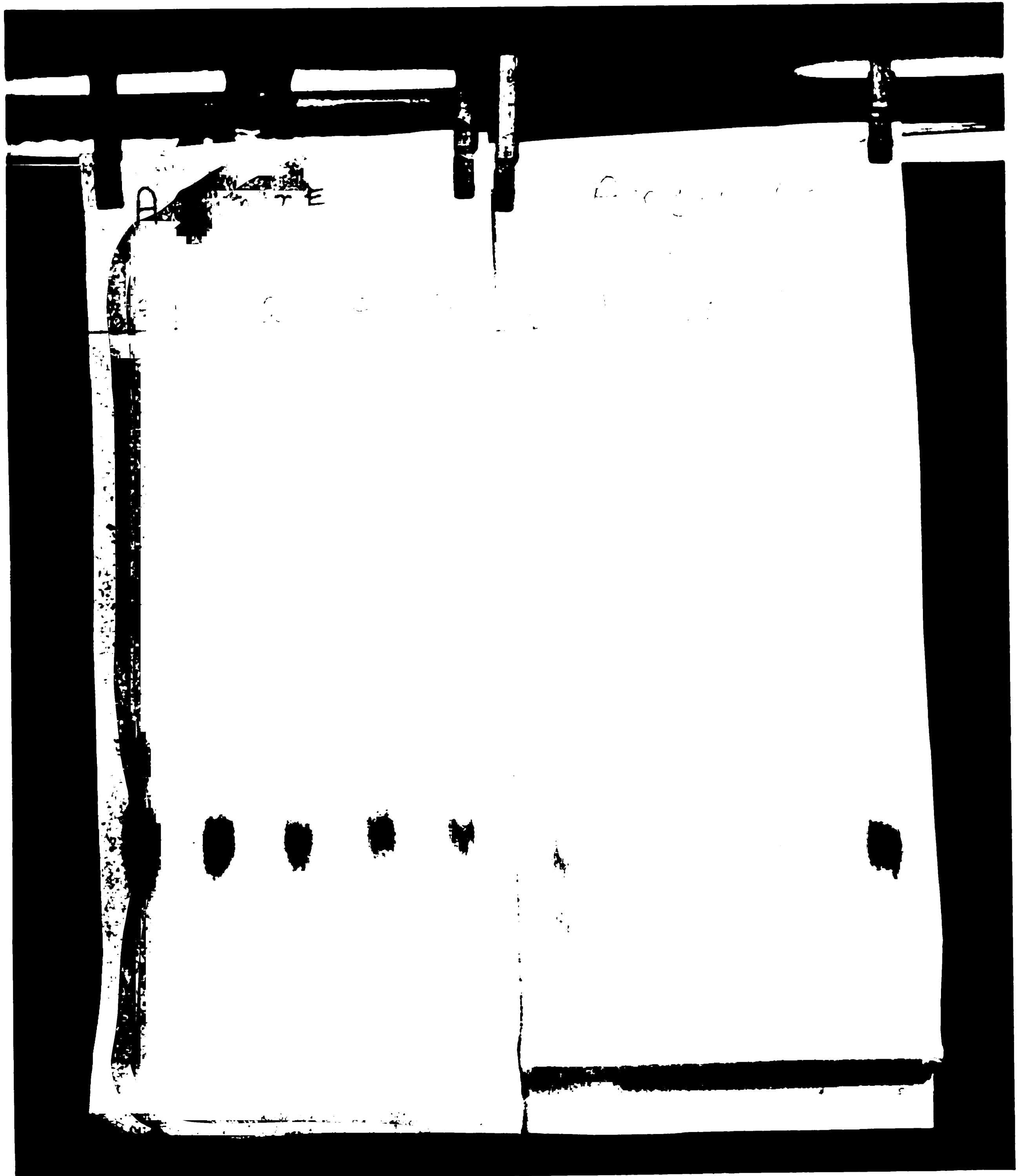


Figure 5. Paper chromatograms of rumen fluid from *in vivo* studies with L-arginine.

Figure 6. Paper chromatograms of rumen fluid from in vivo studies with L-lysine.

again present but decreased in concentration more rapidly than with the lysine and arginine studies. Tryptophan concentrations decreased only slightly during the first three hours and then a gradual decrease occurred until by the tenth hour the tryptophan concentration was markedly reduced. The chromatograms of the samples taken at 14 and 24 hours showed only small amounts of tryptophan. Indole and skatole determinations were made on the samples that had been preserved for the ammonia analyses. These results are found in Table 15. The indole concentration did not

Table 15. Indole and skatole production from in vivo dissimilation studies with DL-tryptophan. mcg./ml. of indole and skatole.

	Endogenous		DL-Tryptophan	
Time	Indole	Skatole	Indole	Skatole
0 hr.	0.4	0	0.3	0.0
1 hr.	1.0	0	0.9	0.0
2 hr.	0.8	0	1.6	0.0
3 hr.	1.2	0	1.8	0.0
4 hr.	1.2	0	2.2	0.0
6 hr.	0.9	0	3.6	0.0
8 hr.	0.9	0	4.8	3.6
10 hr.	0.4	0	4.2	2.0
13 hr.	0.2	0	1.5	0.0
24 hr.	0.3	0	0.4	0.0

increase above the control until two hours after tryptophan administration; then it showed a steady increase up to 4.8 mcg./ml. eight hours after the administration of DL-tryptophan. This maximum was followed by a steady decline in indole concentration. Skatole was detected only in the eight and ten hour samples which correspond to the times of highest indole concentrations.

The individual amino acids in blood plasma from the jugular vein were identified on two-dimensional paper chromatograms by their Rf values. Other methods used were the color reactions of each amino acid when sprayed with the cupric nitrate-ninhydrin reagent of Moffat and Lytle (1959), certain individual tests--proline (isatin), arginine (Sakaguichi) and citrulline (Ehrlich's) and comparison with the chromatograms of bovine plasma amino acids by Coulson *et al*. (1959). These chromatograms were similar to those of Walker (1952) and Coulson *et al*. (1959) and failed to reveal the presence of tryptophan in the plasma. Gordon (1949), however, has reported the presence of tryptophan in bovine serum. Since the plasma amino acid samples in this treatise were prepared by a method similar to that of Walker (1952) and Coulson *et al*. (1959), it is possible that tryptophan was lost during the extraction. Tryptophan was also absent from the plasma amino acid chromatograms of the tryptophan-supplemented animal.

The qualitative estimation of amino acids in blood plasma after intraruminal administration of L-lysine, L-arginine and DL-tryptophan are summarized in Tables 16, 17 and 18, respectively. The lysine and tryptophan studies were on one animal whereas the arginine study was on another. These results are based upon the visual qualitative examination of paper chromatograms of plasma amino acids prepared from jugular blood prior to the administration of the amino acid and one, two and four hours following the amino acid addition to the rumen. Previous studies in this laboratory, involving visual comparisons of paper chromatograms, had indicated little effect on the endogenous levels of the amino acids from the blood plasma of the jugular vein through the first four hours following the first two hours after feeding of the experimental ration.

These results were difficult to interpret due to the limited number of analyses and the poor sensitivity of the analytical technique. The two most striking observations, however, are the lack of effect of tryptophan supplementation on other plasma amino acids and the marked increase in lysine concentration of venous plasma at four hours after the administration of L-lysine to the rumen. Both L-lysine and L-arginine supplementation increased the concentration of several plasma amino acids within one hour following the additions of each amino acid to the rumen.

Table 16. The relative effects on jugular plasma amino acid concentrations of L-lysine addition to the rumen.

Amino Acid	Relative Intensity	Intensity relative to the 0 hr. sample at		
		1 hr.	2 hr.	4 hr.
Aspartic Acid	Very Weak	-[1]	-	-
Glutamic Acid	Strong	+[3]	S[2]	S
Serine	Strong	+	S	S
Glycine	Strong	+	S	S
Threonine	Weak	++[4]	+	+
Citrulline	Moderate	+	S	S
Glutamine	Moderate	+	S	S
Alanine	Very Strong	+	+	+
Tyrosine	Weak	+	+	+
Valine	Strong	++	+	+
Methionine	Moderate	++	+	+
Leucine	Modèrate	++	+	+
Phenylalanine	Moderate	++	+	+
Proline	Weak	-	-	-
Unknown	Weak	S	+	+
Histidine	Weak	-	-	-
Lysine	Moderate	S	-	++
Arginine	Moderate	S	-	-
AABA[5]	Weak	S	S	-

[1] - = less than.

[2] S = equal to.

[3] + = more than.

[4] ++ = more than +.

[5] Alpha amino butyric acid.

Table 17. The relative effects on jugular plasma amino acid concentrations of L-arginine addition to the rumen.

Amino Acid	Relative Intensity	Intensity relative to the 0 hr. sample at		
		1 hr.	2 hr.	4 hr.
Aspartic Acid	Very Weak	S[2]	S	S
Glutamic Acid	Strong	+[3]	S	S
Serine	Strong	+	+	S
Glycine	Strong	+	+	+
Threonine	Weak	+	S	S
Citrulline	Moderate	+	+	+
Glutamine	Moderate	+	+	+
Alanine	Very Strong	+	S	S
Tyrosine	Weak	S	S	S
Valine	Strong	+	+	+
Methionine	Moderate	+	S	S
Leucine	Moderate	+	S	S
Phenylalanine	Moderate	S	-[1]	S
Proline	Weak	S	S	S
Unknown	Weak	S	S	S
Histidine	Weak	S	S	S
Lysine	Moderate	+	+	+
Arginine	Moderate	+	+	+
AABA[4]	Weak	S	S	S

[1] - = less than.

[2] S = equal to.

[3] + = more than.

[4] Alpha amino butyric acid.

Table 18. The relative effects on jugular plasma amino acid concentrations of DL-tryptophan addition to the rumen.

Amino Acid	Relative Intensity	Intensity relative to the 0 hr. sample at 1 hr.	2 hr.	4 hr.
Aspartic Acid	Very Weak	S[2]	-[1]	-
Glutamic Acid	Strong	S	S	S
Serine	Strong	S	S	S
Glycine	Strong	S	S	S
Threonine	Weak	S	S	S
Citrulline	Moderate	S	S	S
Glutamine	Moderate	S	S	S
Alanine	Very Strong	S	S	S
Tyrosine	Weak	S	S	S
Valine	Strong	S	S	S
Methionine	Moderate	S	S	S
Leucine	Moderate	S	S	S
Phenylalanine	Moderate	S	S	S
Proline	Weak	S	S	-
Unknown	Weak	S	S	S
Histidine	Weak	S	S	S
Lysine	Moderate	S	S	S
Arginine	Moderate	S	S	S
AABA[3]	Weak	S	S	S

[1] - = less than.

[2] S = equal to.

[3] Alpha amino butyric acid.

Plasma aspartic acid, proline, histidine and alpha amino butyric acid, however, failed to show a detectable increase. The addition of L-arginine to the rumen resulted in only small increases in arginine on the chromatograms.

Venous serum levels of indole and skatole were less than 0.25 mcg. per ml. at zero, one, two and four hours following the addition of DL-tryptophan to the rumen. These samples, however, were taken prior to the maximum concentrations of indole and skatole in the rumen.

DISCUSSION

Since ammonia is formed in the latter stages of protein catabolism and is the prime nitrogen source in protein anabolism, it is necessary to understand the conditions under which ammonia is released from amino acids. This study was designed for such a purpose.

The experiments *in vivo* using dry cows fitted with permanent rumen fistulas and *in vitro* using rumen liquor and washed suspensions of rumen microorganisms have shown that amino acids are attacked individually by rumen microorganisms. Since the rate of attack upon single amino acids was considerably more rapid and extensive *in vivo* and in rumen liquor than when washed cells were used, it appeared that the method of preparation of the suspension somehow inactivated the enzymes responsible for the deamination. A second possibility for decreased deamination could have been a selection of the non-deaminating population in the washed cell preparations or a lysis or death of the deaminating microorganisms. The first supposition appeared more likely and was the one investigated. However, the individual usage of 48 hr. enriched cultures, pyridoxamine, pyridoxal phosphate, magnesium ion, all potassium buffer, methylene blue or catalase failed to significantly promote ammonia production over the control values and were still low as compared to rumen liquor and

in vivo ammonia production. Lewis (1955) obtained increases in the activity of washed suspensions of rumen microorganisms by using greater care in the maintenance of anaerobiosis, by the addition to the medium of a portion of ammonia-free, Seitz-filtered rumen contents and by the use of a thicker suspension with phosphate buffer at pH 7 containing 0.02% (w/v) glutathione. However, these stimulations were slight and conditions must have differed significantly from those present in rumen liquor. Increasing the number of amino acids to three or four in the suspensions failed to produce greater ammonia levels as might be expected if a Stickland type of reaction predominated. An increased rate of production was obtained, however, in one study in which alanine and proline were incubated together.

The *in vitro* studies demonstrated that amino acids may be divided into three groups with regards to their relative rates of dissimilation. Arginine, aspartic acid, serine, threonine and cysteine were attacked most completely, followed by glutamic acid, phenylalanine, lysine and cystine forming an intermediate group and a third group in which deamination was much less pronounced was tryptophan, delta-amino valeric acid, methionine, alanine, valine, isoleucine, leucine, ornithine, histidine, glycine, proline and hydroxyproline. These findings agree qualitatively with the results of Sirotnak *et al.* (1953) and Lewis (1955) with a few exceptions, notably arginine,

lysine and tryptophan. Both of these authors reported that washed cell suspensions deaminate aspartic acid at a much greater rate than arginine whereas rumen liquor incubated for eight hours in the present investigation demonstrated that arginine contributed approximately five times as much total ammonical nitrogen as did aspartic acid. A marked increase in rumen ammonia levels was also noted when arginine was employed in the in vivo studies. It must be remembered, however, that the in vivo and in vitro rumen fluid studies were not limited to the rumen bacteria but also included the rumen protozoa.

Since Barrentine et al. (1957) and Emery et al. (1958) obtained practical control of bloat with penicillin, a trial was designed to study the effects of penicillin on amino acid dissimilations. Three I. U. of penicillin per ml., the approximate ruminal levels in the study by Emery et al. (1958), had no apparent effect on amino acid catabolism whereas 30 I. U. per ml. markedly inhibited amino acid dissimilation.

The experiment with serine would indicate that specific materials interfering with endogenous metabolism may be useful tools in amino acid dissimilation studies since the addition of arsenate and fluoride addition to the medium increased ammonia production over the control values in eight hour washed cell incubations.

Since delta-amino valeric acid has been shown to be a product from the dissimilation of arginine, lysine, ornithine and proline, it would appear that this amino acid may be an important intermediate in amino acid metabolism by rumen microorganisms. Furthermore, delta-amino valeric acid was found to be present in six of eleven samples of rumen fluid taken two to four hours after feeding. Delta-amino valeric acid arose in arginine, lysine and ornithine fermentations in the absence of any other added amino acid whereas proline appeared to give rise to this amino acid only in the presence of added alanine. Dehority _et al._ (1958), however, reported that proline may be transformed into delta-amino valeric acid in the absence of another amino acid. The fact that this amino acid may be formed in the absence of other added amino acid does not exclude its formation via the Stickland reaction. Hydrogen donators may be formed in the _in vitro_ and _in vivo_ studies in the course of carbohydrate metabolism, from amino acid synthesis, or by the autolysis of the rumen microorganisms themselves.

Both ornithine and arginine were reported by Woods (1936) to be good hydrogen acceptors in amino acid fermentations by _Cl. sporogenes_. Arginine did not appear to act by first being converted to ornithine in the studies by Woods due to the fact that ammonia levels were higher in the reaction flasks containing pairs of amino acids than

could be accounted for as an ornithine reduction. The urea formed in such a reaction was not degraded by _Cl. sporogenes_. In the studies reported here delta-amino valeric acid accumulation closely approximated the formation and disappearance of ornithine. Such findings would indicate that at least part of the delta-amino valeric acid formed in arginine dissimilation studies arose from ornithine. The metabolic pathways by which lysine could yield delta-amino valeric acid are few. Most likely, it appears, is the conversion of lysine to an alpha-keto-delta-amino derivative, which through oxidative decarboxylation would yield delta-aminovalerate.

The fate of delta-amino valeric acid in the ruminant is still unknown. One study by Dehority _et al._ (1958), employing uniformly labeled C^{14} proline, found that the major portion of the non-amino acid activity was present in the volatile fatty acids, particularly the C_5 fraction. By increasing the washed cell incubation time from 24 hr. to 30 hr., a decrease was found in the C_5 fraction with a subsequent increase in the C_3 fraction. An analysis of the C_5 fraction revealed valeric acid and an unknown component. The concentration of this unknown component was five times greater at 30 hr. than at 24 hr. When this isolated unknown material was made alkaline, it could be extracted into ether, indicating its non-fatty acid nature. An organism belonging to the genus

Clostridium has recently been isolated from sewage by Hardman and Stadtman (1960). The over-all reaction catalyzed by this organism was a coupled oxidation reduction process in which two moles of delta-amino valerate were converted to two moles of ammonia and one mole each of valeric, propionic and acetic acids. An intermediate in this dissimilation appeared to be 3 keto-valeric acid and may be the unknown component reported by Dehority *et al.* (1958).

There is very limited information in the literature on the production, absorption and excretion of indole in the normal ruminant. Spisni and Cappa (1954) reported that indole was present in rumen contents of 15 cattle just after slaughter; the amount ranged from 0.09 to 3 mg. per liter. This figure agrees with the levels reported in this study. Indole formation in the rumen has not previously been demonstrated to be due to bacterial action on tryptophan, though it has in monogastric animals (Peterson and Strong, 1953). The high levels of indole formed from the *in vitro* and *in vivo* fermentation of tryptophan very strongly suggest this possibility. Indole production has been demonstrated in pure cultures of rumen bacteria (Gutierrez, 1953; Blackburn and Hobson, 1960a). The mechanism of the over-all reaction of indole formation was established by Woods *et al.* (1947). Employing partially purified tryptophanase preparations from extracts of

Escherichia coli, it was demonstrated that the reaction yielded indole, pyruvic acid and ammonia in approximately equimolar ratios, and that there was no uptake of oxygen.

When a goat was changed from an alfalfa to a _Lepidium_ diet, the indole in the blood rose from zero to 1.0 p.p.m. and this high level at the time of milking caused a high concentration in the milk fat (Conochie, 1953). Conochie (1953) also reported that when indole was given by mouth to a goat three-fourths was excreted in the urine as the indoxyl etheral sulfate. Indoxyl substances were estimated in the urine of 78 cattle by Spisni and Cappa (1954); the amount excreted ranged rom 0.01 to 0.3 g. per 100 ml. urine. When the proportion of grasses in the hay was high, the amount of indoxyl substances in the urine was relatively low, and it increased when the proportion of leguminous plants was high. One can conclude that the higher indole levels on leguminous rations were due to the higher tryptophan content of these plants and/or the greater availability of this tryptophan to the rumen microorganisms.

The results of this study indicated that, in some instances, cadaverine and putrescine may be produced by rumen microorganisms. Putrescine has been reported in a few instances in the rumen liquor of goats and sheep fed on ladino clover or pasture (Shinozaki, 1957). The pharmacology of these two amines has been reviewed by Guggenheim

(1940). A short resume of his findings will be discussed. The occurrence of a second amino group reduces extensively the pharmacological action of alkylamines. Toxic oral dosages are 1.6 g./kg. of putrescine in rabbits and more than 1.7 g./kg. of cadaverine in the dog whereas subcutaneous dosages of either amine of 0.1 g./kg. are toxic to rabbits. The chief site of action of a toxic dose of diamines is the central nervous system and the symptoms are motor paralysis, convulsions, dyspnoic breathing, retardation and arrhythmia of the pulse, and a lowering of the blood pressure and temperature.

Rather convincing evidence now exists which shows that the pattern of amino acids in the diet markedly influences the level of some free amino acids in the blood. In poultry, Charkey _et al._ (1953) and Almquist (1954) observed good correlations between amino acid levels in chick blood and composition of dietary protein. Denton and Elvejehm (1954a, b) reported that the portal and radial vein concentrations of individual essential amino acids in dogs were rapidly increased in proportion to the levels supplied by the test proteins, casein and beef. In the case of the imbalanced protein, zein, which lacks lysine and tryptophan, lysine levels were depressed whereas tryptophan concentrations were well maintained on the zein diet.

The results of the *in vivo* investigations definitely show that individual amino acids differ in their rates of deamination in the rumen. Each of the three amino acids studied--L-arginine, L-lysine and DL-tryptophan--however, had a different effect on rumen ammonia levels. Although the plasma amino acids from the jugular vein were studied only qualitatively, certain trends were notable. The more readily dissimilated amino acids, arginine and lysine, had a positive effect on plasma amino acids whereas tryptophan had very little effect through the first four hours following the amino acid administration. Furthermore, this effect was evident in several of the plasma amino acids, including the essential amino acids; it was no more marked in the amino acid administered except in the lysine concentration of the plasma amino acids taken four hours after lysine administration. The latter phenomenon may have been due to intestinal absorption of the lysine. Although ornithine was present in readily detectable amounts in rumen liquor as early as one hour following arginine administration to the rumen, ornithine was absent on the chromatograms of the blood plasma taken one, two and four hours after the administration of arginine.

These results would indicate that the amino acid administered was either not being absorbed from the rumen, or if absorbed, was being modified before it reached the peripheral circulation. Both Annison (1956) and Tsuda

(1956b) have reported evidence indicating amino acids are not absorbed from the rumen in appreciable quantities. If the blood samples had been taken at longer intervals after feeding, the effects of the intestinal absorption of these amino acids may have been evident. The relative rates of disappearance of these amino acids from the rumen indicated that the half time of ruminal disappearance of amino acid supplements to rations would be in the order of six to eight hours.

SUMMARY

The amino acids serine, aspartic acid, glutamic acid, arginine, lysine, cysteine, cystine, threonine and phenylalanine were readily dissimilated when added to incubating rumen fluid or washed suspensions of rumen microorganisms. Tryptophan, histidine, methionine, ornithine, valine, alanine, leucine, isoleucine, delta-amino valeric acid, glycine, hydroxyproline and proline were dissimilated at lesser rates. The dissimilation rates were more rapid and complete in rumen fluid studies than in washed cell suspension. Three or four amino acids incubated together differed from the summation of the ammonia formed from each amino acid only in the cases where proline and alanine were incubated together. The individual usage of 48 hour enriched cultures, pyridoxamine, pyridoxal phosphate, magnesium ions, all potassium buffers, methylene blue or catalase in washed cell incubations failed significantly to promote ammonia levels over the control values and were still lows as compared to rumen liquor and <u>in vivo</u> ammonia production. Ammonia production and amino acid disappearance, as noted by paper chromatography, were closely correlated.

The D- and L- forms of tryptophan were both dissimilated. Tryptophan yielded indole and skatole. Arginine yielded ornithine, putrescine and delta-amino valeric acid.

Lysine yielded cadaverine and delta-amino valeric acid. Penicillin at 3 I.U./ml. did not inhibit any of the dissimilations whereas 30 I.U. caused a marked inhibition. Tests for amine production from casein hydrolyzate and individual amino acids at pH 4.5, 5.5 and 6.5 were negative, except for cadaverine and putrescine. Arginine produced the highest levels of ammonia in eight hour rumen liquor incubations. The presence of arsenate or fluoride increased ammonia production from serine over the control value. Glutaric acid was not dissimilated _in vitro_ by rumen microorganisms.

Another phase of this study was concerned with the _in vivo_ rumen microbial dissimilation of L-arginine, L-lysine and DL-tryptophan and a comparison of the extent to which the _in vitro_ dissimilations were duplicated _in vivo_. The _in vivo_ dissimilations were in good agreement with the _in vitro_ studies. Arginine and lysine both produced delta-amino valeric acid. Arginine also yielded ornithine. Indole and skatole were formed from tryptophan. Rumen ammonia levels _in vivo_ paralleled what would have been expected from the _in vitro_ studies. The administration of readily dissimilatable amino acids to the rumen had a generally positive effect on the amino acids from jugular blood drawn the first four hours following amino acid administration rather than specifically raising the level of the amino acid administered.

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