STUDIES ON THE AMINO ACID COMPOSITION OF A MICROBIAL FRACTION OF BOVINE RUMEN INGESTA

by

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AN ABSTRACT

Submitted to the School of Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

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The use of urea as a source of non-protein nitrogen in ruminant nutrition has received concentrated attention during the last decade. The question concerning the value of such a compound has been approached in various ways, and is found to be a complex problem. The investigation here recorded is concerned primarily with the amino acid composition of the microbial protein material synthesized from a ration supplemented with urea as against a ration supplemented with a natural protein nitrogen source.

Microbiological assay determinations for the ten essential amino acids were carried out on the liquid ingesta and the isolated protein sediment. Synthesis was accomplished using the in vitro technique.

Since the data obtained indicated no sizable increase in the amount of amino acids as a result of synthesis, comparisons between the two supplements were made on the basis of an amino acid ratio. The author found the quality of the microbial fraction to be of a relatively constant nature. When these ratios were compared with the ratios of some purified proteins and natural feedstuffs, the quality appeared to be fair. Although urea failed to show any superiority over the natural protein along the line of synthesizing capacity, it promoted synthesis equally as well.

The data observed led to the conclusion that the microbial fraction of rumen ingesta is of a constant compo-

sition and provides an adequate amount of building materials to the ruminant. It was also concluded that urea affords a good source of nitrogen for the flora of the rumen, with which they are capable of synthesizing a portion of the protein necessary for the general health and welfare of the animal. The use of urea and such related compounds as nitrogen sources can prove to be very beneficial and economical to the farmers of today and those of the future.

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TABLE OF CONTENTS

PURPOSE OF STUDY

The review of literature which is to follow indicates the great scope of the problem of ruminant nutrition as regards the use of N.P.N. sources as supplements to dietary nitrogen. The greatest quantity of experimental work has been devoted to the study of urea as a partial replacement for ration protein nitrogen. The main interest along this line has concerned an estimation of the amount of microbial protein synthesis occurring and the actual value of urea supplementation as concerns growth, milk production, etc. Few investigations have been made regarding amino acid utilization by the microorganisms involved and the amino acid composition of the mixed rumen contents. One investigator isolated the synthesized protein as a sediment. However, little work has been conducted on the amino acid composition of this synthesized protein from the standpoint of the relative increase of the essential amino acids and the comparison of the protein formed from an N.P.N. source and that synthesized from a natural ration. Therefore, the problem concerned in the following experimental work involved an *in vitro* study of protein synthesis from a soybean-oil meal ration and a urea-supplemented ration with subsequent amino acid analysis of the protein formed. Analysis was made on both the liquid and the dried sediment prior to and following the incubation

period and a comparison made between the two methods. In the first series of trials the liquor was supplemented with urea, a readily available carbohydrate, and yeast extract. In the second series, conducted on the isolated sediment, the additives were urea and glucose with cellulose serving as the substrate.

The author shall attempt to describe the composition of the microbial protein along with an estimation of the increase (or decrease) of each essential amino acid and subsequently estimate the value of the protein synthesized from a urea-supplemented ration as compared with that formed from a natural ration. A comparison will also be made with some feedstuffs, bacterial cells, and purified proteins to demonstrate the relative nutritive value of the synthesized protein.

INTRODUCTION

The complex study of living organisms revolves about many types of relationships which generally involve what is termed the host and the parasite. These relationships may be beneficial or harmful, depending upon the prevailing conditions. The relationship in which both the host and parasite derive benefit is termed symbiosis, and each unit concerned is necessary for the natural continued existence of the associated unit.

The symbiotic association of primary rank in the relative importance to man would undoubtedly be that existing between the ruminant and the associated microflora and microfauna of its alimentary tract. The ruminant is a polygastric mammal, i.e. the digestive tract is composed of four stomachs, each with its particular digestive process. The first of these stomachs is a large sack, found at the lower part of the esophagus, and is termed the rumen. Large quantities of ingesta may be retained in this sacculation for various periods of time. In the case of the bovine, food may be held for some 24 hours. The purpose of food retention is to facilitate pre-digestion of bulk ingesta so that it can ultimately be absorbed from the alimentary tract and utilized by the animal. Any remaining bulk material is returned to the mouth for further mastication and the process repeated. Hence,

it can be seen that the ruminant is able to utilize certain types of energy-containing compounds to a much greater degree than the non-ruminating herbivore. The microorganisms contained in the rumen are the primary agents in this pre-digestion process and it is of interest to examine briefly the conditions which prevail and contribute to the continual proliferation of these microorganisms and, also, to show a general view of the chemistry involved in rumen digestion.

The temperature in the rumen is approximately 39° C and the normal reaction is just below pH 7.0. Since a large proportion of the ingesta is carbohydrate material the acids produced by bacterial action predominate over the alkaline substances formed during fermentation and the reaction tends to become extremely acid in nature. However, this acid condition is neutralized during fermentation by the saliva which has a reaction approximately pH 8.2. The environment is controlled throughout by the constant mixing action caused by the muscular action of the rumen walls and also the passage of gas, formed during fermentation, to the upper part of the rumen. Hence, no stagnation is allowed to occur in any part of the contents. All these factors aid the maintenance and proliferation of a particular type of microbial population. The bacteria and protozoa here encountered are representative of a population the density of which is never met *in vitro* and probably not elsewhere in nature. Such a population arises from the ingesta.

When filled, the rumen contents constitute about one-fifth of the total body weight. As an example, with an animal weighing 1,000 pounds the rumen holds about 25 gallons.

REVIEW OF LITERATURE

In a series of studies on the chemistry of rumen digestion during a 12-hour period Hale (19) found that dry matter in general was digested at an even rate throughout. The predominant action during the first 6 hours was the rapid disappearance of more soluble nutrients, viz. proteins and carbohydrates, from the rumen. Cellulose was slightly disintegrated while lignin and crude fiber remained untouched. The second 6 hours exhibited rapid digestion of cellulose which was paralleled by digestion of both carbohydrate and protein. However, the fact that lignin digestion did not go beyond a few percent was evidence that the chemical composition of the plant ingesta imposes certain limitations upon the ruminant microbial activity. The variable digestion of lignin in the caecum, after its passage from the rumen, exposes cellulose and protein to further disintegration, probably by the iodophile microflora. Herein lies the major function of this organ in the ruminant. It is not as yet clear whether or not the fatty acids observed in the rumen are intermediate or end-products of digestion. Hale (18) also concluded that during the course of a 12-hour digestion period between feedings passage of nutrients from the rumen is affected by (1) the passage of the concentrated rumen contents which remain from previous digestion periods, and

(2) the separation of nutrients from recent plant ingesta with subsequent washing from the rumen. Therefore, it is seen that many nutrients pass from the rumen in both the digestible and indigestible form. Such studies are suggestive of the fact that maximum limits of rumen digestion are normally reached within the 12-hour digestion period. The reaction in the rumen reaches a maximum acidity approximately 6 hours after feeding, after which time the values increase gradually to the preceding level of near neutrality. It is interesting to note here that Monroe and Perkins (32) reported higher acid in ingesta from cattle on pasture than when roughages, corn and silage, were fed.

The claim that non-protein nitrogenous substances could serve as a replacement for a portion of the dietary protein required for maintenance of normal health and growth has frequently been considered during the last half-century. However, this subject did not receive any attention until recently when there arose the possibility of feed ration shortage during the war, and until non-protein nitrogenous compounds such as urea were manufactured in great quantities from atmospheric nitrogen. The belief is that the rumen microorganisms are capable of utilizing the N.P.N. source of nitrogen in the synthesis of their cellular protein which is ultimately utilized by the animal when digestion is completed in the glandular stomach. In other words, the ruminant may be said to live upon the mass of microorganisms formed from

the ingested material rather than on the material per se. It may thus be seen that the substitution of some N.P.N. compound may be a more economical supply of nitrogen for the ruminant, provided the microbial utilization and conversion is efficient both qualitatively and quantitatively.

The question of the value of urea as a N.P.N. source for ruminants has received perhaps the most serious consideration during the latter years of study on this problem. It has been observed that rumen microorganisms can utilize this source of nitrogen provided a readily available carbohydrate is present as an energy source. Urea is believed to be hydrolyzed to ammonia by the inherent microbial enzyme urease, and the ammonia nitrogen in turn utilized by the organisms in the synthesis of their cellular protein. The value of substituting urea for a portion of dietary protein is substantiated by the literature. However, when urea is employed as the sole source of dietary nitrogen, the efficiency is known to be markedly reduced. Agrawala et al. (1) found that calves could not survive on such a purified ration because the organisms could not convert the large quantity of ammonia to protein-nitrogen with sufficient rapidity to reduce the toxic effect of ammonia upon the microbial population. Therefore, while urea is suitable as a feed ingredient because of its economy, lack of odor, high nitrogen content (46.6% N), and biological availability, the amount which can be substituted is limited to a certain de-

gree by the rapidity of its conversion to ammonia in the rumen. It is interesting to note the fact that lambs appear to possess greater efficiency in utilizing urea-nitrogen than do steers.

The first type of study carried out in an effort to examine the question of utilization of N.P.N. compounds, with emphasis upon urea, was feed experiments involving sheep and steers. Hart et al. (23) considered that the best type of experiment for such a problem was one involving long term growth, milk production, wool production, etc. They ran four calves, one receiving a basal ration of 6 percent protein, one receiving an addition of urea, the third receiving an addition of ammonium bicarbonate and the fourth receiving an addition of casein. Each supplement was of such an amount that the total protein was brought up to 18 percent. The study ran for forty weeks. While the animal receiving casein showed more growth, the rate was at certain times approached by the animal receiving urea and bicarbonate. The increment of weight was constant in composition. They concluded with reasonable certainty that the growth record so obtained was proof of the whole or partial utilization of ammonia and urea for synthesis of protein via the intervention of rumen microbes. They found definitely that urea and sodium bicarbonate nitrogen can be utilized by growing heifers when grains or timothy hay supply part of the protein. It was also apparent that bacterial multiplication could be enhanced

by the ingestion of some soluble carbohydrate. Wegner (35) explained the ability of dairy calves to utilize the inorganic nitrogen from urea and bicarbonate for part of the ration protein through the production of protein from this nitrogen by growth of bacteria in the rumen and subsequent digestion of these cells in the fourth stomach and intestines.

Smith and Baker (34) conducted an experimental study on milk production and found that while no significant decrease occurred when urea replaced the nitrogen equivalent of blood meal, the yield immediately fell when urea was removed. Similar studies were run with meat and wool production. As a result of these feeding trials, it was concluded that under favorable conditions urea can be utilized by ruminants for meat, wool and milk production, provided the proportion of nitrogen supplied as urea did not exceed 40 percent. The workers also theorized that urea may prove particularly valuable in areas where grain is especially rich in carbohydrate and poor in protein, since an adequate amount of readily available carbohydrate must be present to balance all nitrogen in the diet. Hart et al. (23) likewise concluded that maximum growth response to urea occurred when the supply of urea-nitrogen was no more than 40 percent of the total dietary nitrogen.

Agrawala et al. (1) conducted studies on the use of urea as sole source of dietary nitrogen. However, as mentioned previously, the conversion of urea to ammonia was too rapid

and became toxic for the microbial population. The purified ration consisted of cornstarch, glucose, cellophane, lard, mineral mix and urea. The nitrogen equivalent (N x 6.25) was 12 percent. Cellophane was found to be an unsuitable substitute for natural roughage because cellulose derivatives are more resistant to microbiological attack and degradation than is the cellulose molecule. In addition, since ruminants are somewhat specific in their natural protein requirements (amino acids) a ration low in this form of protein could lead to the loss of specific ruminal species of microorganisms. Although approximately 90 percent of the N.P.N. disappeared within 6 hours, the indication was that only a small portion of the urea-nitrogen was utilized for synthesis. This was due to the presence of more elementary nitrogen in the purified ration. However, the amount of true protein did increase when the purified ration was fed (33-109 gms.). In this vein, Pearson and Smith, in 1942, calculated from *in vitro* synthesis that in a 75 kg. rumen 450 gms. of protein could be synthesized in 24 hours.

Johnson and co-workers (27) found that upon addition of urea in amounts to produce the equivalent of 12 percent crude protein in a basal ration the result was a nitrogen-retention that was not enhanced by further urea addition, although increased effect was encountered by raising the true protein content of the ration.

Wegner *et al.* (36) found that urea, added to a basal

ration with 1 percent dry matter, completely disappeared within one hour after feeding, having been hydrolyzed to ammonia-nitrogen and/or converted to protein. Although the percent of ammonia-nitrogen in the supplemented basal ration was initially high due to hydrolysis of the added urea, it decreased in 4 to 6 hours to a level approximating that in the basal ration, and in the same time. Protein synthesis was offered as the cause for this disappearance of ammonia-nitrogen. Growth of rumen microorganisms utilizing the added urea was also evidence of an increased protein content. These workers concluded that urea-nitrogen utilization must occur within 4-6 hours after feeding as urea-nitrogen and ammonia-nitrogen are found to be negligible after that time. Duncan et al. (14) showed the mixed proteins synthesized from urea to be similar to those found in the rumen of a calf receiving natural ration.

Harris and Mitchell (22) demonstrated that the addition of urea to a low-nitrogen ration enhanced cellulose digestion and was itself digested as much as 88.8 percent. Their studies showed that sheep fed on rations containing urea and minimal amounts of protein providing only 10 percent of the nitrogen required for equilibrium could be maintained in body and nitrogen equilibrium for more than 100 days. The biological value of urea-nitrogen at N-equilibrium is equal to 62 percent (Casein N = 79%).

In an attempt to demonstrate more conclusive positive

evidence of microbial protein synthesis, Wegner (36) supplemented a low-protein ration of silage and starch (N x 6.25 = 4%) with urea equivalent to 5 percent dry matter. The protein level from the supplemented ration was found to be about 20 percent greater when determined several hours after feeding. Since tests showed the filtrate nitrogen level always to return to the same low level as in the basal ration, this increased total nitrogen was due to protein formation. Hart et al. concluded that it is entirely possible to improve low-protein rations of poor biological value through the use of molasses and/or urea, and that the microbial protein subsequently formed may be of greater value as a ration supplement than some of the protein concentrates in use at the present time.

It is now a well established practice both in America and abroad to employ urea as a partial protein source in a properly balanced ration for ruminants (7). McDonald reported, in 1954, that about 40 percent of the zein used in his studies, contributing about 94 percent of the total dietary nitrogen, was utilized for synthesis of rumen microbial protein. Loosli, in 1949, maintained growth in lambs when urea supplied essentially all the nitrogen, and also demonstrated synthesis of the ten essential amino acids. Hamilton et al. (20) found urea to be satisfactory as a nitrogen source for lambs with the provision that at least 25 percent of the feed-nitrogen be in the form of preformed protein and, further, that the total protein equivalent be under or equal to

12 percent.

In comparing utilization of urea and soybean oil meal nitrogen, Harris and co-workers (21) found the biological value of urea-nitrogen to be 34 while that of soybean oil meal nitrogen was 60 when fed at 12 and 14 percent protein equivalent levels. However, a greater amount of true protein was detected in the rumen of steers receiving urea than in those steers receiving only the low protein ration. The poor urea utilization was attributed to the feeding of a level exceeding that of maximum conversion to true protein by the microorganisms. It has been noted that Smith (33) reported utilization provided the proportion of nitrogen supplied as urea did not exceed 40 percent of the total nitrogen. In connection with this, Belasco (7) assumed it probable that at a protein equivalent level of 43 percent the hydrolysis of urea to ammonia and carbon dioxide exceeded the rate of ammonia utilization by the organisms resulting in decreased synthesis. As a result of research conducted in 1949, McDonald was lead to believe that ammonia, escaping fixation in the rumen, is absorbed into the venous circulation by which it is transported to the liver and subsequently converted to urea. A large portion of this urea is then returned to the rumen as a normal saliva constituent.

In an effort to obtain a more rapid and convenient method for the study of urea utilization and synthesis, the in vitro technique was adopted. Essentially this method in-

volves the removal of rumen contents from a rumen-fistulated steer. Gross material is removed and the resulting liquor is supplemented with the desired substance to be tested. Using conditions which approximate those occurring naturally in the rumen, i.e. 39°C, anaerobic atmosphere, and agitation, the samples are incubated for a period of time and the final sample tested as required. Pearson and Smith, in 1943, were some of the first workers to conduct investigations on urea utilization by means of this technique. They incubated the more liquid portion of the rumen contents for 6 hours with urea and a suitable carbohydrate energy source under a carbon dioxide gas phase in an effort to determine whether one could detect the synthesis of protein from urea. Results of a typical experiment may have been as follows (33):

(1) the total nitrogen remained constant
(2) urea was rapidly converted to ammonia with a subsequent decrease in N.P.N. which occurred mainly, if not entirely, in the ammonia fraction

The value obtained by subtracting the N.P.N. values from those for total nitrogen indicated that the protein synthesis appeared to occur most rapidly during the first 3 hours of incubation. Baker, in 1943, showed that during this three-hour period the microbiological conditions in the rumen liquid were very comparable to those of the initial sample as it came from the rumen. However, these conditions showed a marked difference after six hours due to the autolytic effect pro-

duced. Wegner (35) demonstrated a negligible urea level occurring after 24 hours incubation. An increase in ammonia-nitrogen comparable to the decrease in urea-nitrogen seemed to indicate an initial hydrolysis of urea to ammonia which was followed by the disappearance of the ammonia-nitrogen.

Belasco (8), in studying the effect of adding increasing amounts of urea to this artificial rumen, found that a steady increase in free ammonia resulted with increasing urea concentration. Metabolism of both urea and cellulose increased with increasing levels up to and including the 35 percent protein equivalent level. However, a sharp decrease both in utilization and digestion was noted at the 45 percent protein equivalent level.

Burroughs et al., in 1951, noted an increased cellulose digestion and urea utilization upon the addition of urea to purified protein and protein meals (7). In an in vitro study involving the comparison of urea and protein meals (soybean, linseed, cotton seed, and corn gluten) at comparable nitrogen levels, Belasco (7) demonstrated the superiority of urea as a nitrogen source in promoting cellulytic digestion. In 1:1 urea-protein meal mixtures the percent of urea utilization was consistently higher than in mixtures employing urea alone at a similar total nitrogen level, although the rates of cellulose digestion were similar to those obtained with only urea. Belasco (8) explained this superiority of urea by the fact that urea, being hydrolyzed by rumen bacterial urease, is a

form of readily available nitrogen while the nitrogen from the protein fraction of a meal, which represents a complex polypeptide, is essentially unavailable until cleavage and/or deamination occurs. The increased cellulytic response demonstrated with urea indicates the importance of the availability of ammonia-nitrogen from either protein or non-protein sources in the efficient digestion of roughage. Huffman (25) reported that nitrate can apparently be advantageously and safely substituted for urea, provided sufficient fermentable sugar is given simultaneously.

Hart (23) stated the theory of urea utilization as follows:

> "The bacteria in the rumen find the medium of simple nitrogenous salts and sugar an excellent one in which to grow. Through their multiplication they build proteins which would contain the amino acids necessary for supplements to the proteins of the ration. These bacterial cells pass from the rumen to the fourth stomach where they are digested and become just so much protein for the animal."

Johnson et al. (28) found that all food nitrogen, up to the maximum amount of nitrogen capable of bacterial utilization, would exhibit a biological value characteristic of the mixed microorganisms reaching the glandular stomach. The value appears to be about 60. The consumption of any nitrogen above the required amount should then possess a biological value comparable to that of a non-ruminant of similar requirements.

The question of ruminant nutrition may be regarded as a question of the balancing of nutrients required by the microbes harbored in the digestive tract. The rumen is well suited to the maintenance of a large, prolific population, which is capable of digesting plant constituents and which can further synthesize many nutrient compounds for the host. The microorganisms have readily available access to the nutrients consumed by the host due to their location near the anterior portion of the digestive tract. Further, certain nutrients are transported from the animal body to the omasum by the continuous flow of saliva. This flow also aids in the maintenance of a high water level in the rumen, promoting fermentation.

The main contributors to synthesis are the micro-iodophiles. Macro-iodophiles contribute only very little to the total protein synthesis. Factors such as the volume of the organ, period of retention, and the extent to which optimal conditions prevail, determine the total output of microbial products. Fluids are retained in circulation, affording a permanent medium for microbial activity, by changes taking place between the rumen and reticulum. Ingested plant material is seen to be the natural habitat of this microbial population as well as the functional link between it and the host animal. Decomposition is throughout accompanied by synthesis since the maintenance of the population is a direct consequence of proliferation (5). A source of nitrogen is

essential for this synthesis. This cellular digestion is, therefore, initially bound up with the nitrogen requirements of the microorganisms. Since the medium in which the microorganisms grow is determined by the ration fed, it becomes probable that a varying ration could effect a change in the microflora and thereby a change in the synthetic reactions induced. Bentley (9) demonstrated that this was true by showing the depression of cellulose digestion in steers by feeding starch. On the other hand, B-vitamin synthesis and urea utilization were improved by rations rich in readily available carbohydrate. A natural difference is known to exist between the microflora of animals fed on roughage and those receiving grain-rich rations. Along this line, Gall (16) found the winter and summer ration effect to be the only variable which seemed to influence bacterial population, and the changes were more quantitative than qualitative.

The presence or absence of certain minerals is known to have an effect on ruminant nutrition. Since calcium is found in abundance in roughage, a deficiency is uncommon under natural conditions. Cobalt is the most prevalent deficiency found among ruminants, in many instances being attributed to a phosphorus deficiency. Gall (16) exhibited an increase in bacterial counts of sheep with cobalt supplementation, as well as a marked bacterial type alteration when deficient. Using diminution of N.P.N., when incubated <u>in vitro</u>, as an index of bacterial growth, McNaught (31) observed that rumi-

nant bacteria could tolerate 100 p.p.m. of iron, 10 p.p.m. of copper, somewhat less than 10 p.p.m. of cobalt and 100-1,000 p.p.m. of molydenum. Definite inhibition occurred in the presence of 1,000 p.p.m. of iron, 25 p.p.m. of copper, 1,000 p.p.m. of cobalt, and 2,000 p.p.m. molydenum. The amount of iron associated with microorganisms was found to increase with in vitro incubation. Certain antibiotics may enhance growth of these organisms as well. Knodt (29) found that aureomycin increased the rate of growth of dairy calves and did not apparently effect rumen flora.

No salivary enzyme is possessed by the bovine for the degradation of cellulose, nor is there present any such secretion in the rumen. It is up to the flora alone to perform the function of degradation. Many feeds are known to exert an influence upon these organisms and the ability to digest cellulose. Dried distillers solubles, soybean oil meal, and linseed oil meal appear to be the most helpful, followed by corn molasses, corn, wheat bran, and cottonseed meal (13). Belasco (8) noted that urea gave greater cellulose digestion than did soybean oil meal at equivalent nitrogen levels, and furthermore, urea maintained higher levels of digestion than did any of the protein meals tested at comparable nitrogen levels, especially at the lower levels. Balch (2) suggested a decreased rate of passage through the reticular rumen to be associated with the increased digestion of crude fiber. Certain unidentifiable "cellulytic factors"

have been known to stimulate microbiological activity *in vitro* and have a marked effect on cellulose digestion (9). The factors are apparently present in autoclaved rumen juice, extracts of various plant materials, molasses and yeast extract.

A considerable portion of the crude protein ingested by the ruminant is converted to ammonia by microbial proteolytic enzymes and the ammonia synthesized into microbial protein. As a consequence, a large portion of the protein ultimately used by the ruminant appears to be microorganismal, regardless of the nature of the nitrogenous compounds contained in the ration consumed (27). The active rumen flora will utilize ammonia rapidly as a source of nitrogen in the presence of sufficient readily available carbohydrate and prevents its accumulation. Moir and Williams (25) estimated the conversion of ingested nitrogen to microbial protein to be about 50 percent in sheep. Protein digestion in the rumen can be due only to proteolytic enzymes contained in the food or produced by the microbes. Sym (1938) demonstrated a highly active proteinase, considered of microbial origin, present in the rumen contents (30). McNaught (25) found the dehydrated rumen bacterial cells to contain 44.4 percent crude protein with a digestibility value of 73 and a biological value of 88. However, different nitrogen sources varied markedly in their biological value and in their capacity to promote bacterial growth. She also noted an increase of lysine in incubated samples of from 9.3 to 11.6 mg/100 ml. of rumen fluid, and considered this as evidence of protein synthesis (13). Using

the *in vitro* method of study, Smith (33) found that about 8 mg. N/100 g. of rumen liquid was being converted to protein during incubation. He calculated that if synthesis would proceed at this rate in the intact rumen, about 300 g. of protein, or roughly one-third of the protein requirements of a cow yielding 2 or 3 gallons of milk daily, would be synthesized in one day. He also found the optimum temperature for maximum synthesis to be between 30° and 40°C, with hydrolysis predominating above 40°C. Therefore, synthesis and hydrolysis of protein undoubtedly proceed simultaneously, predominance depending upon prevailing rumen conditions. In an effort to isolate this protein and estimate the amount of true protein, Smith (34) separated a sediment containing the synthesized protein by centrifuging the liquid at 3,000 r.p.m. for one hour. He noted that the weight of sediment and total protein increased in the presence of carbohydrate while N.P.N. decreased, and that there was an increased number of iodophiles accompanied by synthesis of a starch-like polysaccharide. Conversely, in the absence of carbohydrate protein hydrolysis predominated and total protein-nitrogen and bacterial count decreased while N.P.N. increased, thus pointing out the importance of adequate available carbohydrate in the conversion of N.P.N. to protein, and also that an increased iodophile count accompanies synthesis. Typical analytical figures for this sediment as found by Smith are: 0.5 percent moisture, 36.3 percent protein, 46.6 percent polysaccharide, 9.5 percent lipoid material, and 6.2 percent ash. These values are

very similar to corresponding values for feeding stuffs such as linseed cakes. Wegner (35) criticizes the comparability of results secured by the in vitro method, however, since in the rumen a maximum flora is always present while in in vitro experiments the flora must first develop. During this intervening time, chemical changes, such as proteolysis, which do not have time to occur naturally may be taking place. This may be diagrammed in such a manner:

$$NH_3N \underset{\text{Bacterial Proteolytic Enzymes}}{\overset{\text{Bacterial Growth}}{\rightleftharpoons}} \text{Protein}$$

The crude protein of roughages contain from 10 to 15 percent N.P.N. as free amino acids, nucleic acids, purine and pyrimidine bases, etc. However, rumen microorganisms have been considered by some investigators to be relatively simple in the nature of their requirements, and not in need of complex forms such as amino acids. Hamilton et al. (20) found urea satisfactory as a nitrogen source for growing lambs, provided at least 25 percent of the feed nitrogen was in the form of preformed protein and, further, that the total protein equivalent in the ration was not in excess of 12 percent. This latter fact was confirmed by Wegner (37). However, although simple nitrogenous compounds such as urea are undoubtedly utilized for microbial protein synthesis, most workers have discovered that such compounds are not as effective as the nitrogen from natural proteins. This may be

due to the rapid conversion to ammonia, the excess of which becomes unavailable to bacteria for conversion since it is absorbed through the rumen wall.

When one is considering the protein synthesizing power of rumen microorganisms, it is well to remember that microbial proteins are not fixed structures but exist in a dynamic steady state (10). This fact is probably very advantageous to the organism. Since a cell must respond to varying conditions such as growth, infection, etc., the lability of the structures offer an easier ability to adapt to such changes. Protein metabolism involves a steady dynamic state of continuous, equal synthesis and breakdown.

$$\text{Protein} \rightleftharpoons \text{Amino Acids} \rightleftharpoons \text{Catabolic Products}$$

A continuous breakdown and reconstitution of peptide bonds occurs, and synthesis takes place both when amino acids are supplied in the diet and when the animal is fasting.

The major initial reaction occurring during nitrogen metabolism is the loss of the alpha-amino group, due to either oxidation or transamination. While the alpha-amino group ultimately appears in mammals, mainly in urea, the residual carbon skeleton may be reaminated or converted to other products. It is a generally accepted fact that urea arises mainly by the action of arginase on arginine to yield urea and ornithine (10). Glutamic acid, being an important link between carbohydrate and protein metabolism, probably repre-

sents the most significant pathway for the formation of alpha-amino groups in higher animals via the conversion of ammonia. Since the first step in the catabolism of most amino acids is usually deamination, most of the nitrogen of the alpha-amino group appears in urea, uric acid, or allantoin. The chief amino acid involved in transamination is l-glutamic acid. Although certain d-amino acids occur in the natural state, their presence remains to be proved in protein and their biological significance is doubtful. Protein breakdown accounts for the formation of ammonia in the rumen and may also account for the origination of the volatile fatty acids. Although only low concentrations of amino acids exist in the rumen liquor, there is evidence of some protease activity by microorganisms. Duncan et al. (14) exhibited the ability of rumen microorganisms to utilize urea-nitrogen for synthesis of amino acids, and found the amino acid pattern from the purified ration to be basically similar to that obtained from a natural ration, with the exception of histidine. The average percent increase of amino acids from the mixed rumen proteins over a 6-hour period was found to be as follows:

Arginine.....43.35%
Histidine.....39.57
Isoleucine....43.66
Leucine.....41.07
Lysine.....44.83
Methionine....44.07
Phenylalanine.41.96
Threonine.....39.43
Tryptophan....40.45
Valine.....41.04

No consistent difference was observed between 0 and 6-hours of incubation when amino acid composition of microbial protein was expressed as percent of sample. However, total amounts present indicated extensive synthesis.

Black, in 1952, concluded that the essential or "indespensible" amino acids are synthesized by the microorganisms while the tissues of the cow furnish the non-essential amino acids. Ruminants are the least definite in their amino acid requirements of all herbivores. Therefore, even N.P.N. sources of combined nitrogen, such as urea and ammonia, have a pronounced protein replacing value in ruminant nutrition. The only exception to the non-critical problem of ruminant protein requirement is the young of the species in which the synthetic ability is not well developed and to which high quality proteins must be supplied in the ration. One can see, therefore, that the term "non-essential amino acids" is significant only when qualified as to species and age period of the particular animal (3).

The rate of amino acid exchange is different in different tissues, but, in general, the rate of incorporation into intact cells <u>in vitro</u> is of the same order of magnitude as <u>in vivo</u>. Conditions were found by el-Shazly (15) for using washed suspensions of mixed rumen microorganisms which attacked amino acids present in mixtures in much the same relative rates as those attacking the whole rumen liquor. The rapidity with which ammonia is accumulated in the rumen fol-

lowing feeding reflects the high microbial activity. From the fact that amino acids do not accumulate it is apparent that the rate of amino acid uptake exceeds proteolysis caused by the microbial proteinase, or that in addition they may be deaminated free amino acids (30).

Even when the source of dietary nitrogen is wholly protein, an appreciable amount is converted to bacterial protein. This bacterial protein is relatively constant in composition for different conditions in the rumen according to Holmes (24). In comparing it with whole egg protein, it is well supplied with arginine, histidine, valine, and tryptophan, but deficient in leucine, threonine, and phenylalamine, and although moderately rich in methionine as compared with other bacterial protein, it is very deficient in this as well as isoleucine. The value of supplementing a ration with methionine has been shown by Gallup (17) who found the addition of 1.6, 2, and 3 g/day to a urea supplemented low-protein ration to increase the average digestibility of nutrients and nitrogen utilization. Johanson et al. (26) concluded that the fact that rumen bacterial protein is rich in cystine and methionine is of considerable importance as evidence to the theory of protein synthesis prior to digestion and absorption. They also concluded that in these animals the value of the N.P.N. substance, i.e. urea, is greatly enhanced by supplementation with methionine.

Sulfur requirements are interesting to note, since rumi-

nants utilize inorganic sulfur in the synthesis of cystine and methionine.

Alexander (2) found that a low-protein diet is necessary for conversion of ammonia into protein. The power to deaminate amino-acids depends upon the diet (power of rumen microorganisms) and the rate of microbial attack is determined by the solubility of the protein. While the biological value of this microbial protein is fair and may logically be expected to depend upon its amino-acid composition, since it is hydrolyzed to the component amino acids, its inferiority may perhaps be related to the methionine deficiency of "food yeast."

EXPERIMENTAL PROCEDURE

Two rumen fistulated steers were employed in the study. Steer A received a ration composed of 25 pounds of corn silage[1] plus 2 pounds of soybean-oil meal[2] per day. Steer B received a ration of 25 pounds of corn silage plus 2 pounds of a urea-corn mixture[3] per day. Both were fed an additional 25 pounds of mineral mix[4] per day.

Rumen ingesta was collected from each steer about 13 hours after feeding to facilitate removal of ingesta by eliminating much gross material. The fresh sample was filtered through cotton guaze to remove solid material, and the resulting liquor was centrifuged in an International centrifuge to rid the liquor of the smaller food particles and larger masses of protozoa.

In the first series of trials the liquor was centrifuged at 2,000 r.p.m. for 10 minutes. The resulting supernatant was prepared for incubation by the addition of 0.3 percent urea, 1 percent glucose, and 0.5 percent Difco yeast extract. Duplicate 20 ml. portions were removed for the preparation of the amino acids filtrate and duplicate 10 ml.

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1. Corn Silage.....2.72% protein, 64.03% water
2. Soybean-oil Meal.....48.44% protein
3. Corn-Urea Mixture....14.79% urea, 46.13% protein
4. Mineral Mix.....50% trace mineral salt + 50% dicalcium phosphate

portions removed for preparation of the tryptophan filtrate. Duplicate 25 ml. portions were withdrawn for the protein nitrogen determination and duplicate 1 ml. samples removed for determination of dry matter content. The initial sample was then incubated in a water bath at 39° C under CO_2 for a period of 6 hours, after which time test portions were removed as detailed above.

Amino acid hydrolysates were prepared by adding 20 ml. of concentrated HCl and autoclaving at 15# pressure (121° C.) for 8 hours. The hydrolysate was cooled and adjusted to pH 6.8 with 19 N NaOH, diluted to 100 ml. with distilled water and filtered through #40 Whatman filter paper. The resulting filtrate was a 1-5 dilution of the original sample. Storage was made under toluene at refrigeration temperature. Tryptophan hydrolysates were prepared by adding 40 ml. of 3 N NaOH and autoclaving as for the amino acid samples. The hydrolysate was adjusted with concentrated HCl to pH 7.0, diluted to 100 ml. and filtered through #40 Whatman filter paper. The final filtrate represented a 1.-10 dilution of the original sample. Storage was made as above. Protein nitrogen was determined by the Kjeldahl method. The 25 ml. sample was precipitated by the addition of 15 ml. of 10% sodium tungstate and 60 ml. of 0.33 N H_2SO_4. The resulting precipate was separated out by filtering through Reeve Angel #812 fluted filter paper, and the determination run on the precipitate plus the paper. Calculation was then based upon the amount

or protein nitrogen per 100 ml. of liquor (N x 6.25). Percent dry matter was ascertained by drying the liquid samples of known weight in a vacuum oven and calculating the dry matter by loss of moisture.

The amino acid filtrate was assayed microbiologically for dl-methionine,[1] l-lysine,[2] l-arginine, dl-threonine and l-histidine,[3] dl-leucine, dl-valine, dl-phenylalanine, and dl-isoleucine,[4] as well as l-glutamic acid[5] and dl-aspartic acid.[2] Tryptophan assay was carried out according to the method of Keuken, Lyman and Hale.[6] Values were calculated as mg. of l-amino acid per gram of dried rumen material. Any increase in amount was assumed to be the result of microbial synthesis. The assay organisms used were Lactobacillus arabinosis 17-5 (#8014), Streptococcus fecalis (#9790), and Lactobacillus mesenteroides P-60 (#8042), obtained from the American-Type Culture Collection.

In trial II, determination of total N.P.N. was made in order to ascertain whether the N.P.N. content was decreased as a result of incubation.[7]

The second series of trials involved the isolation of synthesized protein material and analysis of this dried sediment. Since trouble was encountered with steer A and samples

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1. Lyman et al., J. Biochem., 166:161 (1946).
2. McMahon, J. R., and Snell, E. E., J. Biochem., 152: 83 (1944).
3. Greenhut, I. T., Schweigert, B. S., and Eluehjem, C. A., J. Biochem., 162:69 (1946).
4. Schweigert, et al., J. Biochem., 155:183 (1944).
5. Lyman et al., J. Biochem., 157:395 (1945).
6. Lyman et al., J. Biochem., 171 (1947).
7. See Appendix I for method.

were not withdrawn, the results of these latter trials are based upon the rumen ingesta obtained from the urea supplemented steer only. In an attempt to promote greater microbial activity *in vitro*, cellulose was incorporated as a substrate for the incubated samples. The substance employed was Solka-floc.[1] The rumen sample was removed as previously described. However, it was used in both the centrifuged and uncentrifuged state. Greater activity was expected from the uncentrifuged liquor due to the additional number of microorganisms.

The sample used for incubation in trial IV was uncentrifuged. The nitrogen source was 0.05 percent urea, the readily available carbohydrate was 0.01 percent glucose and 1.0 percent Solka-floc served as the substrate. The sample was run in duplicate. An initial 0-hour sample was prepared for sediment isolation by centrifuging in the International centrifuge for 10 minutes at 2,000 r.p.m. Following the addition of the above mentioned substances to the uncentrifuged samples, a 25 ml. portion was removed for cellulose determination.[2] The samples were then incubated for 24 hours under CO_2 at 39°C. At the end of this incubation period one-half of the inoculum was removed for sediment isolation and a sample removed for cellulose determination. The remaining half was again brought to volume with an artificial complex[3] and half quantities of urea and glucose added. A cellulose

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1. Purified wood cellulose (SW 40A).
2. Crampton, E. W., and Maynard, L. A., J. Nutr., 15 (1938).
3. McDougall's Modified Synthetic Saliva with trace elements.

sample was withdrawn and incubation carried on for another 24 hours, after which a cellulose sample was again removed.

Isolation of the sediment was accomplished by centrifuging off the gross material at 2,000 r.p.m. for 10 minutes in the International centrifuge and running the supernatant through the Sharples Super Centrifuge for 20 - 30 minutes. The sediment thus obtained was resuspended in a 50/50 absolute alcohol-water mixture and spun down in a Sorval centrifuge at 60,000 r.p.m. for 20 - 30 minutes. The sediment was washed four times in this manner, alternating with absolute alcohol and the alcohol-water mixture. The final sediment was washed with ether and air-dried. The dried material was crushed and the resulting powdery material hydrolyzed for assay. Amino acid hydrolysates were prepared by adding 10 ml. of concentrated HCl per 0.1 gram of sample and autoclaving for 8 hours as described previously. Tryptophan samples were prepared by adding 5 ml. of 4 N NaOH per 0.2 gram of sample and hydrolyzing as above. The hydrolysates were adjusted to the desired pH, diluted to 100 ml. and filtered as before. Microbiological assays were carried out as for the liquid, and calculations made accordingly on the basis of mg/gm. of sediment.

The feeding ration was changed from corn silage to poor quality timothy hay plus the mineral mix for trials V and VI. The samples were neither supplemented nor incubated, but merely strained and precentrifuged in the International centri-

fuge. The time of centrifugation for samples 6 and 7 was 10 minutes at 1,500 r.p.m. while for samples 8 and 9 the time was 5 minutes at 1,000 r.p.m.. Isolation of the sediment and preparation of the filtrates were as described. The sediment from samples 6 and 9 was very dark in color. The supernatant liquid from the Sharples centrifugation was used for the assay of any free amino acids which might have been present.

The ration was again altered for trial VI by changing the corn-urea mixture to a corn-biuret (crude) mixture[1] and feeding this in the amount of 1½ pounds per day along with the poor quality timothy hay. The sample was centrifuged at 1,500 r.p.m. for 5 minutes and a sample set aside for sediment isolation (sample 10). Two samples were then prepared for incubation as follows:

Sample 1	Sample 2
Substrate..2% Solka-floc	2% Solka-floc
Complex....1 l. McDermits (½ strength)+trace elements[2]	1 l. McDermits (½ strength) +trace elements
Inoculum...1 liter centrifuged rumen liquor	1 liter centrifuged rumen liquor
N-source...0.2% urea	0.65% Drackett Assay Protein[3]
Carbohy....0.08% glucose	0.08% glucose

Duplicate 25 ml. portions were withdrawn from each sample for determination of Protein-N and triplicate 25 ml. portions removed for cellulose determination. Incubation was carried

1. 14.7% biuret-urea, ca. 42.4% crude protein.
2. See Appendix I for formulation.
3. Standardized protein from soybeans, Drackett Products Co.

out over a 24-hour period and test portions again withdrawn. Gross matter was removed from the incubated sample and the supernatant spun down in the Sorval centrifuge for 30 minutes. The sediment obtained was washed as previously described. Filtrates were prepared as before and analysis made in the manner already outlined.

TABLE I

AMINO ACID COMPOSITION OF LIQUID INGESTA[1]

Trial No.	Diet Fed	Inc. (HRS)	Protein (N x 6.25) mg/100 ml.	Amino Acid												Total N.P.N.
				Arg	Hist	Isol	Leu	Lys	Met	Phe	Thr	Try	Val	Asp. Acid	Glut. Acid	
II	S.O.M.[2]	0	196.50	3.71	1.17	3.14	4.21	4.42	1.09	2.51	4.49	0.60	3.50	8.18	---	157.45
			231.75	4.88	1.43	3.85	5.27	5.49	1.40	3.29	5.86	0.74	4.33	10.25	---	146.84
		6	477.25	4.75	1.56	4.95	6.23	7.06	1.52	3.89	7.06	0.59	6.40	12.17	---	106.52
			477.25	4.73	1.62	4.95	6.14	7.04	1.51	3.86	7.27	0.58	6.00	11.87	---	117.62
III	S.O.M.	0	196.50	4.82	1.86	5.50	13.42	6.92	0.83	3.83	5.72	0.78	4.67	2.42	8.60	---
			193.00	5.13	1.83	5.60	12.86	6.92	0.89	3.92	6.00	0.85	4.51	2.41	9.01	---
		6	459.75	6.13	2.18	7.00	15.24	9.12	1.46	4.70	6.76	0.68	7.36	2.97	10.99	---
			480.75	6.27	2.13	6.98	15.82	9.31	1.30	4.31	6.78	0.70	7.06	2.82	11.76	---
II	UREA	0	158.00	4.28	1.36	3.45	5.10	5.67	1.22	2.78	5.17	0.69	3.82	9.47	---	145.97
			158.00	4.46	1.47	3.62	5.11	5.43	1.26	3.00	5.36	0.72	4.01	9.17	---	139.31
		6	435.25	4.09	1.50	4.30	5.21	6.04	1.35	3.18	5.54	0.64	5.02	10.91	---	94.05
			456.25	4.16	1.50	4.01	5.07	6.13	1.31	5.31	5.45	0.59	4.76	10.55	---	93.5
III	UREA	0	175.50	4.67	1.79	4.68	12.37	6.84	0.88	3.53	5.09	0.80	3.81	2.10	8.56	---
			161.50	4.23	1.73	4.21	11.19	6.32	0.85	3.57	4.93	0.69	3.82	2.04	8.94	----
		6	484.50	5.89	2.44	6.39	15.19	8.36	1.42	4.86	7.58	0.59	6.55	3.08	12.87	---
			499.00	6.61	2.50	7.22	15.70	8.24	1.42	5.00	8.07	0.59	6.90	3.04	13.18	---

1. Values expressed as mg/gm dry matter (1-Form)
2. S.O.M. - Soybean oil meal ration

TABLE II

AMINO ACID RATIO (BASED ON DRY WEIGHT)

Trial No. / Sample	In. (hrs.)	II		III	
		S.O.M.	UREA	S.O.M.	UREA
Arg[1]	0	6.4	6.2	6.0	5.9
	6	8.0	6.7	9.0	10.6
Hist	0	1.9	2.0	2.3	2.3
	6	2.7	2.4	3.1	4.2
Isol	0	5.2	5.1	6.8	5.9
	6	8.4	6.7	10.1	11.5
Leu	0	7.1	7.2	16.0	15.7
	6	10.4	8.3	22.5	26.2
Lys	0	7.4	7.8	8.4	8.8
	6	11.9	9.8	13.4	14.1
Met	0	1.9	1.7	1.1	1.2
	6	2.6	2.1	2.0	2.4
Phe	0	4.3	4.1	4.7	4.7
	6	6.6	5.2	6.5	8.4
Thr	0	7.7	7.4	7.1	6.7
	6	12.2	8.9	9.8	13.3
Try	0	1.0	1.0	1.0	1.0
	6	1.0	1.0	1.0	1.0
Val	0	5.9	5.5	5.6	5.1
	6	10.5	7.9	10.4	11.4
Aspartic Acid	0	-	-	3.0	2.7
	6	-	-	4.2	5.2
Glutamic Acid	0	13.8	13.1	10.8	11.7
	6	20.4	17.3	16.5	22.1

1. First letters of each amino acid used.

[redacted]

TABLE III

AMINO ACID RATIO (BASED ON WGT.-VOL.)

Trial No. / Sample	Inc. (hrs.)	II		III	
		S.O.M.	UREA	S.O.M.	UREA
Arg	0	6.4	6.1	6.1	5.7
	6	7.9	6.6	8.9	10.5
Hist	0	2.0	2.0	2.2	2.3
	6	2.6	2.4	3.1	4.2
Isol	0	5.0	5.0	6.8	5.7
	6	8.2	6.7	10.2	11.4
Leu	0	6.8	7.1	16.0	15.2
	6	10.6	8.2	22.2	26.0
Lys	0	7.2	7.8	8.5	8.5
	6	11.8	9.8	13.2	14.0
Met	0	1.8	1.7	1.0	1.1
	6	2.6	2.2	1.9	2.6
Phe	0	4.2	4.0	4.7	4.6
	6	6.5	5.2	6.5	8.2
Thr	0	7.5	7.3	7.1	6.5
	6	12.0	8.8	9.6	13.1
Try	0	1.0	1.0	1.0	1.0
	6	1.0	1.0	1.0	1.0
Val	0	5.7	5.5	5.6	4.9
	6	10.3	7.8	10.4	11.3
Aspartic Acid	0	-	-	3.0	2.7
	6	-	-	4.5	5.2
Glutamic Acid	0	13.3	13.0	10.7	11.3
	6	20.1	17.2	16.3	21.9

[redacted]

TABLE IV

AMINO ACID COMPOSITION OF SEDIMENT[1]

Trial No.	IV		V					VI	
Sample	0-hr.	24-hr.	6	7	8	9	10	1(urea)	2(D.A.P.)[2]
Arg	----	----	23.9	28.2	25.7	26.4	25.5	22.3	23.5
Hist	----	----	17.5	20.2	19.1	20.3	19.6	19.4	19.0
Isol	----	----	31.5	35.4	33.3	33.5	32.7	31.2	32.7
Leu	85.6	82.9	74.1	86.6	78.0	78.0	73.8	69.5	76.5
Lys	47.0	47.8	34.2	36.8	34.2	39.3	38.0	31.6	35.5
Met	11.8	9.9	8.8	12.1	9.3	10.4	11.2	9.4	10.6
Phe	24.5	24.6	23.0	27.1	25.4	25.6	23.8	21.0	24.2
Thr	----	----	31.0	34.5	30.3	31.9	29.9	26.7	29.8
Try	2.6	----	3.6	3.7	3.2	3.5	3.2	2.8	2.5
Val	34.8	32.8	31.4	36.2	31.1	32.5	34.7	31.5	35.0
Aspartic Acid	----	----	15.8	17.7	15.5	16.5	16.0	13.7	14.7
Glutamic Acid	----	----	60.7	70.1	64.0	61.7	63.3	55.4	63.2

1. Values expressed as mg/gm. of dried sediment
2. Drackett Assay Protein

TABLE V

AMINO ACID RATIO OF SEDIMENT

Trial No. / Sample	Inc. (hrs.)	IV	V					VI	
			6	7	8	9	10	Urea	D.A.F.
Arg	0	--	6.7	7.7	8.1	7.5	7.9	---	---
	24	--	---	---	---	---	---	7.8	9.4
Hist	0	--	4.9	5.5	6.0	5.8	6.1	---	---
	24	--	---	---	---	---	---	6.1	7.6
Isol	0	--	8.8	9.6	10.5	9.6	10.2	---	---
	24	--	---	---	---	---	---	11.0	13.1
Leu	0	32.8	20.7	23.5	24.5	22.3	23.0	---	---
	24	--	---	---	---	---	---	24.6	30.6
Lys	0	18.0	9.6	10.0	10.6	11.2	11.8	---	---
	24	--	---	---	---	---	---	11.2	14.2
Met	0	4.5	2.5	3.3	2.9	3.0	3.5	---	---
	24	--	---	---	---	---	---	3.3	4.2
Phe	0	9.4	6.5	7.4	8.0	7.3	7.4	---	---
	24	--	---	---	---	---	---	7.4	9.7
Thr	0	--	8.7	9.4	9.5	9.1	9.3	---	---
	24	--	---	---	---	---	---	9.4	11.9
Try	0	1.0	1.0	1.0	1.0	1.0	1.0	---	---
	24	--	---	---	---	---	---	1.0	1.0
Val	0	13.3	8.8	9.8	9.8	9.3	10.8	---	---
	24	--	---	---	---	---	---	11.1	14.0
Aspartic Acid	0	--	4.4	4.8	4.9	4.7	5.0	---	---
	24	--	---	---	---	---	---	4.8	5.9
Glutamic Acid	0	--	17.0	19.0	20.1	17.6	19.7	---	---
	24	--	---	---	---	---	---	19.6	25.3

TABLE VI

AMINO ACID RATIO COMPARISON

Protein	Soybean Meal[1]	Casein[1]	Gelatin[1]	Zein	Lactobacillus Spp.[2]	E.coli.[2]	B.subtilus[2]	Whole Egg Protein[2]	Rumen Bact. "Dry Fed"[2]	Rumen Bact. "Green Fed"[2]	Rumen Mat.[2]	S.C.M. Ration Liquid	Urea Ration (Liq)	Urea Ration (Sed)	Raw Sed.
Arginine	5.6	3.2	88	16	19.6	17.3	1.5	8.4	11.6	9.8	12.4	8.5	8.7	7.8	7.6
Histidine	--	1.2	10	9	8.5	5.5	5.0	2.8	3.4	5.2	4.8	2.9	3.3	6.1	5.7
Isoleucine	--	3.0	17	43	10.0	7.0	6.4	4.1	2.0	2.3	4.5	9.3	9.1	11.0	9.7
Leucine	--	7.6	37	237	10.5	8.3	10.2	4.7	3.2	3.3	6.6	16.5	--	24.6	22.8
Lysine	5.4	6.1	43	--	18.3	12.3	15.0	6.6	4.4	4.2	13.6	12.7	12.0	11.2	10.6
Methionine	1.2	2.6	8.0	23	17.5	2.0	2.0	1.8	0.8	1.0	1.9	2.3	2.3	3.3	3.0
Phenyl-Alanine	--	4.0	20	64	4.5	2.8	3.8	5.5	1.6	1.7	2.6	6.6	6.8	7.4	7.3
Threonine	--	3.0	15	22	15.0	4.7	5.2	2.7	2.1	2.2	5.8	11.6	11.1	9.4	9.2
Tryptophan	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Valine	--	5.4	21	24	9.8	7.7	8.4	4.2	2.6	4.1	5.8	10.5	9.7	11.1	9.7
Aspartic Acid	--	4.9	59	34	---	---	---	---	5.1	5.8	---	4.2	5.2	4.8	4.8
Glutamic Acid	--	16.1	103	257	16	---	---	---	8.2	10.1	---	18.5	19.7	19.6	18.7

TABLE VI (Continued)

1. Values obtained from reprint Proteins & Amino Acids in Animal Nutrition, H. J. Almquist, U. S. Industrial Chemicals, Inc.

2. Basic values obtained from Moir, et al., Australian J. of Biological Sciences. Vol 6, No. 4. pp. 637-644, 1953

ANALYSIS OF DATA

The first trial of series one was not analyzed since the desired data were incomplete.

Table I shows the amino acid composition of the microbial protein as determined by microbiological assay of the fermentation liquor before and after a six-hour incubation period. The values obtained for total N.P.N. show a decrease in that form of nitrogen while the amount of protein increased measurably. This is indicative of protein synthesis as a result of incubation. This fact holds true for both the soybean oil meal and the urea supplemented ration. Leucine, lysine, threonine, valine and glutamic acid appear to increase in a significant amount while tryptophan decreases. However, the increase in each amino acid as such is insignificant when compared with the tremendous increase in protein. The data in table I were calculated on the basis of percent dry matter in the sample. However, since the quantity of dry matter decreased during incubation due to the utilization of the additives, this basis was considered to be questionable. A check on the values was made by calculating the values on a weight-volume basis, i.e. mg./100 ml. of rumen liquor. It was surprising to note that these new values showed a general decrease in amino acids after incubation, and also indicated soybean oil meal to be more satisfactory than urea. The in-

creases in protein values are, however, still vastly out of proportion. The dry-weight values were found to be similar to those obtained by Duncan et al. (14). Since the amino acid nitrogen was not determined in this work, percentage composition calculation was not possible. Also, due to the very slight increase in individual amino acids no separate tabulation was made to show this. Inasmuch as it was desired to set forth a comparison between the values obtained from soybean oil meal and those from urea, and actual percentage values could not be indicated, an amino acid composition ratio was determined. This ratio was determined by using the tryptophan value as the reference value of 1.0. The ratio obtained from the dry matter values was checked against that calculated on the weight-volume basis. Tables II and III show that the ratio is essentially the same for both methods. It also points out the fact that soybean oil meal and urea possess about the same capacity as a nitrogen-source for synthesis. The two trials agree to a reasonable degree in the majority of values. Isoleucine, leucine, lysine, threonine, and glutamic acid appear to show the greatest increase as a result of synthesis.

The second series of trials involved the isolation of the sediment with subsequent analysis. Since samples were not collected from the steer receiving soybean oil meal, the values shown in the tables represent only the microbial protein from the ration supplemented with urea. It must also

be noted that the amino acid values are representative of the raw ingesta, i.e. the material was not incubated for the purpose of demonstrating in vitro synthesis, but merely precentrifuged and then run through the Sharples centrifuge. Calculations are on a weight-weight basis. The two samples which were incubated contained cellulose as a substrate and the percent cellulose digestion was taken as an index of synthesis. Good synthesis was observed in trial IV. The Drackett Assay Protein used in trial VI apparently promoted greater synthesis than did the added urea. As seen in table IV, the values are much higher than those obtained from the liquid. However, this is to be expected since the sediment is assumed to contain the "true" protein. The values for trial V are good replicates, indicating that the protein material is of a relatively constant composition. Amino acid composition is exhibited here by a ratio as in the preceding trials, tryptophan being taken as the reference value. High values were again observed for isoleucine, leucine, lysine, threonine, and glutamic acid. The ratio of composition is set down in table V. Drackett Assay Protein seems to promote greater synthetic activity than does urea. A ratio comparison with some purified proteins, microorganisms, and previously determined rumen bacteria values is exhibited in table VI. The ratios were calculated from tryptophan values in order that all the values should be comparable.

DISCUSSION

The experimental work described in this paper actually involved a dual problem, viz. a comparison of methods for the determination of amino acid composition of rumen microbial protein, and the value of urea as a partial non-protein nitrogen substitute in ruminant nutrition.

The first problem was met by analysis of the rumen liquor and isolation of the protein material as a dried sediment. Analysis of the liquor, while being simple and convenient, involved one main drawback, viz. the question of free amino acids and soluble proteins. Although the assays conducted for the determination of free amino acids gave no significant results, it must be noted that the liquor employed was the supernatant from the Sharples centrifugation which was not hydrolyzed. Therefore, the assay values may very well be in error since, while there were no free amino acids as such, there may be some soluble protein which when hydrolyzed would yield a number of free amino acids. Hence, the liquid values obtained may be inclusive of some of these soluble protein constituents as well as the amino acids from the synthesized protein material.

It is interesting to note, but difficult to explain, the reason for the large increase in protein due to synthetic activity although the increase in amino acids was so slight.

While the tungstic acid precipitation is only empirical, it is a generally acceptable method for the precipitation of protein. Therefore, the protein-nitrogen values are assumed to be correct. The possibility for error remaining is in the question of the validity of the microbiological assay itself. The author considers the assay methods to be very reliable since all samples fell within the standard range and duplicate values were well in accord. Hence, the author considers these factors to be doubtful as sources of error. The reason for the discrepency must, therefore, be attributed to some factor which has not been detectable in this investigation.

Further study of methods involved the isolation of a protein sediment. This method was perhaps more accurate from the standpoint of protein material analyzed. However, problems arose in this case as well. A main source of error may have been in the washing of the sediment. It is known that a sizable quantity of material is lost due to incomplete centrifugation. This loss was preventable by washing with absolute alcohol; however, the strength of the alcohol alone may have caused an alteration in the protein so as to effect the final values. There is present, also, the possibility of inhibition of the assay microorganisms due to a carry-over of alcohol. The sediment obtained was only a small portion of the total, but the values indicate a material of relatively constant composition.

There appeared to be a very slight change in the amino acid composition as a result of synthesis, as in the case of the liquid. The increase encountered in the sample using Drackett Assay Protein as a nitrogen source may have been the result of amino acids supplied by the protein itself. The sizable increase in glutamic acid could also have been due to diffusion of glutamic acid into the cells. When comparing these two methods on the basis of an amino acid ratio, one sees that similar values are obtained, thus indicating quite clearly the fact that the microbial protein material is of a constant composition from the standpoint of the essential amino acids. When these values are further compared with purified proteins and other bacterial cells, one observes that the synthesized and normal microbial protein is quite adequate and satisfactory as a source of essential building material.

The data collected in this particular series of studies indicate that urea may be satisfactorily employed as a partial protein-nitrogen substitute. Although the values obtained show no outstanding increases as a result of incorporating urea into the ration, it is seen that it is equally satisfactory as soybean oil meal. In this case the value would lie in the economy of using urea over such a natural feedstuff as soybean oil meal.

SUMMARY

The possibility of employing urea as a partial source of nitrogen in ruminant feed rations has received much attention in the last few years. Many types of comparisons have been made between urea and natural feedstuffs in an effort to indicate the value of urea for this purpose. The experimental work as set forth in this paper was an attempt at pointing out the value of urea from the standpoint of amino acid composition of the microbial protein as a result of synthesis from a ration supplemented with urea as against a ration supplemented with soybean oil meal. However, difficulties and unforseen factors entered into the work and all trials run were not comparison trials. Therefore, although the author was not able actually to indicate the value of urea as clearly as desired, she was able to roughly show the relative amino acid composition of the protein. Two methods were used in determining the composition and the results obtained indicate that the microbial fraction of the rumen ingesta is of a relatively constant composition.

From the information gleaned by comparison of urea and soybean oil meal, the synthesis-producing capacity appears to be similar. The author, therefore, concluded that urea is as satisfactory as soybean oil meal in nitrogen value and is more economical.

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. As already mentioned, the protein composition of the microbial fraction is relatively constant. When compared with other purified proteins and natural feedstuffs it is seen that some values are very similar. In other words, the protein nutritive value is adequate according to some materials already in use.

In the final analysis, it may be said that urea may well be considered a satisfactory source of non-protein nitrogen in ruminant nutrition. The microbial protein material synthesized seems to be sufficient in amino acids to supply the animal adequately, and the use of urea is much more economical from the standpoint of the farmer. Although much work has been done along this line of study, there remains room for much more. The problem of urea supplementation is a complex problem, and one that can be approached in many ways. In vitro study appears to be the preferred method of study at present; however, there is the possibility of improvement. The field of study is wide open and the farmer is eager to learn all he can about this question. Besides meaning more meat on his beef cattle and more milk from his dairy herds, it means more money in his pocket. The use of non-protein nitrogenous compounds such as urea may certainly be a great boon to the farmer of today, both here and abroad.

APPENDIX

Modified Synthetic Saliva with Trace Elements

$NaHCO_3$	49.0 g
$Na_2HPO_4.12\ H_2O$	47.0 "
NaCl	2.4 "
KCl	2.9 "
$CaCl_2.2H_2O$	10.0 ml. stock soln.
$MgCl_2.2\ H_2O$	10.0 " " "
$FeSO_4.7\ H_2O$	20.0 " " "
$CaCl_2.6\ H_2O$	10.0 " " "
$ZnSO_4.7\ H_2O$	10.0 " " "
$CuSO_4.5\ H_2O$	10.0 " " "
$MnSO_4.H_2O$	10.0 " " "

Add 8 liters distilled water to a 10 liter bottle, add reagents in the order listed, and make up to 10 liters with distilled water.

Stock Solutions / 100 ml.

$FeSO_4.7\ H_2O$	2.0 g.
$CaCl_2.6\ H_2O$	0.4 "
$ZnSO_4.7\ H_2O$	0.2 "
$CuSO_4.5\ H_2O$	0.1 "
$MnSO_4.H_2O$	0.1 "
$CaCl_2.2\ H_2O$	2.6 "
$MgCl_2.6\ H_2O$	6.4 "

Determination of Total N.P.N. (Pearson & Smith, Biochem. J., 37 (1943))

Neutralize aliquots of tungstate filtrate with N NaOH using phenol red as an indicator. Add 10 ml. of 0.6% KH_2PO_4 as a buffer followed by 1 ml. of a 5% water urease solution. Incubate the mixture at 40-45°C. for 20 minutes. Add 2 ml. of N NaOH and distill in a steam current for 10-15 minutes, collecting the distillate in 2% boric acid solution.

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