The cystine and methionine content of Montana range grass
by Earl W Turner

A THESIS Submitted to the Graduate Committee in partial fulfillment of the requirements for the degree of Master of Science in Chemistry
Montana State University

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The leaf proteins of several Montana range grasses are extracted using sodium dodecyl sulfate. The advantages claimed for this method of extraction are simplicity, rapidity, and high yields of a final product containing as high as 80% pure protein. Cystine and methionine are determined on the leaf protein preparations using both chemical and micro biological assay methods. A comparison is made of the results obtained by the various assay methods. Methionine and cystine values obtained by both microbiological and chemical methods are found to be in good agreement and also consistant with results obtained by other methods. Methionine and cystine are determined on Agropyron smithii and Agropyron cristatum at two different stages of growth. The methionine and cystine contents of the younger grasses are considerably higher than of the more mature plants.

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Chairman, Graduate Committee

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I. ABSTRACT

The leaf proteins of several Montana range grasses are extracted using sodium dodecyl sulfate. The advantages claimed for this method of extraction are simplicity, rapidity, and high yields of a final product containing as high as 80% pure protein. Cystine and methionine are determined on the leaf protein preparations using both chemical and microbiological assay methods. A comparison is made of the results obtained by the various assay methods. Methionine and cystine values obtained by both microbiological and chemical methods are found to be in good agreement and also consistant with results obtained by other methods. Methionine and cystine are determined on Agropyron smithii and Agropyron cristatum at two different stages of growth. The methionine and cystine contents of the younger grasses are considerably higher than of the more mature plants.

II. INTRODUCTION

The presence of the sulfur containing amino acids, cystine and methionine, in plant proteins has been known for some time. Since these amino acids are quite easily altered by the action of ordinary hydrolytic reagents and probably by reagents employed as protein extractants, the determination of these acids in plant leaf proteins presents a special problem to the biological chemist. Cystine and methionine are essential to the growth of many organisms. In view of these facts, a more exacting study of methods of determining these amino acids in plant proteins is desired.

In the past, quantitative procedures for the determination of methionine and cystine have not been sufficiently accurate to afford more than approximate results. Although there are a number of chemical methods available for the estimation of methionine and cystine, many of these methods present serious difficulties which leave some doubt as to the reliability of the results obtained. Recently much work has been done in an effort to improve the older methods and to develop new methods of analyses which would give more consistent and accurate results. Of the more recently developed methods microbiological assaying offers interesting possibilities.

In view of these developments and the interesting problems that are presented, it was decided to investigate the desirability of the microbiological as well as the chemical methods of analysis. The leaf protein extracts from two common varieties of Montana range grass, Agropyron smithii and Agropyron cristatum, were selected for this study.

III. HISTORICAL RESUME

The Discovery of Methionine and Cystine:

Two sulfur derivatives have been found as constituents of the protein molecule. These are cystine and Mueller's thio acid (methionine). Cystine was isolated in 1810 by Wollaston and was found to be di,β-thio-α-amino propionic acid. Mörner, Osborne and Guest, Dakin, Osborne, Harris and others found that there was a probability of sulfur compounds in the protein molecule other than cystine. In 1922 Mueller isolated from casein a new sulfur containing amino acid, to which he gave the empirical formula $C_{11}H_{22}SN_2O_4$. Subsequent work on this amino acid by Mueller indicates that the amino acid has the emperical formula $C_5H_{11}SNO_2$ instead of $C_{11}H_{22}SN_2O_4$. In 1928 Barger and Coyne showed that Mueller's thio acid had the constitution αamino-γ-methyl thiol-n-butyric acid, $CH_3-S-CH_2-CH_2-\underset{\displaystyle NH_2}{\underset{|}{CH}}-COOH$ and gave it the name methionine.

Protein Extraction Methods:

One of the most difficult problems in the study of leaf-proteins is the extraction and purification of the protein without subsequent loss of amino acid. The loss of amino acids occurs most readily when there is humin formation. Humin is formed when the hydrolysis is carried out in the presence of carbohydrates.

To determine the protein cystine content it is necessary to hydrolyze the protein with strong acid or with the appropriate proteolytic enzymes. As no method of preparing these proteins has hitherto been available, early workers found it necessary to submit the whole grass to acid hydrolysis or enzymic digestion. This means in effect that the proteins in the leaf material have been treated in the presence of from 3-6 times their own

weight of cellulose, sugars, organic acids, etc., and it is not at all surprising that under those conditions they were unable to attain satisfactory results. However, by extraction of the proteins before hydrolysis fairly reliable results may be obtained.

Chibnall and Lugg have employed a method of extracting proteins from freshly cut grass which involves the use of ether-water as the plasmolyzing agent and a press for squeezing out the soluble protoplasmic proteins. The resultant fluid is treated with acid to flocculate the proteins. The precipitated proteins were purified and subjected to analysis for the various amino acids. The yield obtained compared with the total protein in the leaf was very low yet the assumption was made that the proteins which remained unextracted in the leaf tissue were of the same composition as those which had been extracted. Lugg found only slight variations in the amino acid composition between the various grasses he examined.

Another method for the extraction of leaf proteins is the method of Smith (39). Smith has used sodium dodecyl sulfate, digitonin, and bile salts as the dispersing agent. Preliminary experiments with sodium dodecyl sulfate have been employed at Montana State College Agricultural Experiment Station and it has been found that much higher yields of extracted proteins can be obtained than is possible by the Chibnall-Lugg method. Furthermore, the method seems applicable to dried grasses.

A third method for the extraction of grass proteins has been suggested by Albanese (1) and Mazur and Clarke (29). In this method 90% formic acid solution is used as the extractant. Mazur and Clarke have employed this extractant in their studies on the protein composition of marine algae.

Albanese has extracted leaf proteins from such plants as spinach, cabbage, and celery by this means and has been able to obtain a yield of 90-95% of the total protein. The extracted protein is remarkably free of carbohydrate contamination which is a decided advantage when the protein preparation is to be analyzed for its constituent amino acids.

Hydrolysis of the Protein Material:

Hydrolysis by acids, alkalies or proteolytic enzymes has proved to be the best means of investigating the composition of the protein molecule. In 1820 Braconnot hydrolyzed gelatin by boiling with dilute sulfuric acid. Between 1850 and 1875 Ritthausen, Hlasiwetz and Habermann as well as later investigators have favored hydrochloric acid as a hydrolyzing agent. The action of vegetable enzymes on proteins has been studied especially by Schulze and his co-workers, while about 1900 the action of animal enzymes was extensively studied by Kühne, Kossel, Kutscher and others.

In 1912, Van Slyke found that complete hydrolysis was produced by heating the protein with 3 N hydrochloric acid in an autoclave. The former investigators do not state the quantity of acid used but Van Slyke used thirty-three times more acid than protein. Van Slyke showed further that complete hydrolysis was effected either by heating at 100° for 48 hours with 20% acid or at 150° with 3 N acid. Henriques and Gjaldbäk (1910) found that the hydrolysis of egg albumin was not complete after heating for 12 hours with concentrated acid, but was complete on heating at 150° for 1.5 hours in an autoclave with 3 N acid. They found no advantage in heating at 150° for a longer time; at 180° in an autoclave decomposition of the amino acids occurred

with the formation of ammonia. Zelinsky and Ssadikow (1923) were able to show that complete hydrolysis may be effected with very weak acids. They state that proteins may be completely hydrolyzed by heating in an autoclave at 180° for three to six hours with 4, 3, 2, 1 or even 0.5% hydrochloric acid. It has been found that the amount of cystine recovered from hydrolyzates depends somewhat upon the acid used for hydrolysis. When mixtures of formic and hydrochloric acids were used to hydrolyze insulin some investigators found half again as much cystine as when hydrochloric acid was used alone. Baernstein (5) has advocated the use of HI but this is regarded as inferior to the formic acid mixture by du Vigneaud. Most proteins are certainly not as sensitive to hydrolysis conditions as is insulin. Hess and Sullivan (18) have investigated the rate of liberation of cystine from proteins by acid hydrolysis. Six proteins varying considerably in their cystine content were chosen for the experiment. The proteins were hydrolyzed with 20% HCl, HCl-HCOOH mixtures, and with HI. Regardless of the method of hydrolysis used, the maximum values of all three methods were found to be practically identical. With HI hydrolysis no humin formation resulted. The HI hydrolysis is by far the more rapid procedure and gives maximum values in the shortest possible time. It has the disadvantages, however, that the H_3PO_2 and HI must be driven off before testing for cystine and the hydrolyzate contains only cysteine which prevents the separate estimation of cystine and cysteine. The HCl hydrolysis has the advantage that it is possible to study both cystine and cysteine in the hydrolyzate and to estimate methionine colorimetrically. With HCl and the HCl-HCOOH mixture some humin was formed but there was no evidence of loss of cystine. Hydrolysis

with 20% HCl was found to be more rapid than with the HCl-HCOOH mixture. Sullivan's colorimetric method was used for the estimation of cystine.

Recently many investigators have found that autoclaving with dilute hydrochloric acid gave very satisfactory results. Barton-Wright (8) also Schweigert (37) and Stokes (42) favor this method of hydrolysis. Barton-Wright has used the following procedure for the preparation of protein hydrolyzates for methionine and cystine assays: A one gram sample of finely ground material is hydrolyzed by autoclaving for 5-6 hours at 15 pounds pressure with 25 ml. of 2.5 N HCl. He states that hydrolysis is incomplete in less than five hours. The hydrolyzate is cooled, 2 ml. of 2.5 M sodium acetate solution is added and the pH adjusted to 4.5 with sodium hydroxide and the solution made up to volume.

Methods for Cystine Determination

Sullivan's Method for the Determination of Cystine:

The literature on Sullivan's reaction for cystine has attained formidable proportions and a number of modifications have been proposed, considerable accuracy is claimed for most of them by their authors, though regarded as inadequate by others. The reaction which is highly specific for cysteine gives a red color with 1, 2 naphthoquinone-4-sulfonate in an alkaline reducing medium. Sullivan's procedure for the estimation of cystine consists essentially in reducing the cystine to cysteine and allowing the cysteine to react with the color reagent in an alkaline solution.

Sullivan advocates the addition of a small amount of cyanide before the reagent to stabilize the color. The procedure for cystine consists in first treating the solution with sufficient cyanide then proceeding as with cysteine.

Prunty's modification of Sullivan's method has been used by Chibnall (36) for the determination of cystine and cysteine in leaf proteins. Prunty's modification is much the same as the original Sullivan method except for the addition of a few mg. of zinc dust and a drop of concentrated hydrochloric acid which is used to help maintain a reducing environment in the solution.

Experiences with Sullivan's method were found by Lugg (27) to be unsatisfactory in the following respects: (1) the depth of color is variable and far from proportional to the amount of cysteine and cystine present; (2) even when compared with a standard of the same color results are often irregular; (3) the presence of relatively considerable amounts of other amino acids diminishes the amount of red color due to cysteine and yellow interferes when a pure cystine or cysteine is used for a standard. Lugg suggested two methods which might improve the accuracy of the method: (1) maintain the same pH in the unknown as in the standard; (2) add some amino acid in considerable excess to both the unknown and to the standard. It was found possible to effect both objectives by adding glycine with appropriate amounts of alkali.

Other methods which have been used for the estimation of cystine include Okuda and Baernstein's iodometric determinations. Okuda oxidized cyctine to cysteic acid by bromate in acid solution. Other amino acids interfere with this method of determination. Later he titrated the charcoal decolorized hydrolyzate with KIO_3 in acid solution containing KI, oxidizing cysteine to cystine. Baernstein (2) to avoid losses in charcoal added excess iodine and used gasometric methods to determine the excess iodine.

This, however, led to high values for cysteine. Baernstein (5) recommends the Okuda method on HI hydrolyzates though only cysteine and cystine could be estimated in this way, but humin is absent and there is no necessity to use charcoal or other decolorizing agents.

The Folin method for the determination of cystine has also been widely used. Lugg's modification of the Folin-Marenzi method (26) in principle utilizes the reduction of molybdenum free phospho-10-tungstic by cysteine and insures a rigid control of pH by the use of a citrate buffer at a pH of 5.7. This avoids the disadvantages in the color production of an alkaline reaction. In the original Folin-Marenzi method, precipitation often occurred during the determination and there were no means of correcting for extraneous reducing reagents. In Lugg's modification precipitation is avoided and an ingenious correction is applied for reducing reagents other than -SH- groups. In the presence of $HgCl_2$ cysteine produces no color with phosphotungstic acid and the color developed by other reducers is measured. This method is rapid and capable of a high degree of reproducibility. In Lugg's modification of Sullivan's method great care must be exercised to insure that the solutions, after pouring into the colorimeter cups, are completely reduced before comparison.

Bailey (7) has used various methods for the determination of cystine in proteins and has advocated Lugg's modification of the Folin-Marenzi (26) as a measure of the total disulfide and for the estimation of cystine. He states that Lugg's modification of the Folin-Marenzi method is rapid and capable of a high degree of reproducibility and that the color matching is more precise than Sullivan's method or its modifications. In view of

this information and the foregoing considerations this method would appear to be the most desirable from the standpoint of accuracy and reproducibility.

The factors responsible for errors in the determination of cystine may now conveniently be summarized: (1) errors in the method adopted, e. g., inhibition of color by extraneous reducers as in the Sullivan method; (2) losses during hydrolysis.

Methionine Determinations:

Baernstein (3) (4) has shown that on heating with HI methionine is demethylated and may be estimated as the volatile iodide. In a later paper (6) he has shown that methionine may be estimated after it has been converted to the thiolactone of homocysteine by boiling with HI.

Baernstein obtains practically quantitative results with this method. Kassell and Brand (22) have not found complete recoveries and the hydrolysis with HI have been criticized on the grounds that it causes greater evolution of volatile iodide and H_2S from insulin (which contains no methionine) than does HCl hydrolysis.

McCarthy and Sullivan (30) proposed a method for the determination of methionine based on the formation of red color formed when alkaline solutions of methionine and sodium nitroprusside are acidified. They noted, however, that both tryphtophane and histidine produce a red color under similar conditions. They destroyed the tryptophane with acid hydrolysis and stated that the addition of glycine would eliminate the color produced by histidine.

Csonka and Denton (10) have determined the methionine content of proteins and foods using the McCarthy-Sullivan colorimetric method. They

used a Coleman spectrophotometer to establish spectral transmission curves for the red color formed. Csonka and Denton found that the stoichiometric relationship of 2:1 was not valid when glycine was added to the reaction mixture. They found that glycine considerably increased the transmission. This was first thought to be caused by a reaction between glycine and sodium nitroprusside, however, further investigation revealed that the glycine also decreased the color produced by the methionine nitroprusside reaction. The common ion effect is a plausable explanation and slightly increased amounts of sodium nitroprusside is apparently an advantage. The best approach to the problem would be to separate methionine from the other materials in the protein hydrolyzate since histidine and other amino acids effect the reaction mixture.

Kolb and Toennies (23) were able to precipitate methionine quantitatively with mercury salts from a solution containing no other amino acids. When this was done with a protein hydrolyzate other amino acids were also precipitated, thus the precipitation with mercury salts is of no advantage. Histidine may be precipitated with phosphotungstic acid and it is then possible to use the nitroprusside test for methionine without the addition of glycine.

Grau and Almquist (16) used the McCarthy-Sullivan colorimetric method for the estimation of methionine in feedstuffs. They used both acid and enzymatic methods of hydrolysis and were unable to obtain complete hydrolysis of the protein material, thus found it necessary to assume that the soluble protein was representative of the total protein. Despite incomplete hydrolysis they were able to obtain consistant results using the

McCarthy-Sullivan method.

Microbiological Assays:

The ability of certain microorganisms to grow on a synthetic medium permits the development of a quantitative determination for each of the essential constituents of the medium. With the use of such a chemically defined medium this method of assay has been widely used the last few years for the quantitative determination of vitamins. Recent reports in the literature indicate that microbiological assaying may be a much simpler and more accurate way to determine amino acids than many of the chemical methods in use at the present time.

One of the first microbiological assay methods to achieve reasonable specificity was devised by Schopfer in 1935 for vitamin B_1 using the mold Phycomyces-Blakesleanus. Despite continued study and modification his method has not been widely used.

Snell and Strong (1939) developed a microbiological method for the determination of riboflavin which was rapidly accepted and soon gained widespread use. This was the first method in which lactic acid producing bacteria were used. As knowledge concerning the growth requirements of lactic acid bacteria increased they were utilized for the assay of additional vitamins. With the knowledge gained concerning the vitamin requirements of lactic acid bacteria it became possible to study the nitrogen requirements of the group. Further investigation revealed that a variety of amino acids were necessary for growth. The specificity of the different amino acids was studied and further investigation showed that this requirement, in many instances, could be made the basis for quantitative methods

for the determination of these amino acids.

Nutritive Requirements of Microorganisms:

The ideal medium for microbiological assay should contain all substances necessary or stimulatory to the growth of the microorganisms being used, but be completely free of the nutrilite to be determined. This ideal situation is approached more or less closely by assay methods now in use. An intimate knowledge of the growth requirements and optimum conditions for growth of the test organism is necessary before a satisfactory quantitative assay can be developed.

The general nutritional requirements of lactic acid bacteria may be summarized as follows: (1) energy source - carbohydrate (glucose); (2) nitrogen source - amino acids; (3) accessary substances - vitamins and other growth factors; (4) mineral salts; (5) buffer. For their energy supply the lactic acid bacteria depend upon the presence of a fermentable carbohydrate. Homo-fermentive organisms such as Lactobacillus arabinosus, Lactobacillus delbruckii and Streptococcus faecalis are able to convert glucose almost quantitatively into lactic acid.

Glucose $\longrightarrow$ 2 lactic acid

Heterofermentive organisms such as Leuconostoc mensenteroides and Lactobacillus fermenti produce other products from glucose as well as lactic acid, such as ethanol, CO_2 etc. The optimum pH for most of these organisms is between 6-7 although growth will continue until a pH of about 4 is reached. To prevent the lactic acid formed from inhibiting growth a suitable buffer must be added to the medium. Acetate was found especially suitable for this purpose by Snell, Strong and Peterson and has been widely

used. No extensive study of the mineral requirements of lactic acid bacteria has been made. For some bacteria, variations in the mineral salts have been found to improve growth. Luckey (25) showed, for example, that the addition of potassium salts greatly improved the growth of Streptococcus faecalis. Modification of the minerals used in other media might prove helpful. One is able to obtain what is known as a standard growth curve by adding to the synthetic medium differential amounts of the nutrilite to be determined. The media tubes containing varying amounts of the amino acid being treated are then inoculated with the test organism, incubated, and the lactic acid produced is then titrated with a standard base e. g., 0.1 N NaOH. The standard growth curve may then be obtained by plotting the cc. of base vs. micrograms of amino acid per tube. For the assay of protein hydrolyzates the amino acid being treated for is left out of the synthetic medium and replaced by varying concentrations of the protein hydrolyzate. Since these organisms are able to utilize such amino acids in the form of their peptides, the amino acids may be determined even in incompletely hydrolyzed proteins. The lactic acid is titrated as before and one is readily able to calculate the amount of the amino acid present in the hydrolyzate. It is necessary to experiment with synthetic media of various compositions in order to obtain a "perfect medium" in which the organism will attain maximum growth under optimum conditions. Many references are given in the literature of media which have been found satisfactory for these organisms. Although media given for the same organism may contain the same basic components, the percentage composition may vary considerably according to different references. This variance in composition

is largely due to the difference in purity of the amino acids used by different workers. The difficulty of preparing a standardized medium may be minimized by the use of pure amino acids and vitamins which are now available.

Lactobacillus arabinosus:

Pollack and Linder (35) have shown that a synthetic medium composed of twenty amino acids, salts, glucose, sodium acetate, vitamins and other growth factors will support the growth of Lactobacillus arabinosus. Snell (40) has shown that at least two strains of the original culture of L-arabinosus exist; one needs para-aminobenzoic acid, the other which is a mutant does not.

Shankman (38) has studied the amino acid requirements of L-arabinosus and found the following amino acids to be essential nutrilites:

*Cystine	Tryptophane	Isoleucine	glutamic acid
*Methionine	Leucine	Valine	Threonine

The lactic acid produced by the fermentation was titrated with 0.1 N NaOH and the titration was found to be reproducable within 0.1 ml. using brom thymol blue for the indicator.

Hier (19) has made microbiological analysis of animal proteins employing L-arabinosus, L-casei and Leuconostoc mesenteroides. The following amino acids were determined with L-arabinosus 17-5; glutamic acid, leucine, isoleucine, valine and threonine. Arginine, phenylalonine, and tyrosine were determined with Lactobacillus casei. Histidine and lysine were assayed with Leuconostoc mesenteroides. Microbiological values agree quite well with values obtained by chemical procedures and it is possible for one analyst to determine all the amino acids described by Hier in this paper

in 4 to 5 samples in approximately seven working days. McMahan and Snell (31) indicate that while all the amino acids used in their basal medium are not essential for the growth of the organism, heavier growth is obtained and antagonistic effects occur less frequently when the complete amino acid mixture is used. The media used in this paper were very satisfactory in that there were no indications of antagonistic effect observed in the various samples studied. Kuiken (24) has used Lactobacillus arabinosus 17-5 for the determination of leucine, isoleucine and valine and found that incomplete hydrolysis showed low values for these acids. Alkaline hydrolysis is unsatisfactory because it brings about racemization and only natural occurring forms of these acids are utilized.

Nyman and Gortner (33) have made studies on Lactobacillus arabinosus 17-5 cultures and have found that degraded cultures of L-arabinosus and L-casei can be rejuvenated by a change in transfer medium, a greater frequency of transfer and by incubation of the culture at an optimum temperature of 32°C. They recommend the use of liver tryptone agar and liver tryptone broth as stock culture media. Hegsted (17) investigated the amino acid requirements of Lactobacillus arabinosus 17-5 and found that growth failed when ariginine, cystine, glutamic acid, isoleucine, leucine, methionine, phenylalanine, tryptophane and tyrosine or valine were omitted from the basal medium. Growth did not occur in a mixture of only ten amino acids. Growth measurements were made with both turbidimetric measurements and by titration of the lactic acid produced. Titration usually gave more consistent results.

Streptococcus faecalis and Lactobacillus delbriickii.

Stokes and Gunnes (42) have developed a standard assay procedure for ten essential amino acids (including methionine) applicable to foodstuff and other natural products. Nine of the amino acids can be determined with S-faecalis and phenlyalanine with L-delbrüchii. Although two organisms are used only one standard medium and one procedure is necessary. The amino acid is measured by titration with standard alkali with the exception of histidine only/(l) isomers are available and (d) isomer is inactive. Lower microbiological values were consistently obtained for methionine as compared with chemical methods; it is possible that the chemical values may obtain substances other than methionine.

Leuconostoc mesenteriodes:

The amino acid requirements of Leuconostoc mesenteriodes P-60 have been investigated by Dunn (11). A standard growth curve has been determined for both methionine and cystine. He found that large concentration of salts inhibit the growth of Leuconostoc mesenteriodes so acid hydrolyzates of protein should have most of the acid removed before being neutralized with NaOH to bring the pH up to 7.

Other Organisms:

Dunn (12) has made determinations of methionine in protein hydrolyzates with Lactobacillus fermenti 36*. He found that methionine was essential or accessory for the satisfactory growth of: Lactobacillus casei, Lactobacillus arabinosus 17-5, Lactobacillus fermenti, and Leuconostoc mesenteriodes P-60. d (+) Methionine was found to be completely inactive

in promoting growth of L-arabinosus and Leuconostoc mesenteriodes. It, however, stimulated the growth of Lactobacillus fermenti to an extent entirely comparable to that exhibited by the l (-) antipode. The ability of a microorganism to utilize both optical isomers effectively is an unique property not hitherto recorded in the literature. He determined the methionine content of hydrolyzed casein and silk fibroin with this organism. The basal medium C of composition given by Dunn (10) was used.

Barton-Wright (8) has had very satisfactory results using L-arabinosis for the assaying of cystine and Leuconostoc mesenteriodes for methionine.

IV. STATEMENT OF THE PROBLEM

1. Variety of range grasses used:
   (a) Agropyron cristatum
   (b) Agropyron smithii
   (c) Boutelcua gracillis
2. Proteins extracted from the dried grass by means of:
   (a) Sodium dodecyl sulfate, Smith (39)
   (b) Formic acid extraction, Albanese (1)
3. Extracted proteins were purified, hydrolyzed and subjected to analysis for both methionine and cystine.
4. Cystine estimated by Lugg's modification of Folin-Marenzi method (26).
5. Methionine estimated by McCarthy-Sullivan colorimetric test Csonka and Denton (10).
6. Microbiological assays were carried out on the leaf protein extracts using L-arabinosus for cystine and Leuconostoc mesenteroides for methionine.

V. EXPERIMENTAL WORK

Preparation of Samples:

The grass samples were collected from Experiment Station plots located at Bozeman, Montana. The grasses were grown under as nearly identical conditions as possible. The grasses were cut once in the spring and again in midsummer after they had reached the early bloom stage.

List of Grasses and Dates of Cutting:

Agropyron cristatum	5-10-46	7-15-46	
Agropyron smithii	5-10-46	7-15-46	
Bouteloua gracillis	- - - -	7-15-46	8-29-46

The grasses after cutting were brought to the laboratory and spread out to dry. They were dried as quickly as possible in a warm room and all weeds and other extraneous material were carefully removed by hand picking.

After drying the grasses were ground in a Wiley mill until they passed through a 40-mesh screen then stored in air tight glass jars.

Total nitrogen determined by the Kjeldahl method was multiplied by the factor 6.25 to give the crude protein content.

Protein Extraction:

The first method used for protein extraction was that of Albanese (1) which is the formic acid extraction method. Preliminary experiments by Albanese (1) demonstrated that the formic acid extraction of fresh vegetables after acetone extraction yielded high nitrogen recovery with an unavoidable impurity of a polysaccharide nature. He observed that the addition of two volumes of ethanol to one volume of formic acid extract of

spinach leaves resulted in a quantitative precipitation of these carbohydrates with only a negligible loss of nitrogen.

Since the polysaccharides appear to be the principal carbohydrate contaminants of the fractions resulting from the formic acid extraction of vegetables, Albanese has concluded that the solubility of the proteins and the insolubility of the polysaccharides in the formic acid-ethanol mixture offers a simple method for the purification of the protein moiety.

Albanese states that formic acid extraction may not be applicable to some types of feedstuff, however, it was decided to try the method to see what results could be obtained. Bouteloua gracillis cut August 22, 1946, was used to test this method. After cutting, the fresh grass was frozen by storage in the freezing compartment of an electric refrigerator set at 0°C. This served to break down the cell walls, thereby increasing the permeability to solvents. 1195 g. of grass was treated in this manner, then chopped up, placed in a five gallon food jar and covered with acetone for two days. This treatment with acetone tended to remove much of the remaining moisture and many of the soluble plant pigments. After standing two days the acetone was decanted off and the grass was wrapped in a piece of cheese cloth and submitted to a continuous acetone extraction for 24 hours. This extraction was carried out at 53°C.

Since excessive decomposition of formic acid at its boiling point prevented the use of continuous extraction technique, the acetone extracted grass was treated as follows: The cloth sack and its contents were removed from the extraction chamber and placed in a current of air for several hours to drive off the acetone. A sample was then taken of the acetone ex-

tracted grass for a total nitrogen determination and the remainder of the grass was placed in 2 l. beakers covered with 90% formic acid and placed on a hot plate, the temperature of which was carefully maintained between 75-80°C. The formic acid extraction was continued for 24 hours, then repeated until nearly all of the protein and carbohydrate material had been removed. After the extraction was complete, the formic acid was decanted off and the fibrous residue transferred to a 2 gallon enameled pail and mixed mechanically for thirty minutes with three liters of 95% ethanol to facilitate handling and subsequent drying. This residue after drying was light brown in color and woody in appearance and texture. A sample of this dried material was collected for total nitrogen determination. The alcohol filtrate was brought to volume of 2 l. and added to the combined formic acid fractions which had previously been concentrated in vacuo to 1 liter. The mixture then was allowed to stand two hours at room temperature and the precipitated carbohydrates filtered off using fluted filter paper. The carbohydrate material was dried and preserved for nitrogen determination. The filtrate was reduced in vacuo to remove all the ethanol and formic acid, then taken to dryness in a large evaporating dish at about 60°C. This final residue which constituted the protein material was a dark brown to black in color.

The dark color may have been caused by plant pigments which were not removed by the hot acetone extraction or by the presence of humin material. In an effort to further purify the preparation, it was subjected to hot acetone extraction for 24 hours in a continuous extraction apparatus. This last extraction was of very little value and no visible purification was observed.

Extraction of Leaf Protein by use of Sodium Dodecyl Sulfate, Smith (39).

The following grasses were subjected to this type of extraction:

A-cristatum 5-10-46 7-15-46

A-smithii 5-10-46 7-15-46

B-gracillis 7-15-46 8-29-46

The extractions were carried out on air-dried grasses after they had been ground to pass through a 40-mesh sieve.

Reprecipitated sodium dodecyl sulfate was used for this extraction. A sample of 50 g. was placed in a glass jar and about 200 cc. of a 2% solution of sodium dodecyl sulfate added until the jar was about one-half full. The jar was placed in a shaking machine in a horizontal position to give the maximum swirling motion, and shaken for three hours. The jar was then removed and the material filtered through a piece of cheese cloth squeezing out the excess liquid. This extraction was carried out three times for 3 hours each using fresh sodium dodecyl sulfate solution each time. The filtrate was centrifuged after each extraction and the sludge thus obtained was submitted to further extraction. After the final extraction, 95% ethanol was added with rapid stirring to the clear filtrates and then allowed to stand until precipitation was complete. The supernatant liquid was decanted off and the protein material was purified by a continuous acetone extraction for 24 hours.

After the acetone extraction the protein preparation was placed in a vacuum oven at 50°C. for 3 hours until the material was thoroughly dry. The final product was then ground up in a mortar to facilitate homogeneous sampling. The product was white to light gray in color with a slight greenish

tint. Kjeldahl nitrogen determinations were run on:

(a) original grass sample

(b) sludge remaining from centrifuging

(c) residue after extraction with sodium dodecyl sulfate

(d) purified protein.

Hydrolysis of Protein:

In view of the satisfactory results obtained by Barton-Wright (8), Schweigert (37) and Stokes (42) using dilute solutions of HCl, it was decided to use the following procedure: One gram sample of finely ground material was hydrolyzed by autoclaving for six hours at 120°C and 15 pounds pressure with 25 ml. of 2.5 N HCl. The hydrolysis was carried out in 50 ml. flasks fitted with glass caps. After hydrolysis the solution was allowed to cool and the pH adjusted to 6.8 using the Beckman pH meter. The solutions were then brought to a volume of 100 ml., filtered, covered with a thin layer of sulfur free toluene and stored in the refrigerator. All the leaf proteins as well as casein samples, which were used to check the different assay methods, were hydrolyzed by this method.

Colorimetric Method for Cystine, Folin and Marenzi (14); Lugg (26); Kassel and Brand (22).

Theory: Phospho-18-Tungstic acid (Folin's reagent) was used for the colorimetric determination of cystine. The blue color developed depends upon the reduction of phospho-18-tungstic acid by cysteine. Cystine is first reduced by sodium sulfite to give cysteine. The phospho-18-tungstic acid is prepared essentially by treating sodium tungstate with phosphoric acid. The colora-

tion developed by a reducer may vary with the time allowed for the reaction, the concentration and amounts of the reacting materials, the pH of the reacting mixture, salt concentration and the type and order in which the materials are added.

Evidence has been adduced by Lugg (26) to show that the reaction between cystine and sulphites in cold aqueous solution is not a reduction in the ordinary sense of the term, but that it is most probably expressed by the equation $R - S - S - R + H_2SO_3 \longrightarrow RSH + RHS_2O_3$ where $R - S - S - R$ and $R\,S\,H$ represent cystine and cysteine respectively. Then $2\ R\,S\,H$ + reagent $\rightleftharpoons$ $R - S - S - R$ + reduced reagent. By estimating cysteine and cystine in acid solution all the disadvantages of alkaline color development may be avoided.

The disadvantages of acid color development include slight loss in color and diminution in the rate of development. For all around utility a pH of 5.7 has been selected, and to minimize color development by extraneous reducers and for the sake of economy only small amounts of reagent are employed. A standard is prepared using cystine plus sulfite. When extraneous reducers are present their effects must be found by artifice. In the presence of $HgCl_2$ cysteine will give no color, thus the blank used for comparison was prepared exactly the same as the unknown except for the addition of $HgCl_2$ which removes the cysteine leaving only extraneous reducers to effect color development.

Lugg's (26) modification of the Folin and Marenzi method (14) is designed for use with the visual colorimeter and he has adopted a rather ingenious method of correcting for extraneous reducers. The correction for extraneous

reducers may be very greatly simplified by the use of the spectrophotometer.

Kassell and Brand (22) have adapted Lugg's-Folin method to the use of the Pulfrich Photometer. Perhaps the method used in this thesis should more correctly be referred to as the Kassel and Brand rather than the Lugg method, although they are essentially the same from the standpoint of the reagents used to bring about the color development.

Reagents:

All reagents used were the same as those specified by Lugg (26).

Phospho-18-tungstic Acid Reagent:

This was prepared according to the directions of Folin and Marenzi (40) with the separation of any molybdenum present according to their plan. It may be looked upon as containing per liter 100 g. of the salt $Na_2WO_4 \cdot 2H_2O$, 172 grams of H_3PO_4 and 16.1 g. of LiOH. The sodium tungstate should be prepared by dissolving the necessary amount of precipitated WO_3 in the necessary amount of NaOH solution. If the reagent fails to comply with the following requirements it must be discarded:

(1) It must give no coloration with tyrosine or tryptophane at any pH.

(2) At pH 5.7 and 8.4, 4 mg. of cystine in the presence of 2 cc. of reagent and 1 millimole of sulphite must give not less than 3.95 times the coloration developed by 1 mg. of cystine.

(3) At pH 5.7 and 8.4 and in the absence of zinc and ammonium salts, 80 mg. of cystine with 2 cc. of reagent must give almost exactly seven times the coloration developed by 2 mg. of cystine.

The reagent prepared by the following method met all these requirements: 50 grams of sodium tungstate and 100 cc. of water were added to a 500 cc. Florence flask, shaken until the tungstate dissolved, then added slowly with cooling 10 ml. of 85% phosphoric acid. The solution must not be allowed to become warm from the heat of reaction. Hydrogen sulfide was passed into the phosphotungstic acid solution at a very moderate rate for 20 minutes. At the end of the first 3-4 minutes 5 ml. of H_3PO_4 was added without interrupting the H_2S current. (This enables one to obtain the molybdenum sulfide in a somewhat less finely divided condition so it can be filtered more easily.) At the end of 20 minutes the solution was filtered using a No. 40 Whatman filter paper. When conditions are right, the filtrate should be clear and have greenish color. The filtrate was then transferred to a separatory funnel and 150 cc. of 95% ethanol added with shaking. The bottom solution contained all the phosphotungstic acid in a supersaturated solution. This solution was then withdrawn into a weighed 300 ml. flask and water added until the weight of the contents was 150 grams. The solution was boiled a few minutes over a micro burner and tested with lead acetate paper to be sure all the hydrogen sulfide had been removed. The flame was cut down and 10 ml. of 85% phosphoric acid was added. The flask was then boiled gently with refluxing for one hour. The solution was filtered and a few drops of bromine were added and the solution was boiled until all the blue color was gone, then boiled vigorously several minutes to remove the bromine. The flask was then covered with a beaker and cooled under running water. A lithium phosphate solution was prepared by placing 12.5 g. of lithium carbonate and 25 ml. of phosphoric acid in a liter beaker, then slowly adding 125 ml. of water, the resulting solution was then boiled to remove CO_2, cooled, and then added to the phos-

photungstic acid solution and the total volume brought to 500 ml. with distilled water.

Sodium Acetate Solution:

A 4 molar sodium acetate solution was prepared containing 544 g. of the salt $C_2H_3O_2Na \cdot 3H_2O$ per liter of solution.

Citrate Buffer Solution:

This contains 105 g. of crystalline citric acid $C_6H_8O_7 \cdot H_2O$, 52.5 g. of NaOH, 13.6 g. of $ZnCl_2$ and 26.8 grams of NH_4Cl per liter. It was prepared by shaking the citric acid and NaOH with about 500 cc. of water with constant cooling until entirely dissolved. The $ZnCl_2$ was dissolved in 20 cc. of water and added to the cold solution, and this followed by the NH_4Cl dissolved in about 200 cc. of water. The mixture then diluted to 1 liter.

Buffered Sulphite Solution:

9.5 grams of sodium metabisulphite ($Na_2S_2O_5$) dissolved in about 70 cc. of water, 15 cc. of 4 M sodium acetate solution was added and the mixture diluted to 100 cc. Lugg states that this solution will keep for months in a stoppered bottle, however, it was found necessary to prepare this solution fresh daily in order to obtain a reliable standard curve.

Mercuric chloride:

A 0.1 M solution of mercuric chloride was prepared containing 2.72 grams of $HgCl_2$ per 100 cc. of solution.

Standard Cystine Solution:

0.5 grams of l (-) cystine was dissolved in 50 cc. of 1 N HCl and dil-

uted to 500 cc. to provide a .1 N HCl solution containing 1 mg. of cystine per ml. The solution was covered with a thin layer of toluene and stored in the refrigerator. Solution was diluted 1:10 to give .1 mg. of cystine per ml. when used.

Estimation in General:

All estimations were carried out in standard 100 cc. flasks. Standard was prepared at the same time as unknown. Seven minutes were allowed for the color development, then the solution was diluted to the mark and read at 650 mu. in a Coleman spectrophotometer. Aliquots of the unknown cystine solution were brought to a pH of 5.7 with sodium acetate solution.

Preliminary work was done to determine what effect the order of addition of the reagents had on the color development and standard curves thus obtained. The rate of color development was studied. The effect of using fresh sulphite reagent was investigated. Hydrolyzed casein samples as well as leaf proteins were assayed by this method. The casein was used to provide literature checks. Vitamin test casein was first used, however, after a number of assays it was found to be unsatisfactory and casein prepared by the Hamerstein method proved to be of better quality than the commercial product. Assays were run with cystine added to the sample both before and after hydrolysis to determine cystine recovered.

Experimental Procedures:

The order of addition of the reagents was found to be very important. The reagents were added from a burette to the reaction flask, the flask well shaken after each addition. The only order given by Lugg (26) was found

to be satisfactory and any change in the order of the addition of the reagents resulted in unsatisfactory color development and inconsistency in the standard curves obtained. To obtain a standard curve the following reagents were added in given order to a 100 ml. volumetric flask with shaking after each addition.

10 cc. of citrate buffer
2 cc. phospho-18-tungstic acid reagent
2 cc. sodium acetate
Standard cystine solution (.1 mg./ml.)
1 cc. buffered sulphite

The color was allowed to develop 7 minutes and the flask diluted to the mark. A standard blank was prepared same as above omitting the cystine solution. A standard blank was found necessary inasmuch as the sulphite reagent tends to reduce the phosphotungstic acid. The amount of color due to the reduction depends upon the age of the sulphite solution. The range of the standard curve was from 0.1 mg. to 0.8 mg. of cystine. At first 8 levels of concentration were determined for the standard curve. Later, however, it was found that four points were sufficient to provide reliable accuracy. Standard cystine was run at 0.1, 0.2, 0.4, and 0.8 mg. of cystine with reliable uniformity in the results. A standard cystine curve is given on Page 54, Figure 2.

In order to determine the rate of color development the following scheme was used: Using 10 ml. of standard cystine and the order of addition indicated above, the color was allowed to develop four minutes, then 2 ml. aliquots were taken every minute for seven minutes. Each aliquot was immed-

iately diluted to 8 ml. with distilled water to halt the color development and read at 650 mu. After the first reading the tubes were allowed to stand 30 minutes, then read again. The optimum color development was found to occur at 7 minutes. The dilution halted the color reaction and there was no change in the readings taken at the end of the 30 minute period. A curve illustrating the rate of color development is given on Page 53, Figure 1. It has been thought advisable by some investigators to decolorize the protein hydrolyzate before testing for cystine, various decolorizing charcoals are recommended for this purpose.

Several adsorbant charcoals were tested and found to be unsatisfactory for this purpose since in addition to decolorizing the hydrolyzate, a practically quantitative adsorption of cystine was observed. 300 mg. of Darco was added to 10 ml. of standard cystine solution, the mixture shaken and allowed to stand for 1 hour, filtered and the cystine determined on the filtrate. 98% of the cystine was adsorbed. This was tried with several other charcoals which were available and all showed similar results. So in view of these observations, it was decided advisable to forego the decolorization. The hydrolyzates ranged from light amber to brown in color and on dilution of the aliquot sample to 100 ml. this color was greatly diminished. By using the following scheme for preparing the unknown blank correction is made for the color of original solution as well as that due to extraneous reducers.

Unknown	Unknown Blank
10 cc. citrate buffer	10 cc. citrate buffer
2 cc. color reagent	2 cc. color reagent
2 cc. sodium acetate	2 cc. sodium acetate

(Continued on page 34).

(Continued from page 33).

Unknown	Unknown Blank
Aliquot of unknown (titrated to pH 5.7 with sodium acetate)	1 cc. mercuric chloride
1 cc. buffered sulphite	aliquot of unknown (pH 5.7)
	1 cc. buffered sulphite

Seven minutes was the time allowed for color development and the volume was then diluted to the 100 ml. mark. The same size aliquot is taken for the unknown and the blank so that both contain the same concentration of extraneous reducers. The 100 ml. flask containing the reaction mixture was thoroughly mixed and aliquots transferred to calibrated colorimeter tubes, the readings taken at 650 mu.

The unknown blank was placed in the colorimeter, the scale indicating the per cent transmission was set at zero and the unknown solution then matched against this blank. The amount of cystine was then calculated from the per cent transmission with reference to the standard curve.

It was found that the age of the sulphite reagent greatly effected the slope of the standard curve and that by using freshly prepared sulphite solution practically the same curve was obtained each time.

Colorimetric Determination of Methionine using Sodium Nitroprusside Reagent, (30), (16), (10).

Experimental Procedures:

An aliquot of acid hydrolyzed protein was transferred quantitatively to a 25 ml. volumetric flask and sufficient conc. HCl added to bring the acid concentration up to 4-5% HCl (which is a suitable concentration for the precipitation of the basic amino acids with phosphotungstic acid). An aqueous solution of phosphotungstic acid containing 1 gram per 1 ml. was added drop-

wise until precipitation was complete, then a few drops in excess. Solution was then made to a volume of 25 cc. with distilled water, allowed to stand in an ice bath for two hours, after which it was centrifuged for ten minutes and placed in the ice water bath again for one-half hour. The supernatant liquid was then filtered through a number 40 Whatman paper. After the filtrate had reached room temperature, an aliquot containing not more than 300 mg. of the original protein material was transferred to another 25 ml. volumetric flask and 5 N NaOH added dropwise to a pH of 4 using the Beckman pH meter. The solution was then made to volume with distilled water.

An aliquot of 4 ml. or less of this filtrate was transferred to a centrifuge tube. To this 2 ml. of 5 N NaOH was added and sufficient quantity of distilled water to bring the total volume to 6 ml. Then 1 ml. of freshly prepared 1% sodium nitroprusside was added and the contents of the tube thoroughly stirred. The tube was placed in a water bath for 8 minutes at 40°C., then in an ice water bath for 5 minutes after which 2.5 ml. of 20% HCl was added slowly with vigorous shaking. The shaking was continued in the bath until the characteristic color development in about 1 minute, then stirred mechanically for another minute at room temperature. The solution at this point often becomes slightly turbid so it was found necessary to centrifuge at high speed for 5 minutes and then transfer the solution to a calibrated colorimeter tube and read at 510 mu in the Coleman spectrophotometer.

A standard methionine curve was obtained by treating a standard solution in (.1 mg. of methionine per ml.) exactly the same manner. Distilled water was used for the blank and the curve plotted with log of per cent transmission vs. mg. of methionine. The range of the curve was from .25 mg.

to 1.25 mg. of methionine with readings taken at 6 levels. By using freshly prepared sodium nitroprusside reagent the curve was found to be exactly the same each time (within limits of experimental error) with the slope of the curve remaining constant. A standard methionine curve is given on Page 67, Figure 6. The methionine content of acid hydrolyzed leaf proteins, vitamin test casein, and freshly prepared casein was determined by this method.

Microbiological Determination of Cystine Using Lactobacillus-arabinosus 17-5:

All microorganisms used for these tests were obtained directly from American Type Culture Collection, Washington D. C. The greatest care was taken in handling the stock cultures to prevent any contamination. Immediately upon receiving the cultures, transfers were made to liver-tryptone-agar stabs with weekly transfers being made from the agar stab to liver tryptone broth, then to the agar again the following week. This process was repeated every 7 days as recommended by Nyman and Gertner (33) for increasing the activity of L. arabinosus 17-5. A similar medium prepared from bacto peptone and bacto tryptone with riboflavin added was found to be even more effective than the liver-tryptone medium in stimulating the growth of L. arabinosus 17-5 and Leuconostoc mesenterioides P-60.

All amino acids and vitamins used in preparing the standard media were of the highest purity obtainable and where possible the dl synthetic amino acids were used rather than natural occurring product because of the higher purity of the synthetic preparations.

Water used for making up the synthetic media was distilled from pyrex glass to eliminate any effect due to heavy metal ions. The redistilled water was stored in a 5 gallon pyrex glass bottle and tightly covered to keep out dust.

All glassware used was cleaned either with Dreft or trisodium phosphate and thoroughly rinsed with distilled water. Chromic acid cleaning solution was avoided to prevent any effect due to chromate ions. All transfers were carried out in an inoculating room where dust and air currents were kept at a minimum to prevent contamination by spore forming organisms.

The standard mediumrecommended by Barton-Wright (38) was used with certain modifications which were found to be necessary.

Preparation of Stock Solutions:

The stock solutions described below were preserved in the presence of a thin layer of sulphur free toluene and stored in a refrigerator. All stock solutions were prepared with glass distilled water.

l-cystine: 0.5 grams of l-cystine was dissolved in 300 ml. of water and 2 ml. of concentrated HCl was added, the final volume made up to 500 ml. and stored in a refrigerator. This cystine solution was diluted before use to give a standard solution containing 10 micro-grams of cystine per ml.

Glucose, sodium acetate, sodium chloride and ammonium sulphate were weighed out as required.

Biotin: An ampoule containing 25 ug. of the free acid was made up to 250 ml. with water. One ml. of salt solution A was added prior to making up to volume. The solution contained 0.1 ug. of Biotin per ml.

p-aminobenzoic acid: 0.1 gram of p-aminobenzoic acid was dissolved in 100 ml. of water containing 1 ml. of glacial acetic acid.

Thiamine, Nicotinic acid, Calcium d-pantothenate and Pyrodoxine: A separate stock solution of each of these substances was prepared by dissolving 0.1 g. in 100 ml. of water.

Folic Acid: Concentration of this vitamin was 2 ug/ml. dissolved in 50% ethanol.

Riboflavine: One ml. of glacial acetic acid was added to 25 mg. of riboflavine and the solution diluted to 1000 ml. with water solution contained 25 ug. per ml.

Adenine, guanine and uracil: A single stock solution of these three substances was prepared containing 1 mg. of each per ml. Solution was brought about by heating with a few drops of concentrated hydrochloric acid.

Xanthine: The concentration of this solution was also 1 mg. per ml. Solution was effected by a few drops of strong ammonia.

The various stock solutions of vitamins with the exception of biotin were renewed at weekly intervals, riboflavin and pyrodoxine were prepared fresh each time and the adenine, guanine, uracil and xanthine solutions were renewed at two-week intervals. All stock solutions were preserved under toluene in a refrigerator.

Inorganic Salt Solution A: This was composed of 25 g. of K_2HPO_4 and 25 g. of KH_2PO_4 in 250 ml. of water.

Inorganic Salt Solution B: This solution contained 10 g. of $MgSO_4 \cdot 7H_2O$, 0.5 g. of $MnSO_4 \cdot 4H_2O$ and 0.1 g. of $FeCl_3$ in 250 ml. of water. Addition of a few drops of concentrated hydrochloric acid was necessary to prevent the formation of any precipitate in the mixture.

Preparation of Inoculum:

The inoculum was prepared by making a transfer from the agar stab culture into a bacto-peptone, bacto-tryptone broth (composition of this special media is given on Page 44.) This was incubated for 18-20 hours at 37°C. It was then centrifuged aseptically and, after the supernatant liquor had been poured off and replaced by 10 ml. of 0.9% sterile saline solution, it was stirred with a platinum wire and centrifuged again. This washing operation was repeated again with another 10 ml. of saline solution. After the third centrifuging and replacement with 10 ml. saline solution, 2-3 ml. of this suspension was diluted to 100 ml. of saline solution, thoroughly mixed to insure a uniform suspension and this dilute inoculum was used for subsequent inoculation.

The fermentations were carried out in pyrex test tubes which were calibrated to the 10 ml. mark. Aluminum caps 2.5 x 5 cm. which fit loosely over the mouth of the tube were used instead of cotton plugs. The aluminum caps were easy to wash and they saved a great deal of time which would otherwise have been spent rolling plugs.

Assay Procedure:

The following amounts of amino acids, vitamin supplements, purine and pyrimidine bases and mineral salts, when made up to a volume of 500 ml., gives sufficient basal medium for 100 tubes, 5 ml. media per tube.

Amino Acids		Vitamin Supplements etc.	
l (+) glutamic acid	400 mg.	Aneurine (stock sol. dil. 1:10)	2.0 ml.
dl aspartic acid	800 mg.	Calcium d-pantothenate (dil. 1:10)	2.0 ml.
l (+) lysine HCl	200 mg.	Pyridoxine solution (dil. 1:10)	2.0 ml.

(Cont'd page 40)

(Cont'd from page 39.)

Amino Acids		Vitamin Supplements Etc.	
dl threonine	200 mg.	Riboflavine solution	16.0 ml.
dl valine	200 mg.	Nicotinic acid solution (dil. 1:10)	4.0 ml.
dl isoleucine	200 mg.	p-amino benzoic acid soln. (dil. 1:10)	1.0 ml.
dl alanine	200 mg.	Biotin solution	5.0 ml.
l (-) leucine	100 mg.	Adenine + guanine + uracil solution	10.0 ml.
dl methionine	100 mg.	Xanthine solution	10.0 ml.
dl phenylalanine	100 mg.	Glucose	20.0 g.
l (+) arginine HCl	50 mg.	Sodium acetate $3H_2O$	33.2 g.
l (+) histidine HCl	50 mg.	Sodium chloride	5.0 g.
l (-) tyrosine	40 mg.	Ammonium sulphate	3.0 g.
dl tryptophan	80 mg.	Inorganic Salt Solution A	5.0 ml.
		Inorganic Salt Solution B	5.0 ml.

This mediumrecommended by Barton-Wright (8) was used for a number of assays and found to be inadequate in several respects. Using this basal medium large blanks were obtained and the growth response was not proportional to the amount of cystine added. This was found to be due partly to the impurity of one of the amino acids used. The l (+) glutamic acid first used was found by a colorimetric determination to contain 0.11% cystine which was responsible for the high blanks. Even before this difficulty was discovered it was found that there were several limiting factors other than cystine. The vitamin concentration was found to be too low and the addition of several other amino acids were found to be stimulatory to the growth of L-arabinosus. The difficulty of high blanks due to impurity of the l(+) glutamic acid was

corrected by using l (+) glutamic acid prepared by Merck which contained no appreciable amount of cystine. After numerous assays the following medium was selected.

For 500 ml. Basal Medium

Amino Acids		Vitamins and Supplements, Etc.	
l (+) glutamic acid	400 mg.	Thiamine	3 ml.
dl aspartic acid	800 mg.	Calcium d-pantothenate	3 ml.
l (+) Lysine HCl	200 mg.	Pyrodoxine	3 ml.
dl threonine	200 mg.	Riboflavine	50 ml.
dl valine	200 mg.	Nicotinic acid	5 ml.
dl isoleucine	200 mg.	P-amino benzoic acid	2 ml.
dl alanine	200 mg.	Biotin	25 ml.
l (-) leucine	100 mg.	Folic Acid	2 ml.
dl methionine	100 mg.	Adenine + guanine + uracil	15 ml.
dl phenylalanine	100 mg.	Xanthine	15 ml.
l (+) arginine HCl	50 mg.	Glucose	20. 0 g.
l (+) histidine HCl	50 mg.	Sodium acetate $3H_2O$	33.2 g.
l (-) tyrosine	40 mg.	Sodium chloride	5.0 g.
dl tryptophane	100 mg.	Inorganic Salt Solution A	5 ml.
l (-) proline	50 mg.	Inorganic Salt Solution B	5 ml.
dl serine	100 mg.	Note; The vitamin solutions were used in	
dl norleucine	100 mg.	the same concentration as the stock	
dl norvaline	100 mg.	solution (not diluted 1:10).	
glycine	100 mg.		

The amino acids were weighed out and dissolved in 100 ml. of water. Addition of two to three drops of concentrated hydrochloric acid and gentle warming was found necessary to bring about complete solution. Tryptophane was dissolved separately by warming in the presence of a few drops of concentrated hydrochloric acid, tyrosine by warming with .1 N NaOH solution. The remaining components were then added to the amino acid mixture and the pH adjusted to 6.8 with a few drops of concentrated NaOH solution. The whole was made up to a volume of 500 ml with water. This gave a mixture having twice the concentration of the final assay medium. Five ml. of the mixture was transferred to each tube. Thirty-one tubes were retained for the blanks and the standard cystine curve. The tubes were set up in triplicate and to them added serially 5 ml. water (= blank), 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, ml. of standard cystine solution containing 10 ug. of cystine per ml. To each tube sufficient distilled water was added to bring the final volume to 10 ml. The remaining tubes were used for the assay of the hydrolyzates. It was found desirable to carry out each assay at four levels of concentration and it was most convenient to do this by adding 1, 2, 3 or 4 ml. of the protein hydrolyzate to 5 ml. of basal medium, then bringing the solution to a final volume of 10 ml. with distilled water. After all solutions had been added the tubes were shaken until thoroughly mixed and autoclaved at 15 lbs. pressure, 118.5° C. for ten minutes. A higher temperature or longer period of autoclaving caused carmelization of the media which made it worthless for microbiological assaying. The tubes were run in triplicate at each level of concentration.

After autoclaving the tubes were allowed to cool then inoculated using a 5 cc. syringe (which had previously been sterilized by autoclaving at 120°C for 30 minutes). Two drops of inoculum were transferred to each tube. The concentration of the inoculum was found to be a very important factor, if the inoculum was too concentrated or if there was a clumping of the organisms, erratic growth curves resulted.

The tubes were then placed in an incubator for 36-40 hours at 37°C. The temperature as well as the length of the incubation period were found to have a very definite effect on the standard curve obtained. Barton-Wright as well as several other investigators have recommended 32° C. for 72 hours which proved to be unsatisfactory. It was found that 37° C. for 36-40 hours gave a standard growth curve which was much more reliable and had a steeper slope. It was necessary to stop growth after about 40 hours in order to stop the lactic acid development which exceeded the buffering capacity of the medium when longer incubation periods were used.

After incubation the contents of the tubes were quantitatively transferred to 125 ml. flasks and lactic acid formed by the fermentation was titrated with 0.1 N NaOH using brom thymol blue as the indicator. A standard curve was then drawn by plotting ug. of cystine vs. cc. of 0.1 N NaOH.

Grass leaf proteins, vitamin test casein, and freshly prepared Hammerstein casein were assayed by this method. Cystine was added in known amounts both before and after hydrolysis to determine the recovery.

Leuconostoc mesenterioides P-60 was also used in assaying for cystine. The method used and the basal media for Leuconostoc mesenterioides are given in the following section, The Microbiological Assay of Methionine. A stan-

dard curve for cystine using Leuconostoc mesenterioides is given on page 60, Figure 5. The liver-tryptone-agar and broth medium used to carry stock cultures was prepared according to directions given by Nyman and Gortner (33) and contained the following:

Liver tryptone-agar (500 ml.)		Liver-tryptone-Broth (500 ml.)	
Tryptone	5.0 grams	Tryptone	5.0 grams
glucose	5.0 grams	Glucose	1.0 gram
K_2HPO_4	1.0 grams	K_2HPO_4	2.5 grams
$CaCO_3$	1.6 grams	Yeast extract	1.0 gram
Agar	9.0 grams	Liver extract	50.0 ml.
liver extract	60.0 ml.		
inorganic salts A	3.0 ml.		
inorganic salts B	3.0 ml.		

pH was adjusted to 7 and 10 ml. dispensed in each tube. Leuconostoc mesenterioides was carried on the same medium except an agar slant was used rather than stab culture. Another medium which was even more effective in stimulating growth had the following composition:

Per Liter Media		For Stabs	
2.5 g.	Bacto peptone	1%	yeast extract
2.5 g.	Bacto tryptone	1%	glucose
1.0 g.	Bacto yeast	1.5%	agar
10.0 g.	Sodium acetate		
10.0 g.	Glucose		
5.0 ml.	Inorganic salt solution A		
5.0 ml.	" " " B		
2.0 mg.	Riboflavin		

pH adjusted to 7.

For the preparation of Hamerstein casein the method given by Mitchell (32) was used. A two quart sample of skimmed milk was adjusted to a pH of 4.63 using 0.05 N HCl, stirring rapidly with a mechanical stirrer. The resulting precipitate was washed repeatedly with distilled water by decantation and then dissolved by adding sufficient 0.1 N NaOH to bring the mixture to a pH of 6.3. The mixture was filtered through a suction filter using filter cloth and the precipitate discarded. The casein was reprecipitated using 0.5 N HCl at a pH of 4.63, filtered, washed, redissolved and precipitated again. The precipitate was washed free of chlorides with distilled water, then dehydrated by alcohol and ether. The resulting product was white in color and appeared to have greater purity than the commercially produced vitamin test casein which had been used earlier.

Microbiological Determination of Methionine Using Leuconostoc mesenterioides P-60.

Microbiological method used for methionine was that given by Barton-Wright (8) using Leuconostoc mesenterioides P-60. Very little modification of his method was found necessary and the techniques used were the same as those described for cystine. The lactic acid developed by the fermentation was titrated against 0.1 N NaOH using brom thymol blue indicator. Few difficulties were encountered and except for slight changes, the basal medium suggested by Barton-Wright was used. All stock solutions were prepared as before.

Assay Procedure: The following amounts of amino acids, vitamin supplements, etc. gave sufficient media for 100 tubes.

Volume Made Up to 500 ml.

Amino Acids		Other Components	
dl- -Alanine	1000 mg.	Glucose	20.0 g.
l (+) Arginine HCl	250 mg.	Sodium acetates $3H_2O$	19.9 g.
dl Aspartic acid	800 mg.	Ammonium chloride	6.0 g.
l (-) cystine	100 mg.	Sodium chloride	5.0 g.
l (+) glutamic acid	500 mg.	Adenine + guanine + uracil soln.	15.0 ml.
glycine	100 mg.	Xanthine solution	15.0 ml.
l (+) histidine HCl	100 mg.	Aneurine solution	3.0 ml.
dl-Isoleucine	200 mg.	Pyridoxine solution	3.0 ml.
l (-) Leucine	100 mg.	Calcium-d-pantothenate solution	3.0 ml.
l (+) Lysine HCl	125 mg.	Nicotinic acid solution	5.0 ml.
Norleucine	100 mg.	Biotin solution	50.0 ml.
Norvaline	100 mg.	Folic acid solution	5.0 ml.
dl Phenylalanine	100 mg.	p-amino benzoic acid solution	2.0 ml.
l (-) proline	100 mg.	Riboflavine	3.0 mg.
dl serine	100 mg.	Inorganic salt solution A	5.0 ml.
dl threonine	500 mg.	Inorganic salt solution B	5.0 ml.
dl tryptophan	100 mg.		
l (-) tyrosine	100 mg.		
dl valine	100 mg.		

The amino acids were weighed out and dissolved in 100 ml. of water. the remaining components of the media were added, the pH adjusted to 6.8 with sodium hydroxide the whole made up to 500 ml. with water. This solution had twice the concentration of the final assay medium. Five ml. were transferred to each tube.

The standard methionine solution contained 10 ug. per ml. For the set up of an assay the necessary number of tubes were retained for blanks and the determination of the standard methionine curve. As in cystine the total volume of each tube was made up to 10 ml. with water.

Each assay was carried out at four levels of concentration. The hydrolyzates were diluted to contain an appropriate amount of methionine. The concentration levels that were used were 1.0, 2.0, 3.0, and 4.0 ml. of hydrolysate.

After the addition of the standard amino acid solution or hydrolysates and the adjustment of the volume of the tubes to 10 ml., the tubes were covered with aluminum caps and sterilized by autoclaving for 10 minutes at 15 pounds pressure, 118.5° C. The tubes then allowed to cool and inoculated with Leuconostoc mesenterioides. The same procedure was used in preparing the inoculum as in the cystine determination. The tubes were then incubated at 37° C. for 36-40 hours. The lactic acid formed was titrated with 0.1 N NaOH using brom thymol blue indicator.

Leaf protein preparations, vitamin test casein, and freshly prepared Hamerstein casein were assayed by this method. A standard methionine curve using Leuconostoc mesenterioides P-60 is given on Page 68, Figure 7.

TABLE NO. I

Results of Formic Acid - Ethanol Extraction of Bouteloua gracillis Cut 8-23-46

Weight of fresh grass analyzed	1195.0 g.
Per cent moisture on air dry basis	38.1 %
Per cent nitrogen in air dry sample	1.31%
Weight loss by acetone extraction of fresh grass	420.1 g.
Per cent nitrogen of grass after acetone extraction	1.39%
Weight of residue after formic acid extraction	306.7 g.
Per cent nitrogen in residue after formic acid and acetone extraction	0.51%
Per cent nitrogen in carbohydrate residue	0.56%
Per cent nitrogen in formic acid soluble fraction	2.55%
Per cent nitrogen in protein extract	4.04%

Summary of Nitrogen Distribution After Extraction

Weight of fresh grass analyzed	1195.0 g.
Weight of nitrogen in sample	9.69 g.
Per cent protein found (% N x 6.25)	8.19 %
Nitrogen extracted grams	1.74 g.
per cent extracted	18 %
Formic acid soluble nitrogen grams	5.80 g.
per cent of total nitrogen	60 %
Nitrogen in acetone soluble fraction	.30 g.
per cent of total nitrogen	3.1 %
Nitrogen in carbohydrate residue	.16 g.
per cent of total nitrogen	1.7 %
Nitrogen in residue after formic acid + acetone extraction	1.55 g.
per cent of total nitrogen	16 %

TABLE NO. II

Results of Sodium Dodecyl Sulfate Extraction

Bouteloua gracillis (cut 8-29-46)

Weight of air dry sample	200.00 g.
Per cent nitrogen in sample	1.42 %
Weight of residue after extraction	157.96 g.
Per cent nitrogen in residue	0.63 %
Per cent nitrogen in purified protein preparation	8.04 %

Nitrogen Distribution After Extraction

Weight of air dry sample	200.00 g.
Per cent protein found (% N x 6.25)	8.89 %
Total weight of nitrogen in sample	2.84 g.
Weight of nitrogen extracted	.71 g.
per cent of total nitrogen	25. %
Weight of nitrogen in residue after extraction	.99 g.
per cent of total nitrogen	35. %

The Following Grasses were Subjected to Sodium Dodecyl Sulfate Extraction

Grass Extracted	Wt. of Air Dry Sample	% N in Sample	Wt. N in Sample	Wt. N Extracted	%of Total in Purified Protein	%N in Protein preparation
Agropyron smithii (cut 5-10-46)	50.0 g.	3.99 %	1.99 g.	.85 g.	42.7 %	12.53 %
Agropyron smithii (cut 7-15-46)	50.0 g.	1.54 %	.77 g.	.29 g.	37.6 %	11.23 %
Agropyron cristatum (cut 5-10-46)	50.0 g.	4.34 %	2.17 g.	.82 g.	37.6 %	12.71 %
Agropyron cristatum (cut 7-15-46)	50.0 g.	1.19 %	.60 g.	.23 g.	37.6 %	11.51 %

TABLE NO. III

Record of Hydrolysis

Protein Sample	Grams/100 cc. Hydrolyzate	Date Hydrolyzed	No. of Hydrolyzate
Bouteloua gracillis (cut 8-23-46)	1 g./100 cc.	11-26-46	8A + B
Bouteloua gracillis (cut 8-23-46)	1 g./100 cc.	1-21-47	6A + B
Agropyron smithii (cut 5-10-46)	1 g./100 cc.	1-21-47	1 A + B
Agropyron smithii (cut 5-10-46)	1 g./100 cc.	3-2-47	21
Agropyron smithii (cut 7-15-46)	1 g./100 cc.	1-21-47	2A + B
Agropyron smithii (cut 7-15-46)	1 g./100 cc.	3-2-47	22
Agropyron cristatum (cut 5-10-46)	1 g./100 cc.	1-21-47	3A + B
Agropyron cristatum (cut 5-10-46)	1 g./100 cc.	3-2-47	23
Agropyron cristatum (cut 7-15-46)	1 g./100 cc.	1-21-47	4A + B
Agropyron cristatum (cut 7-15-46)	1 g./100 cc.	3-2-47	24
Vitamin Test Casein	1 g./100 cc.	11-22-46	5A
Vitamin Test Casein	1 g./100 cc.	1-21-47	9A + B
Vitamin Test Casein	1 g./100 cc. + 10 mg. cystine + 10 mg. methionine	11-22-46	5B
Vitamin Test Casein	1 g./100 cc.	3-2-47	25
Freshly prepared Hamerstein Casein	1 g./100 cc.	2-21-47	18A + B
Freshly prepared Hamerstein Casein	1 g./100 cc.	3-2-47	26

VI. DISCUSSION OF EXPERIMENTAL RESULTS

Discussion of Protein Extraction:

The results obtained using A. A. Albanese's method (1) are given on Table I, page 48. The per cent of total protein extracted was 18% and the per cent protein of the final product only 25%. It appears that this method was not suitable for the extraction of grass proteins.

Albanese found that application of the formic acid-ethanol method to dried seed meals or pulverized dehydrated vegetables was unsatisfactory owing to the formation of gels and loss of dispersed protein achieved in these preparations. In his experiments 60 to 70% of the total nitrogen was removed after 5 extractions with formic acid, and he states that the adaptability of the method to foodstuff other than fresh vegetables is limited by the particle size and physical characteristics of the product.

The formic acid treatment brought about a certain amount of hydrolysis of the plant protein and carbohydrate material which resulted in considerable humin formation. Due to the high lignin and carbohydrate content of the grass used, a colloidal dispersion resulted which did not precipitate out on addition of ethanol. Since the separation of the protein depends upon the insolubility of the carbohydrates in the formic acid-ethanol mixture, the fact that these materials were not rendered insoluble by the addition of ethanol indicates that the carbohydrate-protein relationship was such as not to be separable by this technique.

Sodium dodecyl sulfate extraction proved to be very satisfactory. The results of several extractions are given on Table II, page 49. No effort was made to effect a quantitative separation, yet yields as high as 43% were obtained. The per cent of total protein extracted varied somewhat with the age

of the grass. In general, the later the cutting the lower the yield. This could be due to several factors, one of which might be the decrease in protein content and the increase in lignin and carbohydrate material in the more mature grasses, which would make complete extraction more difficult. The function of lignin in the plant is apparently to give strength and rigidity to the cell walls. The unlignified tissue of the younger grasses is usually soft, contains a higher percentage of water and has a much lower breaking strength which would tend to make extraction much less difficult. The final products contained as high as 80% pure protein material which is considerably better than results obtained by the formic acid-ethanol extraction. The purity of the final preparation was lower in the more mature grasses. Lugg (28) used acetone ether, water for the plazmolyzing agents and was able to extract only 27.2% of the total protein from Medicago-sativa (lucerne) with a final product containing approximately 80% protein.

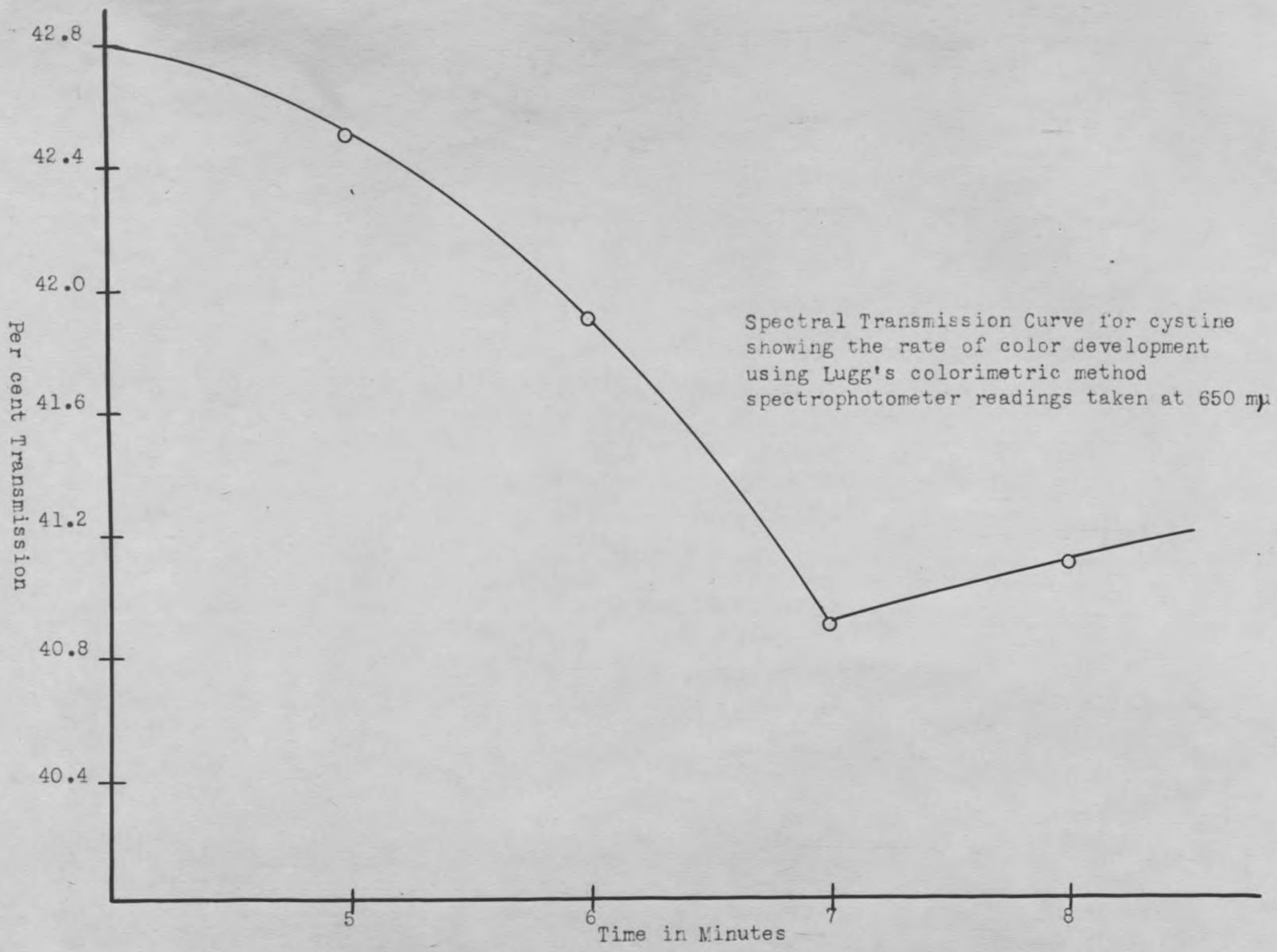
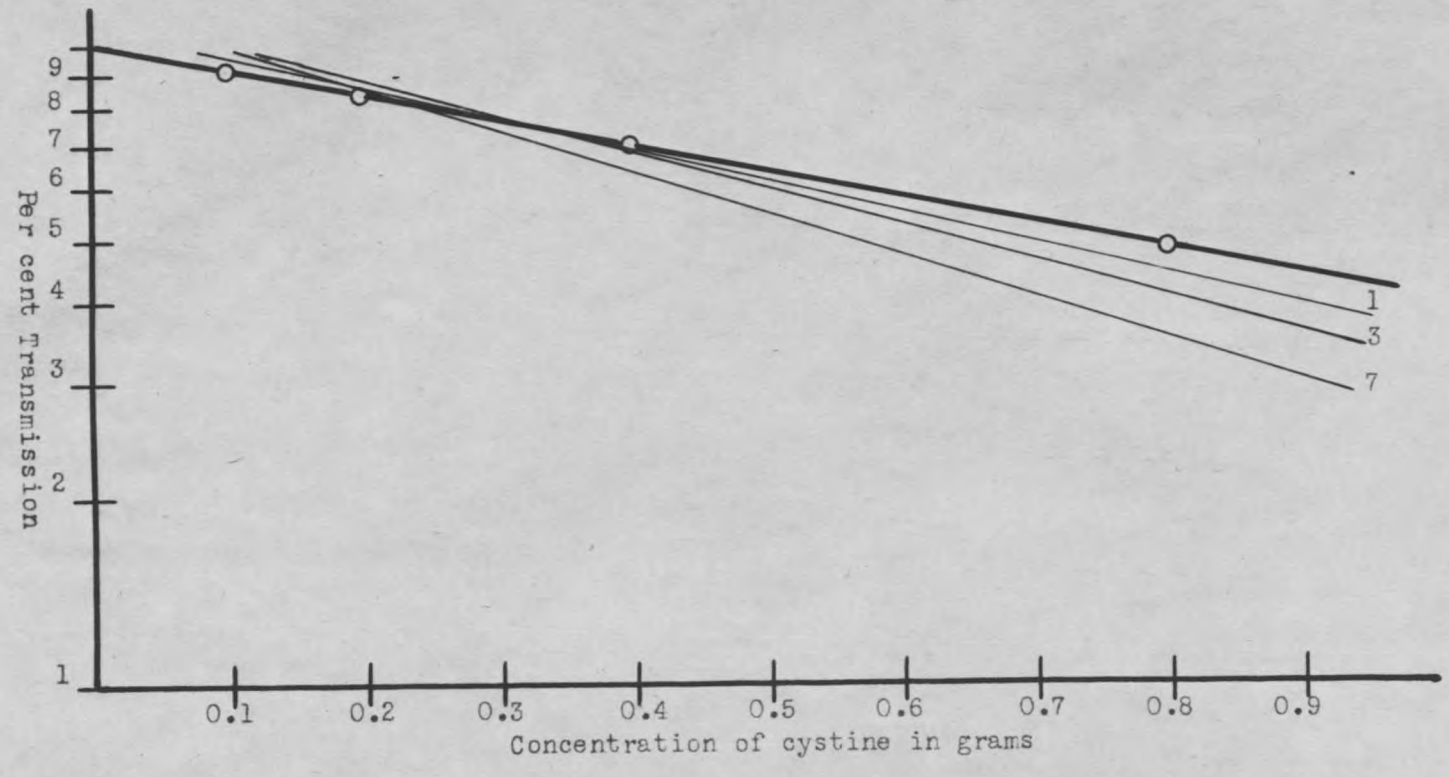


Figure 1

Spectral Transmission Curve for cystine showing the rate of color development using Lugg's colorimetric method spectrophotometer readings taken at 650 mμ

Figure 2



Curve obtained using freshly prepared sodium sulfite reagent.

Curves obtained when 1, 3, and 7 day old sodium sulfite solutions were used.

Spectral Transmission Curve for cystine using Lugg's colorimetric method.
Spectrophotometer readings taken at 650 mμ

TABLE NO. IV.

Results of Cystine Determinations Using Lugg's Modification of Folin-Marenzi Method

Protein Extract	A-smithii		A-cristatum		B-gracillis	
Date grass was cut	5-10-46	7-15-46	5-10-46	7-15-46	8-29-46	8-29-46
Hydrolyzate number	21	22	23	24	8A	8B
Date of determination	3-20-47	3-20-47	3-20-47	3-20-47	12-29-46	12-30-46
Per cent cystine reported on the basis of 16% N.	1.11 1.11 1.17 1.17	1.05 1.05 .96 .96	1.03 1.03 1.02 1.02	0.78 0.78 0.80 0.80	0.50 0.62 0.54 0.60	0.64 0.66 0.56
Average value %	1.14	1.01	1.03	0.79	0.57	0.62

Casein	Vitamin Test		Freshly Prepared Hamerstein Casein		
Hydrolyzate Number	5-A	5-B Cystine added	18A	18B	26
Average Value %	0.31	1.27	0.47	0.50	0.50

Per Cent Cystine Recovery; cystine added after hydrolysis of protein:
Hydrolysate 8B - 95% recovery; Hydrolysate 21 - 93% recovery

Per Cent Cystine Recovery; cystine added before hydrolysis:
Hydrolysate 5B - 87% recovery

NOTE: All percentages of methionine and cystine reported in this thesis are on the basis of 16% nitrogen.

Discussion of Cystine Determinations:

Lugg's modification of the Folin-Marenzi method for the determination of cystine presents numerous difficulties. Molybdenum must be carefully removed from the phosphotungstic acid reagent to avoid reactions with tyrosine, tryptophan and phenolic groups generally. Most hydrolysates which have to be analyzed for cystine contain other substances which contribute to the coloration developed. Those soluble products arising from the digestion of carbohydrate material with mineral acid will also reduce the reagent. There is reason to believe that any sufficiently soluble sulphhydryl compound will reduce the reagent and the same may be said of any disulphide which will react with sulphite to form a sulphhydryl compound. It is clear then that the reaction with phospho-18-tungstic acid is not only unspecific for cystine among the sulphhydryl compounds, but it is further unspecific for the sulphhydryl compounds themselves. Correction has been made for these extraneous reducers by preparing an unknown blank containing mercuric chloride which inhibits color development due to cysteine and the cysteine content is then obtained by difference. The pH of the color reaction must be carefully maintained at a pH of 5.7 and the quantity of the reagents added must be carefully controlled. Many of these difficulties have been discussed by Lugg (28).

Figure 1, page 53, illustrates the importance of controlling the length of time for color development. Temperature has apparently little effect on the rate of color development and all reactions were carried out at room temperature.

Figure 2, page 54, illustrates the importance of using fresh sodium sulfite solution. A change of slope of the standard curve is observed when old sulfite solution is used, however, with freshly prepared solution the same standard curve may be repeated each day.

The results of cystine determinations using Lugg's modification of the Folin-Marenzi method are given on Table No. IV, page 55. The values obtained for cystine in vitamin test casein agree quite well with those given by Black and Bolling (9). The value given by Black and Bolling for the percentage cystine in casein is 0.36 $\pm$ 0.04%. Calculated to 16.0 per cent nitrogen, the value shown on Table IV is 0.31% cystine calculated on the basis that the pure protein contained 16% nitrogen.

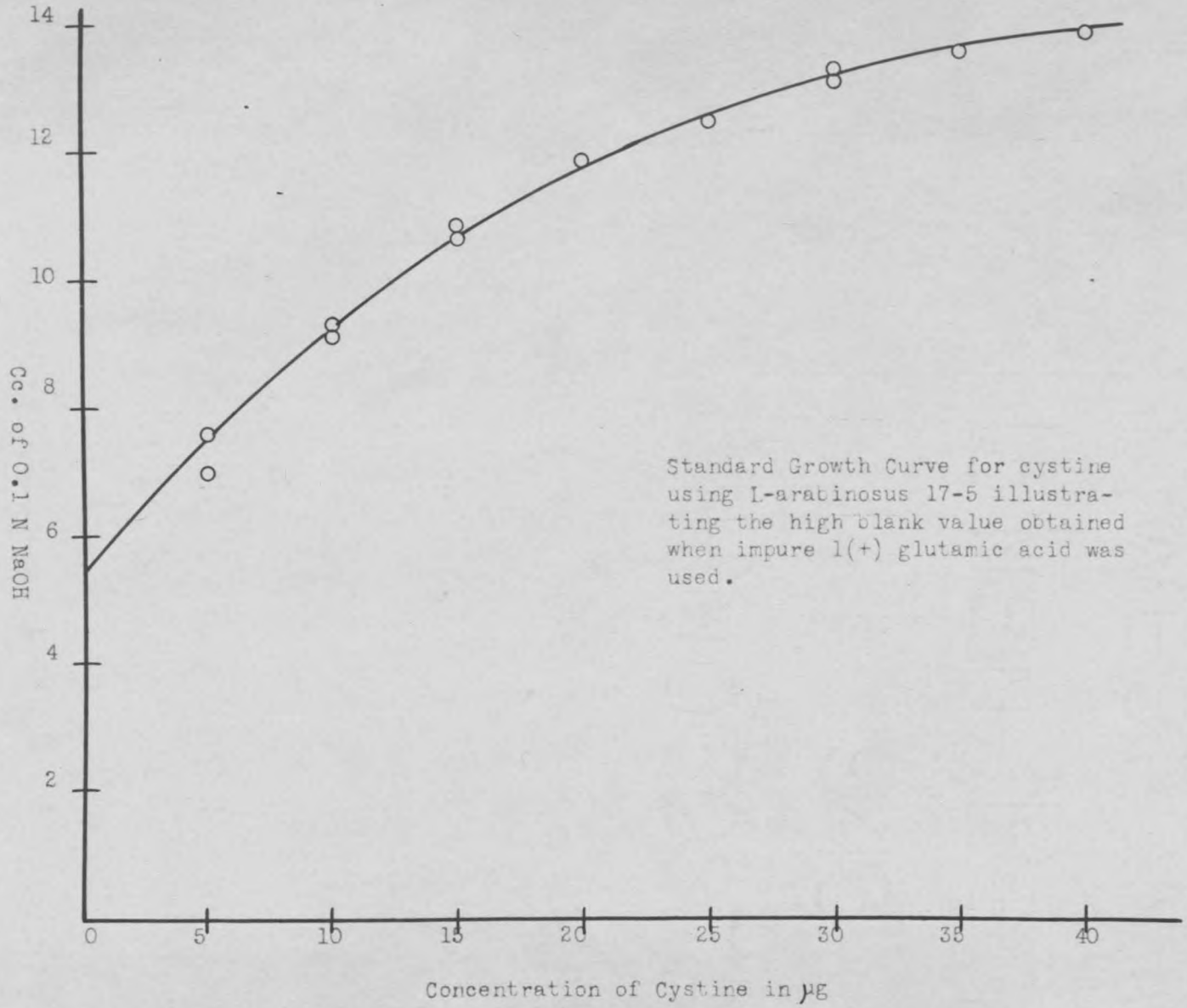


Figure 3

Standard Growth Curve for cystine using L-arabinosus 17-5 illustrating the high blank value obtained when impure l(+) glutamic acid was used.

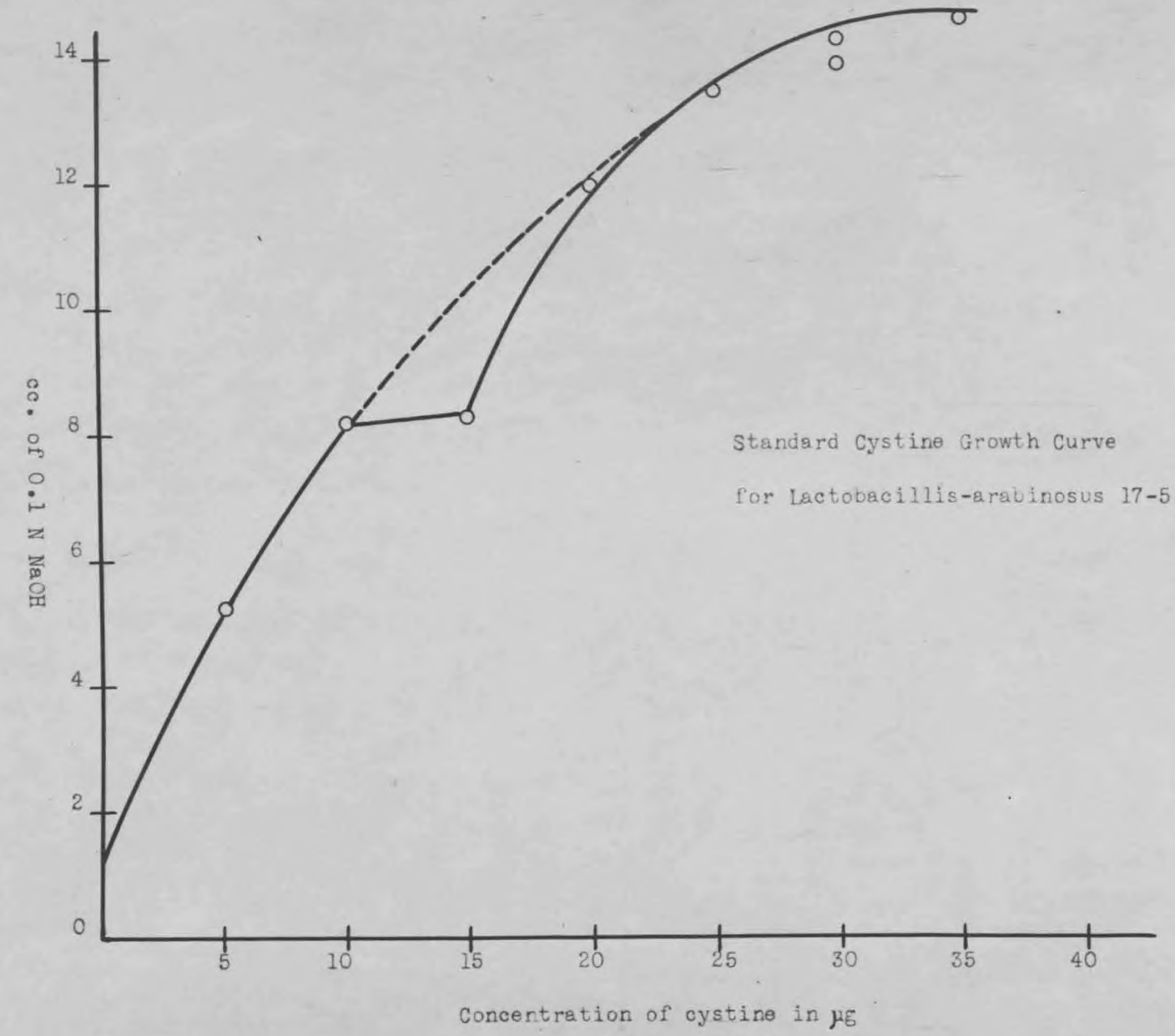


Figure 4

Standard Cystine Growth Curve

for Lactobacillis-arabinosus 17-5.

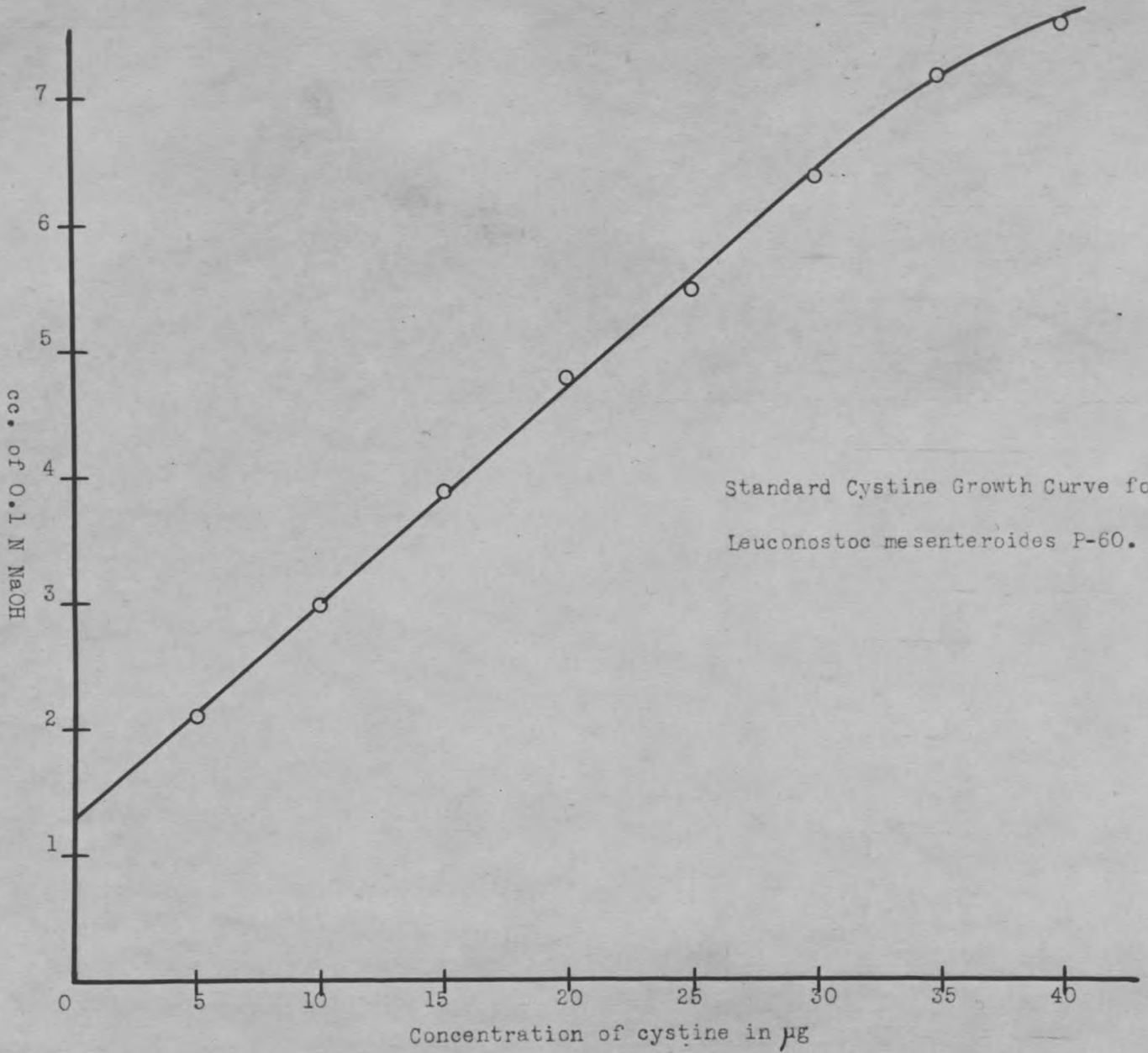


Figure 5

Standard Cystine Growth Curve for

Leuconostoc mesenteroides P-60.

TABLE NO. V

Results of Cystine Assay Employing Lactobacillus-arabinosus 17-5

Protein extract	A-smithii					A-cristatum		
Date grass was cut	5-10-46		7-15-46			7-15-46		
Hydrolyzate number	21	1A	2A	2B	22	4A	4B	24
Date of assay	3-25-47	4-10-47	2-26-47	3-5-47	3-25-47	3-5-47	3-5-47	3-25-47
	1.62	1.19	1.99	1.90	1.31	1.08	1.35	1.11
	1.69	1.25	1.95	1.90	1.55	1.25	1.35	1.53
	1.75	1.19	1.95	1.84	1.55	1.28	1.35	1.50
	1.88	1.26	1.73	1.84	1.61	1.08	1.14	1.39
	1.75	1.26	1.66	1.91	1.61		1.25	1.18
		1.07			1.49			1.29
		1.06						1.29
								1.22
								1.25
								1.29
Average Value %	1.74	1.18	1.86	1.88	1.52	1.17	1.29	1.30

Protein extract	A-cristatum	B-gracillis	Hamerstein Casein	
Date grass was cut	7-15-46	8-29-46		
Hydrolyzate number	24	6-B	18A	18B
Date of Assay	4-10-47	3-5-47	3-8-47	3-8-47
	1.33	1.14	.47	.47
	1.11	1.15	.39	.31
	1.01	1.12	.32	.39
	.89	1.06		.47
	.76	1.14		.31
	.81			
Average value %	1.18	1.12	0.39	0.39

Note: Per cent cystine recovery; cystine added after hydrolysis.
Hydrolyzate #21 - 101% recovery.

TABLE NO. VI

Results of Cystine Assay Using Leuconostoc-mesenteroides P-60						
Protein Extract	A-smithii		A-cristatum			Hamerstein
Date grass was cut	5-10-46	7-15-46	5-10-46	5-10-46	7-15-46	Casein
Hydrolyzate number	1-A	22	3-A	23	24	18A
Date of assay	4-14-47	4-14-47	4-14-47	4-19-47	4-19-47	4-19-47
	1.37	1.33	1.42	1.21	.97	.46
	.84	1.20	1.48	1.08	.90	.53
	.83	1.07	1.29	1.11	.79	.52
	1.11	1.19	1.23	.94	.74	.59
	1.32	1.13		1.33	.71	.59
	1.17	1.01		.89	.66	
		.94		.88	.66	
				.79		
Average Value %	1.11	1.12	1.35	1.03	0.78	0.54

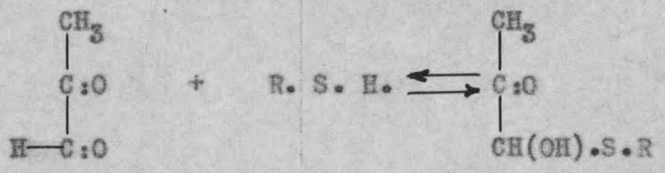
Cystine Assay Using Lactobacillis-arabinosus 17-5:

A great deal of difficulty was encountered in obtaining a suitable basal media for this organism and numerous modifications were tried before a suitable medium was finally obtained. The most difficult problem involved was the elimination of high blanks which resulted in erratic standard curves and high cystine values. Figure 3 illustrates a typical standard curve with a high blank and shows how the titrations vary in tubes containing equal concentrations of cystine. It appeared to be quite obvious that the high blank was undoubtedly due to the impurity of some amino acid in the medium. By trial and error elimination, it was found that the l (+) glutamic acid being used was responsible for this difficulty. When l (+) glutamic acid of a better quality was used the high blank disappeared and a more stable growth curve was obtained. A colorimetric determination was made on the l (+) glutamic acid which had been found impure and it was discovered that this product contained 1.1 mg. of cystine per gram. This resulted in an additional 5 ug. of cystine per assay tube and would easily account for the high blank obtained.

It was necessary to increase the vitamin concentrations to approximately ten times the concentrations given in the literature and to add other growth stimulating substances. Although the standard curves which were finally obtained showed some inconsistencies, the results were considerably better when the medium given on page 41was used. Figure 4, page 59, shows a standard growth curve using this new media.

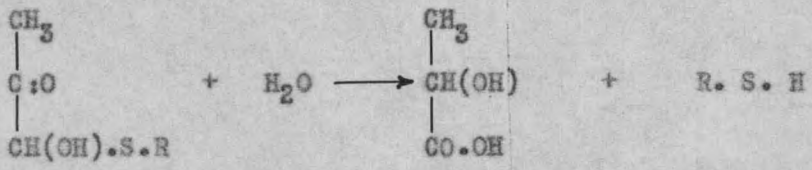
A very interesting observation should be mentioned in regard to the cystine growth curves obtained in this experiment. For every cystine curve

obtained there was a point of inflection at a cystine concentration of 15 ug. Curiously enough this observation has never been reported in the literature. Although the reason for this change in slope is unknown, several explanations might be given. The most reasonable explanation seems to be that after the cystine concentration reaches a certain level, another acid producing mechanism goes into effect increasing the rate of acid production. It is known that cystine acts as an enzyme activator. Cystine is necessary for the synthesis of glutathione (-glutamyl-cysteinyl glycine). Quastel (21) presents evidence for the combination of glutathione with methyl glyoxal and propose a hypothesis of glyoxalase action which includes the formation of an addition compound composed of methyl glyoxal and glutathione:



Methyl glyoxal + Glutathione Addition compound

The addition compound is then decomposed with the formation of lactic acid and glutathione



Addition compound Lactic acid Glutathione

It seems entirely possible that some acid producing mechanism such as this may go into effect when the cystine concentration reaches a certain level. If this assumption is true, it would account for the point of inflection found in standard cystine growth curves. Table No. V, page 61, shows the

results obtained by assaying cystine with L-arabinosus 17-5. The values are in all cases higher than those obtained by other methods and until a more thorough study has been made of the cystine metabolism of L-arabinosus not much value can be attached to these results.

Cystine Determination Using Leuconostoc mesenterioides P-60:

The same basal medium was used for the cystine assay as the methionioine assay employing Leuconostoc mesenterioides P-60. Due to lack of sufficient time only two cystine assays were carried out using this organism. However, very satisfactory growth curves were obtained (Figure 5, page 60) and the results appear to be in good agreement with values obtained by other methods. Table No. VI, page 62, shows the results obtained by this method. In all microbiological assays average results from several levels of concentration must be used because of lack of uniformity of the growth in the individual tubes.

Cystine Determinations in General:

Table No. IX, page 74, shows the composite results of all cystine assays. L-arabinosus values are shown in red type and indicate the high results obtained by this method. All results are arranged in chronological order showing the relative age of the hydrolyzates. Table III, page 50, gives the time of hydrolysis of each hydrolyzate and the letter designation used for each. Assays carried out by Lugg's colorimetric method and using Leuconostoc mesenterioides are in fairly good agreement. On hydrolyzate No. 23 and No. 24, the same value was obtained by both methods. A-smithii apparently has predically the same cystine content at both cuttings. A-cristatum shows

a definite decrease in cystine in the more mature grass. The cystine value varies very little with the age of the hydrolysate.

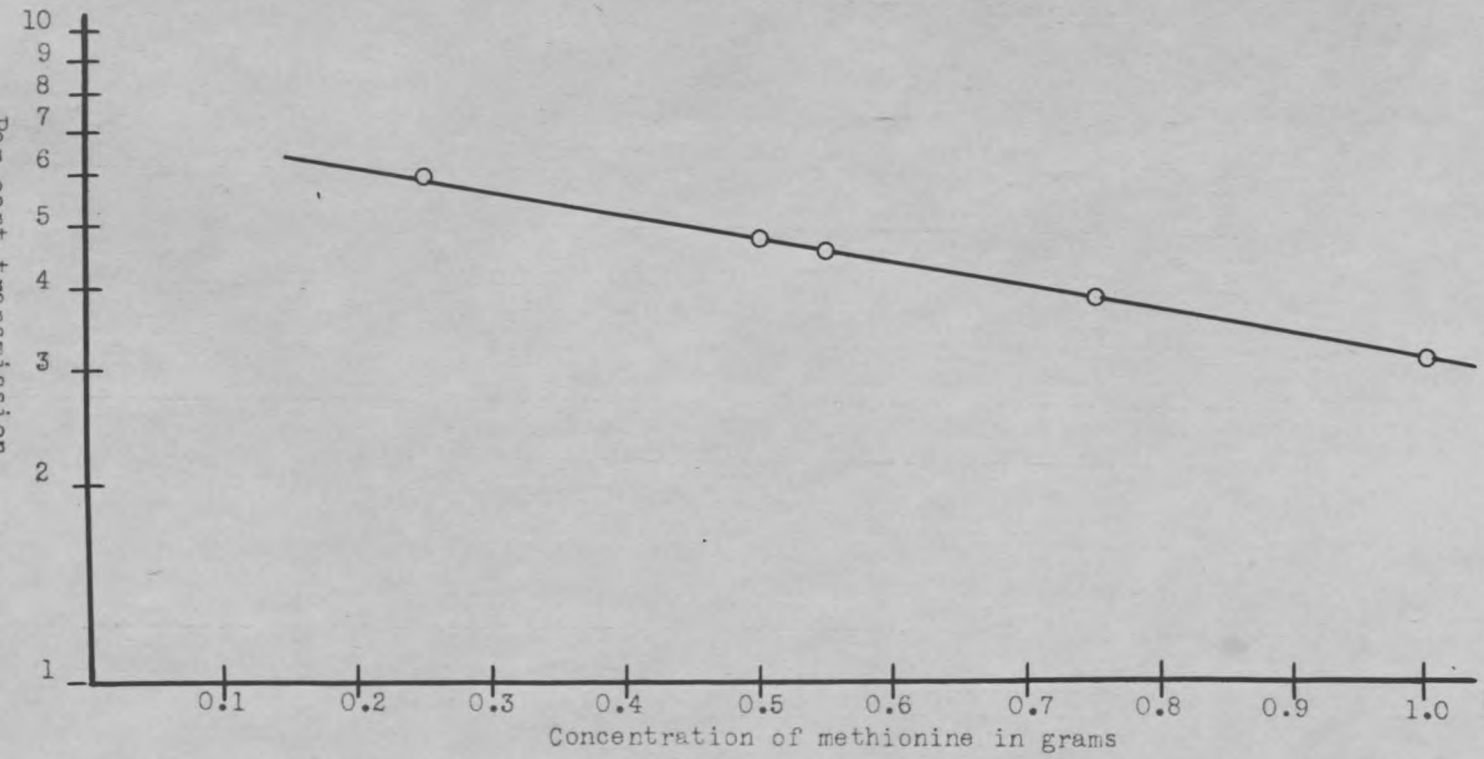


Figure 6

Spectral Transmission Curve for Methionine

using the Csonka and Denton colorimetric method.

Spectrophotometer readings taken at 510 mμ

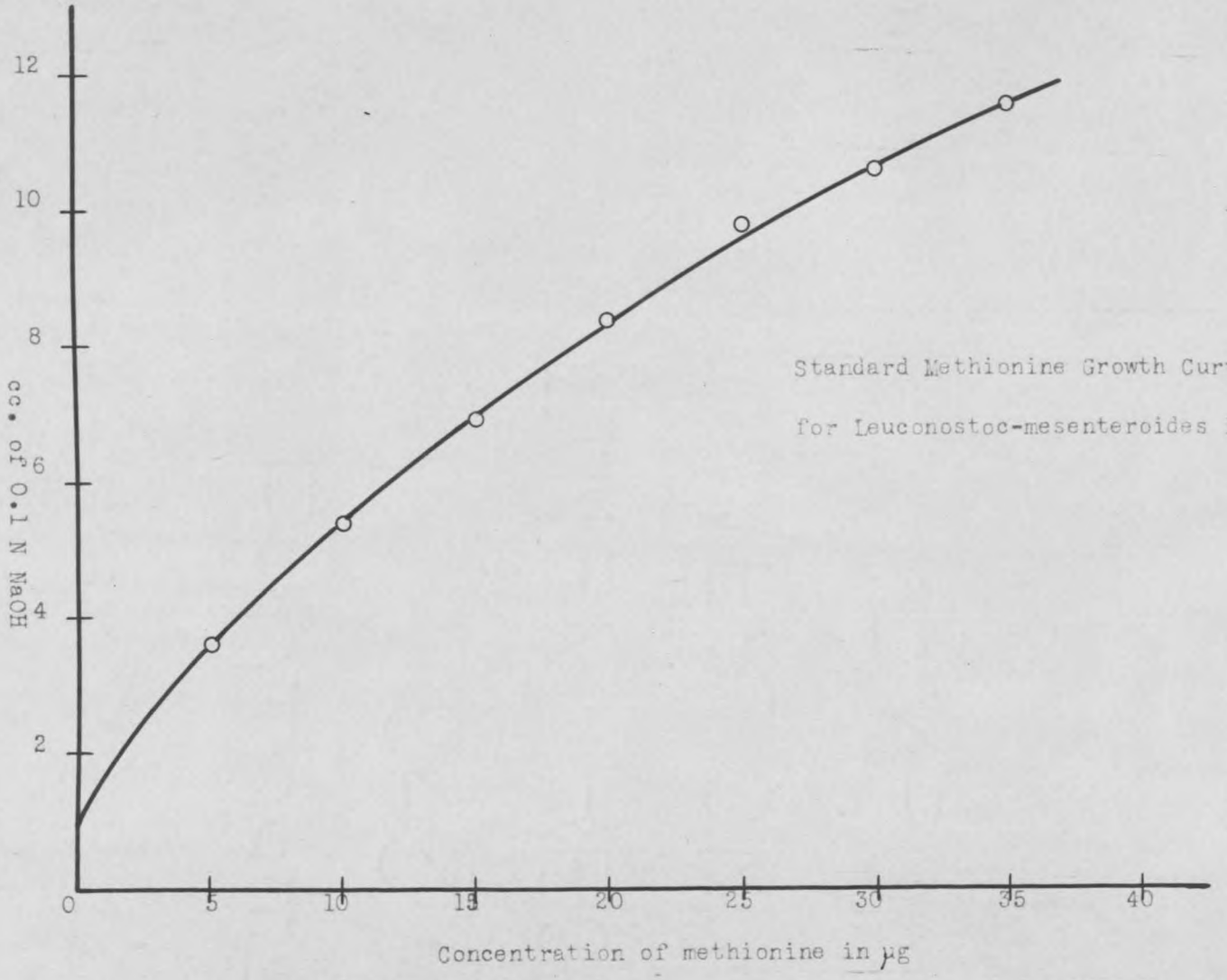
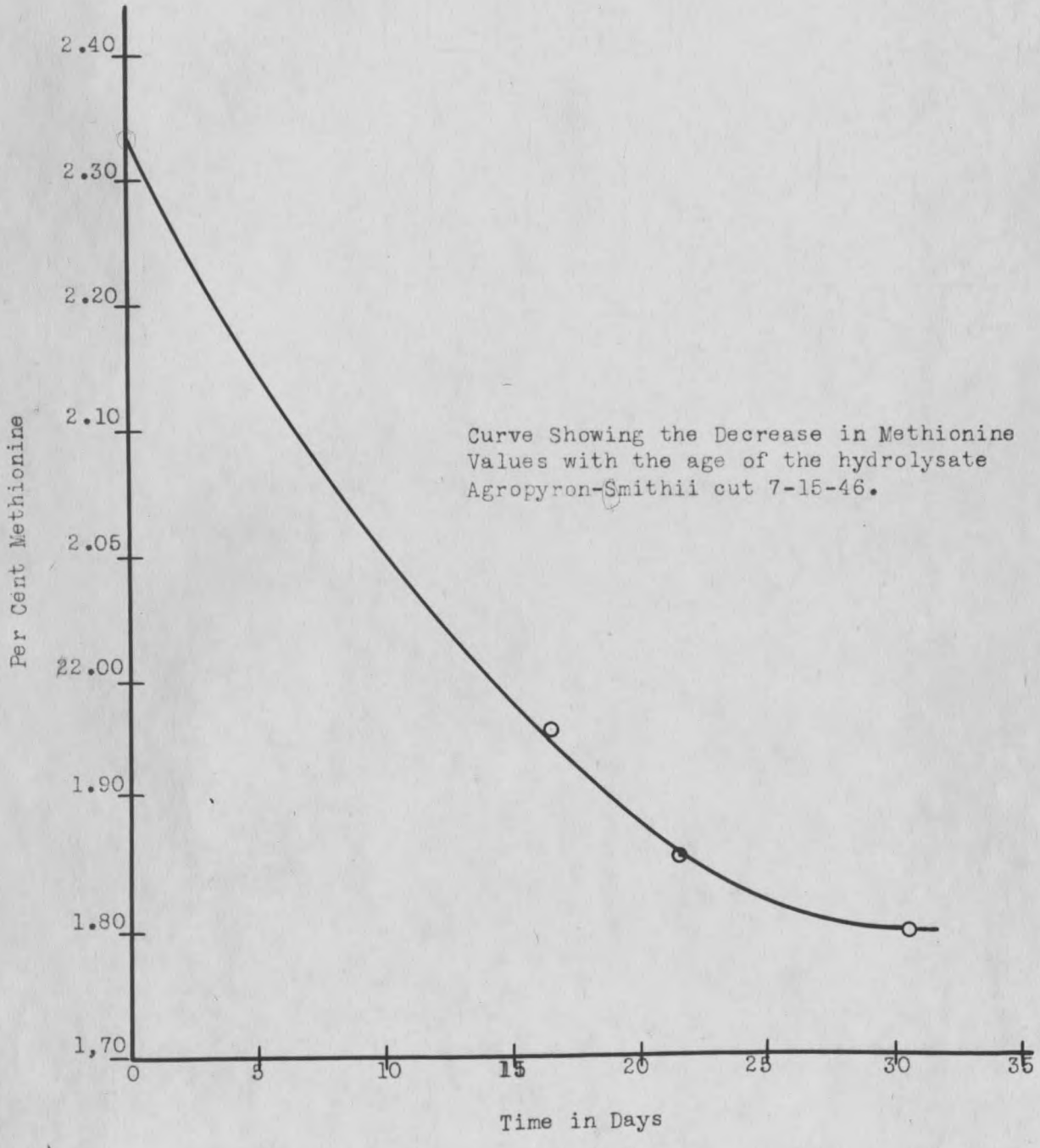


Figure 7

Standard Methionine Growth Curve

for Leuconostoc-mesenteroides P-60.



Curve Showing the Decrease in Methionine Values with the age of the hydrolysate Agropyron-Smithii cut 7-15-46.

Figure 8

TABLE NO. VII

Results of Methionine Determination Using Csonka and Denton's Method

Protein extract	A-smithii	A-cristatum		B-gracillis		Vitamin Test Casein		Hamerstein Casein
Date grass was cut	5-10-46	5-10-46	7-15-46	8-23-46	8-23-46			
Hydrolyzate number	21	23	24	8-A	8-B	5A	25	26
Date of assay	3-24-47	3-24-47	3-24-47	1-4-47	1-9-47	1-3-47	3-18-47	3-18-47
	1.60	1.89	1.74	2.38	2.34	3.07	3.44	3.34
	1.71	2.26	2.14	2.24	2.14	2.63	3.10	2.93
	1.85	2.44	2.03	2.34	2.08	3.12	2.75	3.00
	2.49	2.40	2.14			3.12		
	2.47	2.51	2.10			3.12		
		2.45						
Average Value %	1.99	2.33	2.03	2.32	2.15	3.01	3.09	3.09

TABLE NO. VIII

Results of Methionine assay Using Leuconostoc-mesenteroicides P-60.

Protein extract	A-smithii								
Date grass was cut			5-10-46			7-15-46			
Hydrolyzate number	1-A	1-A	1-A	21	21	2A	2A	22	22
Date of assay	3-18-47	4-10-47	4-18-47	3-18-47	4-5-47	3-18-47	4-10-47	4-5-47	4-18-47
	2.18	1.66	1.21	2.37	2.12	2.14	2.00	1.99	1.62
	2.18	1.79	1.21	2.36	2.12	2.32	1.90	1.93	1.62
	1.71	1.72	1.25	2.37	2.02	2.51	1.72	2.06	1.52
	1.71	1.88	1.60	2.27	2.03	2.34	1.85	1.95	1.83
		1.79	1.53	2.26	1.99		1.67	1.90	1.81
		1.76	1.56	2.27	2.07		1.90	1.90	1.95
		1.79	1.69		2.09		2.00		1.85
		1.83	1.69				1.72		2.01
			1.70				1.81		2.02
							2.10		
							1.81		
Average Value %	1.95	1.78	1.49	2.31	2.06	2.33	1.86	1.96	1.80

Protein extract	A-cristatum								Hamerstein Casein
Date grass was cut			5-10-46			7-15-46			
Hydrolyzate number	3A	3A	23	23	23	24	24	24	26
Date of assay	4-5-47	4-18-47	3-18-47	4-5-47	4-18-47	3-18-47	4-5-47	4-18-47	4-5-47
	2.22	1.29	2.10	2.39	1.35	2.70	2.08	1.28	3.04
	2.33	1.38	2.04	2.39	1.26	2.32	2.25	1.33	3.04
	2.10	1.38	1.91	2.39	1.31	1.68	2.11	1.28	2.95
	2.03	1.76	1.91	2.29	1.79	1.99	2.09	1.43	2.81
	2.10	1.72	1.74	2.33	1.76	1.86	2.11	1.26	2.95
	2.08	1.60		2.33	1.79	1.86		1.44	2.84
	1.98	1.72							2.92
	2.00	1.79							2.93
	1.98	1.76							3.36
Average value %	2.09	1.60	1.96	2.35	1.54	2.07	2.13	1.34	2.98

Methionine Determination Using Csonka and Denton's Method.

No special difficulties were encountered with this method and the results appear to be quite reliable. The standard curve is given in Figure 6, page 67. The curve is a straight line and is reproducible. The results of this method are given on Table VII, page 70. A-cristatum has the higher methionine content of the grasses examined and it was found that the methionine values decrease with the maturity of the grass. The values obtained for the methionine content of casein are in good agreement with each other and with values given in the literature. Black and Bolling (9) give 3.10% methionine in casein which agrees very well with the results obtained in this experiment.

Methionine Determination Using Leuconostoc mesenterioides P-60:

No special difficulties were encountered in growing this organism on synthetic medium and few modifications were necessary in the medium presented by Barton-Wright (8). The acid production of this organism was not as great as that shown by L-arabinosus 17-5,Q however, the standard growth curves obtained, Figure 7, page 68, gave very smooth curves with little variance between assay tubes of the same concentration.

Table VIII, page 71, shows the results obtained by this method. The per cent methionine in casein was slightly lower than those obtained by the colorimetric method. Although some variance was found between different hydrolyzates of the same protein material, much greater differences were observed in different assays of the same hydrolyzate carried out on different dates. The methionine values drop off very sharply with the age of the hydrolyzate. Figure No. 8, page 69, sharply illustrates this fact. On Table

IX, page 74, the assays are arranged in chronological order and it may be observed that there is a great deal of change occurring in the methionine content with the age of the hydrolyzate. This could be due to either of two factors: (1) bacterial action destroying the methionine; (2) the methionine may be unstable in the hydrolyzate solution and be decomposing chemically. This change in methionine content has never been reported in the literature, perhaps due to lack of observation on the part of the different investigators concerned.

The hydrolyzates were neutralized to a pH of 6.8 and autoclaved immediately thereafter at 15 pounds pressure, 118°C., for 20 minutes then cooled and covered with a layer of sulfur free toluene and stored in a refrigerator. Later microbiological examination using staining techniques revealed no bacterial growth which would indicate that factor (1) was probably not responsible for the loss of methionine. In view of these observations, it would be highly desirable to make a more thorough investigation of the changes which take place in protein hydrolyzates. Pharmaceutical concerns are now marketing protein hydrolyzate for therapeutic use. Thus, such an investigation might have practical as well as scientific value.

TABLE NO. IX

Summary Table Showing Average Values of Methionine and Cystine in Grass Protein Hydrolyzates

Protein / Date grass was cut		Agropyron-smithii 5-10-46			7-15-46		Agropyron cristatum 5-10-46			7-15-46		B-gracillis 8-29-46			Hamerstein Casein		
Number of Hydrolyzate		1-A	21	2A	2B	22	3A	23	4A	4B	24	6B	8A	8B	18A	18B	26
Date	Method	Average Values for Cystine															
12-29-46	Colorimetric												0.57				
12-30-46	Colorimetric													0.62			
2-26-47	L-arabinosus			1.86													
3-5- 47	L-arabinosus				1.88				1.17	1.29		1.12					
3-8- 47	L-arabinosus														0.39	0.39	
3-20-47	Colorimetric		1.14			1.01		1.03			0.79						
3-21-47	Colorimetric														0.47	0.50	0.50
3-25-47	L-arabinosus		1.74			1.52					1.30						
4-10-47	L-arabinosus	1.18									1.18						
4-14-47	L-mesenterioides	1.11				1.12	1.35										
4-19-47	L-mesenterioides							1.03			0.78				0.54		
		Average Values for Methionine															
1- 3-47	Colorimetric																
1-4-47	Colorimetric												2.32				
1-9-47	Colorimetric													2.15			
3-18-47	Colorimetric																3.09
3-18-47	L-mesenterioides	1.95	2.31	2.33				1.96			2.07						
3-24-47	Colorimetric		1.99					2.33			2.03						
4-5-47	L-mesenterioides		2.06			1.96	2.09	2.35			2.13						2.98
4-10-47	L-mesenterioides	1.78		1.86													
4-18-47	L-mesenterioides	1.49				1.80	1.60	1.54			1.34						

Table IX, page 74, shows that of the grasses analyzed, the amount of methionine and cystine varies with the variety as well as the age of grass. A-cristatum has a higher methionine content and from the data presented, A-smithii apparently has a higher amount of cystine than the other grasses analyzed. The more mature the grass, the lower the cystine content and although there is less difference, the methionine content also decreases with the age of the grass. The methionine content varies between about 1.95 and 2.33%; cystine 1.14 to 0.79%. For a better understanding of animal nutrition it becomes quite obvious that a more complete study of range grass proteins would be highly desirable. J. W. H. Lugg of Australia and A. C. Chibnall of England have made very valuable contributions in the study of the amino acid composition of leaf proteins. J. W. H. Lugg has made quite a thorough investigation of the sulfur containing amino acids as they occur in the grass proteins found in Australia and some attempt has been made to correlate his findings with animal nutrition. As yet very little of this work has been done in this country. Some recent developments have been made in the amino acid composition of certain dietary proteins and it is to be hoped that this work may be carried forward and thus increase our knowledge of protein nutrition.

VII. SUMMARY

1. Sodium dodecyl sulfate is especially suitable for the extraction of leaf proteins. High yields may be obtained by this method and the final preparations contain as high as 80% protein.
2. Until a more complete study has been made of the cystine metabolism of Lactobacillis arabinosus 17-5, this microorganism should be condemned for the assay of cystine.
3. The adaption of Lugg's modification of the Folin-Marenzi method for use with the Coleman spectrophotometer gives consistent results which are in good agreement with values obtained by other methods.
4. Recovery of cystine added to the protein hydrolyzate was found to be 95-98% using Lugg's colorimetric method. It was also found that as high as 13% of the cystine was destroyed by acid hydrolysis.
5. Leuconostoc-mesenterioides P-60 is very satisfactory for the microbiological assay of cystine.
6. The estimation of methionine by Csonka and Denton's modification of the McCarthy Sullivan method is very satisfactory and the results are in good agreement with literature values obtained by other methods.
7. Leuconostoc-mesenterioides P-60 may be well adapted for the microbiological assay of methionine.
8. Methionine is unstable in protein hydrolyzates prepared and stored under sterile conditions. The loss of methionine in such hydrolyzates is proportional to the age of the hydrolyzate.
9. The methionine and cystine content of Montana range grasses varies considerably with the different varieties assayed.

(Summary Continued)

10. The amount of methionine and cystine in the grasses analyzed was found to be consistently lower in the more mature grass. The early spring cuttings show a higher percentage of both methionine and cystine.

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