MICROBIOLOGICAL DETERMINATION OF ASPARTIC AND GLUTAMIC ACIDS IN VARIOUSLY-FERTILIZED ALFALFA

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CHAPTER I

MICROBIOLOGICAL DETERMINATION OF ASPARTIC AND GLUTAMIC ACIDS IN VARIOUSLY-FERTILIZED ALFALFA

Introduction

In the past years claims have been made by several investigators (1-4) that the fertility of the soil may affect the amino acid distribution of plant proteins grown on that soil. Other investigators (5-10) have found a uniform distribution of amino acid in plant and animal proteins regardless of treatment. In view of the contrasting observations as to the effect of treatment on amino acid distribution of plants, experiments were initiated in 1951 with the cooperation of the Agronomy Department at this station to study this problem. The primary purpose of these studies was the elucidation of any effect which soil-borne mineral elements might have upon the metabolism of a plant. Hollis (10) at this station using rye, oats and alfalfa and studying several amino acids, found no appreciable alteration in the amino acid pattern of the proteins with certain fertilizers. From other previous work at this station glutamic and aspartic acids were selected as the amino acids to be studied by the microbiological procedure in plant proteins grown on variously-fertilized soil.

Variations were encountered in the assay of glutamic acid in alfalfa and were attributed to the aspartic-glutamic acid competitive inhibition which has been described by several investigators (11-15). Early workers (11, 12, 14-16) studied and designed a microbiological procedure for the determination of glutamic acid. This procedure was found to be inadequate for application to sample materials containing high levels of aspartic acid. Accordingly, experiments were initiated for the study of possible improvements of the glutamic acid assay in the presence of high levels of aspartic acid.

It was also observed in this laboratory, as well as in others, that position differences of the tubes during sterilization and incubation in the microbiological procedure cause variation in the values obtained. Investigators (17-19) have found such variations in the procedures of the sterilization and incubation processes. It was thought desirable to design a statistical study of the effect of tube position during autoclaving and incubation upon the variations in the microbiological procedure. With the aid of the Oklahoma State University Statistical Department a statistical approach was designed to evaluate the error due to the position effect.

The results of continued work on the project on the effect of fertility on amino acid distribution of a plant protein are reported in this thesis. Results were obtained and are reported on the improvement of the glutamic acid assay of high-aspartic materials. Tube position effects in

the autoclave and incubator were determined statistically. Results were tabulated, and an analysis of variance was made. The implications of these findings are also discussed in this thesis.

CHAPTER II

GENERAL PROCEDURES

Source of Materials

The alfalfa (*Medicago sativa*, var. Oklahoma Common) which was used in this study was grown on Waynesboro loam soil of low fertility located at the Southeastern Oklahoma Soil Improvement Station about two and one-half miles north of Heavener in LeFlore County, Oklahoma. This experiment was established in September, 1953, as a randomized block, split-plot design with the following treatments:

R_1 - 750 pounds rock phosphate (33% total P_2O_5) per acre

R_2 -1500 pounds rock phosphate (33% total P_2O_5) per acre

P_1 - 250 pounds superphosphate (20% P_2O_5) per acre

P_2 - 500 pounds superphosphate (20% P_2O_5) per acre

K_1 - 100 pounds KCl (60% K_2O) per acre

Mg - 400 pounds magnesium sulfate (9.87% Mg) per acre

B - 40 pounds Borax (11.3% B) per acre

S - 50 pounds sulfur per acre

BMgS - all the above three trace elements

At the time of establishment of this experiment all of the above treatments were applied. The application was maintained annually with the exception of rock phosphate, which was applied only at the time of establishment of the experiment.

Three hay cuttings were obtained in 1955. The yield data, along with the chemical and physical properties of the soil and other information, is reported by Thomas (20).

Collection and Treatment of Materials

Random samples representing each fertilized plot were collected from each of three cuttings of alfalfa in 1955. These samples were brought to this laboratory and placed in the deepfreeze with a minimum amount of time permitted to elapse between cutting and freezing. The leaves were separated from the stems and both fractions were dried at 60°C. in a forced-draft oven. The fractions were weighed separately, and the leaf-stem ratios were determined (Table IV). These were ground in a Wiley Mill and stored in glass air-tight bottles until the analyses were performed.

Procedures for Amino Acid Analyses

Two grams of material were weighed, and the total nitrogen was determined by the Kjeldahl procedure according to the A.O.A.C. method (21).

Hydrolysates were prepared by heating 2.000 grams of alfalfa with 3N HCl in an autoclave for 15 hours at 120°C. After 15 hours of autoclaving, the hydrolysates were removed, adjusted to a pH of 5.5 with 6N KOH by the use of a Beckman pH Meter Model H2, filtered, brought to a predetermined volume and stored at 4°C. At the time of analyses, the hydrolysates were adjusted to a pH of 6.0, appropriately diluted, and the general microbiological procedure as set forth by Henderson and Snell (22) was employed with certain modifications.

Assay racks containing 60 tubes with 10 rows were employed. Two rows of standard solutions of the amino acids were dispensed at levels of 0.0-0.2-0.4-0.6-0.8-1.0 ml. by the use of a Cannon dispenser (23). Aliquots of the hydrolysates were dispensed in the same manner. Water was added in such a way that each tube contained a total volume of 1 ml. Basal media containing all of the necessary growth factors except the one which was being assayed were dispensed at 1 ml. levels so that each tube contained a final volume of 2 ml.

Basal Media

The basal medium used (Appendix) was essentially the same as that recommended by Henderson and Snell (22), with certain modifications. The specific modifications of the medium will be discussed in detail in subsequent sections.

In studies in which L. arabinosus was used for the assay of glutamic acid, asparagine was substituted for aspartic acid at approximately one-fourth the concentration of aspartic acid normally used. This was done to minimize the lag in the standard curve which has been described by several investigators (11-15).

An "all-potassium" modification of the media (Appendix) which was described by Sirny (24) was employed in preliminary work. From results obtained in the laboratory, it was deemed necessary to modify this medium to a high-potassium, low-sodium modification (Appendix). This medium was used with L. arabinosus while the "all-potassium" medium was used for L. mesenteroides.

The medium was prepared the day that it was used. The pH was carefully adjusted with a pH meter to 6.0, and the media were dispensed by the use of a Cannon automatic dispenser (23).

Assay Organisms

Two strains of organisms were used in this study. Leusonostoc mesenteroides P-60 (ATCC 8042)[1] was used for the determination of aspartic acid. Lactobacillus arabinosus 17-5 (ATCC 8014) was used for the determination of glutamic acid.

Every two weeks the organisms were transferred to an enriched basal media, so that the organism was kept in more active state, ready for routine use. Prior to use in assay work, the organism was transferred to 5 ml. of liquid culture medium. This was incubated at 37° ± 1°C. for 10 to 18 hours. Before use, the cells were centrifuged, and the supernatant removed. The cells were resuspended in 5 ml. of sterile 0.9 per cent potassium-chloride solutions, and the centrifugation repeated. This procedure was termed a "washing procedure" and was used for the purpose of removing most of the culture medium. After decanting, the cells were resuspended in the 20 ml. of sterile 0.9 per cent potassium chloride. The inoculum was then ready for use. A drop of

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[1] Identified as Pediococcus cerevisiae by C. S. McCleskey, J. Bacteriol., 64, 140 (1952).

this suspension was added to each assay tube by the use of a sterile syringe.

Sterilization and Incubation

Because of known variations due to autoclaving, a standardized procedure was followed. Prior to use, the autoclave was preheated at 120°C. for several minutes. The pressure was released rapidly, and the racks were placed in the autocalve. Again the temperature was brought to 120°C. for 5 minutes. After 5 minutes the pressure was released at a slow rate over a period of one minute. The racks were removed and allowed to cool. One drop of prepared inoculum was delivered to each tube as previously described. After inoculation the racks were placed into the incubator for 60-72 hours at 37° ± 1°C.

Titrations

After the prescribed 60-72 hours of incubation, the racks were removed from the incubator and prepared for titration. The acid produced during the growth period was titrated electrometrically with approximately .05 N KOH with the aid of quinhydrone, 1 M. calomel electrodes, and a Cannon automatic titrator. The automatic titrator was adjusted so that for each 100 counts 4ml. of approximately .05 N KOH was delivered. The galvanometer was adjusted so that when it was nulled, the solution was pH 7.3. This was obtained by the use of a properly prepared buffer system.

CHAPTER III

THE INFLUENCE OF FERTILIZATION ON THE GLUTAMIC AND ASPARTIC ACID CONTENT OF ALFALFA

Introduction and Literature Review

The primary effort of agriculturists in recent years has been to improve quality as well as to increase the quantity of various crops. This is in contrast to the effort of earlier years in which emphasis was placed on quantity alone. In the case of high-protein products, quality usually refers to the essential amino acid distribution in the protein. One of the problems still facing the animal nutritionist is that of obtaining sufficient protein of high quality to stimulate growth. Any alteration of environmental condition of the soil which will affect the quality of the total protein or of a particular protein in the plant would be desirable.

Several investigators (1-4) have reported that they observed changes in the amino acid composition of protein affected by fertilizer treatments. Other investigators (5-9) have found a uniformity in the amino acid content of protein in plants and meats regardless of the treatment.

Hollis (10) at the Oklahoma Station studied the effect of phosphorus, nitrogen and nitrogen-phosphorus combination

on the methionine, lysine, threonine, glutamic acid, and isoleucine content of the protein in cereal grass and alfalfa. He found that fertilization did not affect the amino acid content of the protein in cereal grass. The results with alfalfa showed that fertilization did not affect the amino acid composition of either the leaves or the stems. A difference was observed in the amino acid composition of the leaf protein and stem protein of alfalfa. The stem protein contained less methionine, less threonine, less glutamic acid and less isoleucine than the leaf protein. As the plant matured, the leaf-stem weight ratio decreased. It was considered likely that the change indicated a shift in the relative proportions of the various proteins in the whole plant.

Lugg and Weller (9) found no variation in the amino acid content of leaf proteins of plant species grown under different environmental conditions. Similar results were also obtained by other investigators (6-8, 10).

The effect of fertilizer on plants might be considered comparable to the effect of diet on animals. If fertilizer affects the amino acid distribution in plant protein, it might be expected that a change in the diet of an animal might alter the amino acid distribution of animal protein. This was not the case when Lyman and Kuiken (5) found that the amino acid distribution of animal tissue was the same regardless of the type of diet and species of animals from which it came.

The effect of mineral fertilizers on plant species has been studied by several investigators (1-4, 10). Blue et al. (1) showed that plants grown on different soil types did not have the same amino acid content. They reported that the increased variation of the amino acid content of plants grown on the same soil was due entirely to the small amount of inorganic elements which had been applied. The most significant increase in amino acids without increases of total nitrogen, resulted from the application of manganese and boron. They stated that these results seemed to substantiate the hypothesis that these two trace elements, manganese and boron, function in the conversion of the carbohydrates into protein.

Sheldon et al. (2) reported that the tryptophan content of soybeans, alfalfa, and red top clover, grown on quartz sand to which a full nutrient solution was added showed a pronounced variation when magnesium, manganese, boron, and iron were each withheld separately. When magnesium was withheld from soybeans grown in nutrient solution, the tryptophan content was reduced from 3.1 to 1.8 milligrams per gram of dry forage. When boron was withheld, the tryptophan in the forage was lower. Calcium and phosphorus also appeared to promote the production of certain amino acids. In general, it was found that the amino acid composition of a plant varied considerably as the concentration of certain elements varied within the soil.

Tisdale et al. (25) found considerable difference in the ability of two strains of alfalfa to synthesize both

methionine and cystine with a given amount of sulfate. The methionine and cystine concentration increased with an increased sulfate ion concentration. Sheldon et al. (3), working along this same line, showed that in sand cultures, the methionine content of alfalfa increased in concentration as the sulfate concentration was increased to a certain level but decreased if this level of sulfate was exceeded. Field trials on low-fertility soil showed that magnesium sulfate as a fertilizer caused an increase in the concentration of methionine. When barnyard manure and sulfur were added, an even greater concentration of methionine was observed. In further experiments by these investigators (4) sulfur applied at a rate of 200 pounds per acre on soil in which the phosphate level was adequate doubled the concentration of methionine in sudan grass. In all of these experiments the total nitrogen remained constant.

Mertz and co-workers (26) found that alfalfa contained all the common 18 amino acids even when grown on sulfur-deficient soils, and synthesis of the plant protein continued at the normal rate. As the sulfur concentration was decreased, the methionine and cystine content decreased, causing a decrease in the rate of protein synthesis. This caused an accumulation of the other amino acids which could be converted to asparagine for storage. This was substantiated by the results which showed an increase in the aspartic acid content of alfalfa grown on sulfur-deficient soil. They concluded that important changes in the protein makeup

of alfalfa take place during growth of the plant on sulfur-deficient soils.

Further evidence of a change in the relative amounts of protein in alfalfa was demonstrated by Mertz and Matsumoto (27). They produced a sulfur deficiency in alfalfa plants which became more severe at each successive cutting. Data were presented on ten amino acids in the leaf and stem which confirmed the findings reported previously (26). These data on the ten amino acids were obtained on normal and sulfur-deficient leaves and stems of alfalfa. Larger amounts of aspartic acid were found to be stored in sulfur-deficient stems than in the leaves. Results showed that in the sulfur-deficient leaves and stems only three amino acids (the sulfur-amino acids and glutamic acid) decreased in the stem, but fifteen amino acids decreased in the leaf; the largest change in amino acid content was in the leaf.

In the work of Mertz and Matsumoto (27) the alfalfa leaf protoplasm was extracted and fractionated into cytoplasmic solution and green particulate matter. The cytoplasmic solution was further fractionated by the use of trichloracetic acid into a cytoplasmic protein fraction and a cytoplasmic nonprotein nitrogen fraction. Results on the alfalfa leaves indicated that the green particulate matter nitrogen and cytoplasmic protein decreased in the sulfur-deficient leaf while the amide nitrogen increased. After the separation of cytoplasmic protein fractions in normal and sulfur-deficient leaves and stems of alfalfa, amino

acids were determined by microbiological procedures. No significant trends or changes in amino acid composition of these fractions were observed. A chromatographic analysis of the separated nonprotein fractions of normal and sulfur-deficient alfalfa leaves was conducted. Results indicated that several free amino acids were present in the normal leaf. These included aspartic acid, asparagine, alanine, arginine, glutamic acid and glutamine. In the sulfur-deficient plant the free amino acids were dominated by asparagine and aspartic acid. Arginine, along with other amino acids was detected in small quantities. A microbiological assay was conducted on the cytoplasmic nonprotein nitrogen fraction for arginine, aspartic and glutamic acid. Results showed an aspartic to glutamic acid ratio in the control plant of 3:1 but in the sulfur deficient plant of about 15:1. Also in this study an electrophoretic analysis was conducted on the isolated protein fraction. Even though there was no apparent amino acid difference in the composition, a difference was shown in the relative proportions of the various cytoplasmic proteins. The difference was a decrease of one protein component with a corresponding gain of another protein component in alfalfa grown on sulfur-deficient soil. The authors suggested that the major cytoplasmic protein was an expendable protein which was depleted when the plant was under stress.

In view of the unsettled question of whether or not fertilizer has an effect upon the amino acid distribution

of plant protein, studies were outlined to further the investigation started by other workers at this station. Alfalfa plots established by the Agronomy Department were available. These plots had received various fertilizer treatments which included phosphorus, potassium, boron, sulfur and magnesium at various rates. Glutamic and aspartic acids were selected for study because of previous work done at this station. The microbiological assay procedure was selected to determine their concentrations in the alfalfa protein quantitatively. This work was designed as a preliminary survey for possible detailed work in the future.

Specific Procedures

Source of materials used in this study, field treatments, handling of samples and general microbiological assay procedures have already been described under General Procedures. Certain modifications of the procedures were employed for the microbiological analyses of alfalfa stems and leaves for aspartic and glutamic acid and require further description.

Modifications of the Media

The basal medium used (Appendix) was essentially the same as recommended by Henderson and Snell (22), but with certain modifications. An "all-potassium" modification of this medium was studied by Sirny *et al.* (24), and has been extensively used in microbiological work done at this

station. This modification was employed for preliminary analyses, but because of the recent demonstration of a sodium requirement in L. arabinosus by Mills (28) and because of a beneficial effect of sodium observed in the glutamic acid assay, the low-sodium modification originally recommended by Sirny et al. (24) was adopted for most of these studies. This modification involves replacement of the sodium citrate with potassium citrate, resulting in a medium which is relatively high in the potassium ion and which also contains a small quantity of the sodium ion from the sodium acetate in the original medium. A further modification for the glutamic acid assay involved replacement of the D L-aspartic acid in the medium with L-asparagine at one-fourth the concentration of aspartic acid. Because of certain difficulties perhaps attributable to the hydrolysis of asparagine to aspartic acid on long standing in acidic solutions, it was decided to incorporate the asparagine into the medium in dry form just prior to use. Following this change, improvement in assay results was obtained; however, specific experiments to directly implicate the hydrolysis of the asparagine as the cause of the earlier difficulties were not conducted. This procedural modification was employed for the major part of the assay work. Another modification employed for both aspartic and glutamic acid assays involved the adjustment of the initial pH of the medium as well as the sample hydrolysates to pH 6.0.

Standard Solutions

L-aspartic acid and L-glutamic acid (A R grade) each at 50 micrograms per ml., were employed for the standard solutions. Because of the inhibition of growth of micro-organisms caused by excess salt in the medium, it was felt desirable to add also to the standard solutions an amount of salt approximately equivalent to the amount found in the diluted sample hydrolysates. This eliminated major differences between standard and sample tubes due to salt content. Potassium chloride was the salt added.

Results

The glutamic and aspartic acid composition of leaves and stems of alfalfa are reported as grams of amino acid per 100 grams of protein in tables according to treatment. Each value shown in the Tables I, II, V and VI is a result of two or more determinations. Results from Tables I, II, V and VI for each sample were averaged within treatments and cuttings. These values are shown as bar graphs in Figures 1-6. The bar graphs show the relationship of aspartic and glutamic acid in the leaves and stems as affected by fertilizer treatment. The leaf-stem ratios are reported in Table IV. Per cent protein values are shown in Table III for stems and Table VII for leaves. The per cent protein is based on 16 grams of nitrogen per 100 grams of protein. The first cutting of alfalfa in 1955 was lost due to adverse weather conditions. Leaf analyses for glutamic and aspartic acid for the fourth

cutting have not been completed, and certain values for the third cutting are also not presently available. This phase of the work is incomplete because of the initiation of studies on improvement of assay procedure.

These latter studies comprised a major portion of the research work conducted and are presented in these studies.

Glutamic Acid in Leaves and Stems

The bar graphs shown in Figures 1-6 were prepared by arranging stem values in ascending order and arranging the leaf values in the same treatment order. Results in Figure 1 indicate that the stem values for glutamic acid range from 4.5 per cent grown on P_1K treated plots to 7.6 per cent grown on K treated plots. Leaf values ranged from 5.2 per cent grown on plots treated with R_1P_1SK to 6.6 per cent grown on check plots. Treatments which exerted the greatest effect upon glutamic acid content of the stem protein were R_1P_1MgK, R_1P_1K, $MgSR_1P_1B$ and K alone. Treatments which caused the greatest increase upon the per cent glutamic acid in the protein of the leaf content were Ck, R_1K, $MgSR_1P_1BK$, R_1P_1S, R_1P_1BK and R_1P_1B. In the third cutting, shown in Figure 2, stem values for glutamic acid range from 4.9 per cent grown on plots treated with $MgSR_1P_1B$ to 6.9 per cent grown on plots treated with R_1. The values for glutamic acid in the leaves range from 5.0 per cent grown on plots treated with R_2P_2K to 7.9 per cent grown on plots treated with P_1. Treatments which exerted the largest effect upon glutamic acid content of stems in Figure 2 as compared to no treatment were R_1P_1BK, CkK and R_1. Treatments

which exerted the largest effect upon glutamic acid content of the leaf as compared to no treatment were P_1 and R_1. In the fourth cutting shown in Figure 3 glutamic acid in the stems of alfalfa ranged from 4.6 per cent grown on plots treated with potassium to 6.9 per cent grown on plots treated with R_1P_1BK. Values are not available for the glutamic acid in the leaf for the fourth cutting. Largest effects with respect to the check plot were due to R_1P_1 and P_1BR_1K, which gave increases, and R_1 which gave a decrease.

Aspartic Acid in Leaves and Stems

The values for aspartic acid in leaves and stems of alfalfa are shown in Figures 4-6. The aspartic acid values for the stems were arranged on the graphs in ascending order. The values for the leaves were arranged according to the same treatment order. In the second cutting, shown in Figure 4, the aspartic acid in the stems ranged from 13.9 per cent grown on plots treated with R_1P_1B to 18.7 per cent grown on plots treated with R_1P_1Mg. The largest increase with respect to check plots was due to treatments R_1P_1S, P_2K, R_1P_1BK and R_1P_1Mg. The aspartic acid values in the leaves ranged from 7.3 per cent grown on plots treated with R_1P_1B to 11.6 per cent grown on plots treated with R_1P_1. A majority of the values for aspartic acid in the leaf are below the check plot values. The treatments which show an increase are R_1P_1, R_1P_1BMgS, P_2 and R_1P_1Mg. In the third cutting shown in Figure 5 the aspartic acid in the stems ranged from 13.2 per cent grown on plots treated with R_1P_1BK to 18.9 per cent grown on plots treated with R_1P_1.

The majority of the treatments gave values close to the check values. R_1P_1SK, R_1P_1K and R_1P_1 gave an increase. R_1P_1BK, R_1P_1B and R_1P_1S showed a decrease when compared with the check plots. The aspartic acid values in the leaf shown in Figure 5 ranged from 9.1 per cent grown on plots treated with R_1P_1BMgS to 13.0 per cent grown on plots treated with P_2. P_2, $MgSK$, R_1P_1B and P_1K show the greatest increase above the check plot value. MgS, R_1P_1B and R_1K showed the greatest decrease. In the fourth cutting shown in Figure 6 aspartic acid values for stems range from 11.4 per cent grown on plots treated with R_1 to 15.2 per cent grown on plots treated with $R_1P_1B_1$. The large increases above the check values were due to R_2, R_1P_1BMgS, R_1P_1S and R_1P_1B with a majority of the treatments below the check value. Aspartic acid values for leaves on the fourth cutting are not available.

Discussion of Results

No systematic trends due to treatments were observed in the glutamic and aspartic acid content of the leaves and stems of alfalfa in either of the three cuttings. There are variations in the amino acid values reported in this study for both leaf and stem proteins of alfalfa. Whether or not the variations found in the study are true variations caused by fertilizer treatment or variations caused by experimental error is not readily ascertained. It is felt that the values reported for aspartic acid content of both leaves and stems of alfalfa are relatively reliable. The

glutamic acid values, however, are not reliable because of the inherent weakness in the procedure used in the assay work. This weakness is attributed to the inhibition by aspartic acid of the utilization of glutamic acid by L. arabinosus. The resulting values are 20-30 per cent lower than what are probably the true values. It was found in the improvement studies reported in this thesis that the aspartic-glutamic acid inhibition varied between assays. Therefore, no correction factor can be applied to glutamic acid assay values which were performed without the aid of the improved procedure. The values obtained under improved assay conditions are not reported in this thesis because they cannot be compared with earlier values obtained by use of the older procedure or compared with previous glutamic acid investigations. Any variation found in this study should not necessarily be interpreted as a change in the amino acid content of a particular protein. It might be interpreted as a shift in the relative proportions of protein between leaves and stems or within themselves as a unit. If the variations are due to treatment rather than experimental error, then a possible explanation is in order. Figures 1 and 2 indicate that the check plot is high in leaf glutamic acid and low in stem glutamic acid. The reverse of this relationship occurs when potassium is added to the soil alone. This might indicate that potassium plays a role in the translocation of glutamic acid from the leaf to the stem. In Figures 1, 2 and 3 a decrease in the number of

treatments which are below the check value might indicate that as successive cuttings are removed from each treatment plot, the cations of the soil are depleted which would cut down the metabolic rate, thereby impairing the translocation mechanism. Combinations of rock phosphate, superphosphate and potassium seem to exert the greatest variation when compared to the check plots.

The aspartic acid content of the leaves and stems is shown in Figures 4, 5 and 6. Again the same relationship which was reported in the glutamic acid values is evident. This relationship is that there is a decrease in the number of treatments which are below the check value. As outlined before, this might indicate an impairing of the translocation mechanism when the cations of the soil are depleted. A decrease in aspartic acid content between cuttings in the stems was noticed. It is felt that this decrease between the different cuttings is a true decrease and not influenced by experimental error. This observed decrease in aspartic acid content may be due to the accumulation of free aspartic acid during the early stages of growth. It is likely that this variation in the amount of free aspartic acid at different times of cutting may account for the decrease of aspartic acid between cuttings. However, it must be recognized that there are many other possible explanations for these variations found in this study, and that the data obtained is not such that definite interpretations can be made. Aspartic acid seems to respond to rock and superphosphate and potassium in a manner similar to glutamic acid.

The results for glutamic acid obtained in the stems range from 4.5 per cent to 7.6 per cent, which is lower than the 7.1 - 7.9 per cent obtained by Hollis (10), but compare favorably with 5.9 - 6.5 per cent values reported for Leguminosae by Lugg (8). The results for glutamic acid obtained in the leaves ranged from 5.9 to 7.9 per cent which is lower than the 8.8 - 9.7 per cent reported by Hollis (10). A possible reason for the values being lower than those of Hollis is that the values obtained for glutamic acid in a sample which contained high levels of aspartic acid may depend as much on the aspartic acid levels as on the glutamic acid levels. Hollis did not report aspartic acid values, so an accurate evaluation of the aspartic effect in his work cannot be made.

The aspartic acid values in this study on leaves and stems compare favorably with values obtained by Mertz et al. (26). This study was designed as a continuation of the preliminary surveys to determine if possible whether or not soil borne elements execute their influence on protein metabolism. After the survey of possible effects of soil borne elements on protein metabolism, closely controlled fertility greenhouse studies with a complete characterization of the fertilizer effect on the nitrogen fractions should be employed. This should consist of a separation of the main nitrogen fractions such as the protein fraction, free amino acid, amide nitrogen, etc. More precise chemical methods should be employed for the quantitative determination of amino acids. Instead of a large number of treatments,

fewer treatments should be used and these treatments should be selected so as to cause large variations. Phosphorus, potassium and sulfur appear to be the most likely minerals to be studied in further work.

Summary

No trends due to treatment were observed in the glutamic and aspartic acid content of the leaves and stems of alfalfa in either cutting. Variation between leaf and stem and within leaves and stems, along with variation between cuttings, were observed. These variations were suspected as being attributable to relative shifts of the amino acid within the plant. Rock phosphate, superphosphate and potassium seem to exert the greatest variation on both glutamic and aspartic acid in the leaves and stems of the alfalfa. Recommendations for further work are made and discussed.

TABLE I

Effect of various fertilizer treatments on aspartic acid content of alfalfa stems*

Treatments	Number of Cutting (2)				(3)				(4)			
				Average				Average				Average
Check	13.4	16.0	13.1	14.0	15.9	15.4		15.6	13.2	12.1	14.5	13.3
K	12.4	17.9	13.3	14.4	16.7	14.8	17.1	16.2	10.7	13.5	15.8	12.2
R_1P_1B	14.5	14.2	13.0	13.9	15.0	15.2	12.7	14.3	12.9	12.5	10.8	12.0
R_1P_1BK	19.2	16.3	19.3	18.3		16.1	14.1	15.1		12.7	13.6	13.2
P_2	17.5	13.1	18.2	16.3	15.6	16.2	15.4	15.7	12.7	14.1	14.3	13.7
P_2K	11.7	24.4		18.1	16.4	17.9	16.0	16.7		12.1	11.8	12.0
P_1	17.2		14.7	15.9	17.2	16.8		17.0	12.4	12.8	12.0	12.4
P_1K	17.0	16.5	16.2	16.5	16.4	14.3	17.5	16.0	10.6	12.0	13.5	12.0
R_2		15.1	14.7	14.9	15.1	16.8	16.2	16.0	12.5	10.8	11.6	11.6
R_2K	15.1	15.0	15.2	15.1	15.1	17.4	16.7	16.4	12.6	17.5	11.7	14.0
R_1P_1S	17.9	18.2	17.2	17.7	15.0	15.4	13.7	14.7	12.9		15.9	14.4
R_1P_1SK	15.9	14.5	16.6	15.7	16.9	18.2	16.5	17.2	12.1	11.1	12.9	12.0
R_1	13.3	16.4		14.8	15.4	19.6	13.6	16.2	12.2		10.5	11.3
R_1K	16.9	14.5	17.1	16.1	17.6	16.2	13.8	15.9		12.4	10.8	11.6
R_1P_1	12.9	15.1	18.6	15.5	21.2	19.7	15.9	18.9		12.2	13.5	12.9
R_1P_1K	14.2		15.8	14.9	23.4	16.7	16.5	18.8	13.5	11.5		12.5
R_1P_1Mg	17.7	20.4	18.2	18.8	19.8	15.7	14.6	16.7		13.0	12.0	12.5
R_1P_1MgK	18.6	15.8	16.1	16.8	17.0	16.1	15.8	16.3	12.2	14.1	12.0	12.7
R_1P_1BMgS	15.2	15.3	16.8	15.7	14.1	15.6	17.5	15.8	12.8	14.2	15.0	14.0
R_1P_1BMgSK	15.3	15.0	14.9	15.1	16.2	13.7	17.7	15.9	12.9	10.1	11.5	11.5

*Values reported as grams amino acid per 100 grams of protein.
Blank spaces represent incomplete analyses.

TABLE II

Effect of various fertilizer treatments on glutamic acid content of alfalfa stems*

Treatments	Number of Cutting (2)				(3)				(4)			
				Average				Average				Average
Check	5.4	4.6	5.0	5.0	4.9			4.9	6.3	5.3	6.3	6.0
K	7.4	9.4	6.1	7.6		8.6	4.6	6.6		4.6		4.6
R_1P_1B		4.7	8.3	6.5	5.9	5.1	6.8	5.9	6.2	5.6	5.0	5.6
R_1P_1BK	6.7	7.4	5.2	6.4		5.6	6.8	6.2		6.6	7.2	6.9
P_2	4.1	5.4	7.4	5.6	5.2	6.2	4.8	5.4	5.9	6.2	4.2	5.5
P_2K	4.4	6.1		5.2	6.2	3.7	5.0	5.0		6.1	6.8	6.4
P_1	5.5	4.7		5.1	5.1	6.1		5.6	4.7	6.3	6.2	5.7
P_1K	5.1	4.2	4.2	4.5	5.7	4.6	5.6	5.3		6.8	5.8	6.3
R_2	6.0	4.6	5.2	5.2	7.4	5.0	6.3	6.2	5.1	4.5	6.3	5.3
R_2K	7.4	5.1	5.0	5.8	5.1	5.4	4.5	5.0	6.2	7.4	4.6	6.1
R_1P_1S	5.5	10.1	3.6	6.4	5.5	5.8	5.9	5.7	6.6		5.0	5.8
R_1P_1SK	5.3	6.3	4.2	5.3	6.7	5.5	6.9	6.4	6.8	4.8	6.4	6.0
R_1	4.6	9.2	5.6	6.5	5.1	7.0	8.7	6.9	5.0		4.9	5.0
R_1K	6.0	6.6	6.2	6.3	4.6	6.9	5.7	5.7	5.2	5.9	6.3	5.8
R_1P_1	4.8	8.4	3.6	5.6	5.6	5.4	5.9	5.6		5.5	6.2	5.9
R_1P_1K	6.5	10.7	4.4	7.2	4.4	6.2	4.5	5.0	5.7	5.3		5.5
R_1P_1Mg	4.4	8.1	7.0	6.5	5.9	5.5	4.4	5.3		5.4	5.0	5.2
R_1P_1MgK	4.2	7.2	9.6	7.0	4.1	5.7	3.5	4.5	5.5	5.8	6.5	5.9
R_1P_1BMgS	5.3	8.0	8.5	7.3	5.6	6.0	5.6	5.7	6.1	5.5	5.4	5.7
R_1P_1BMgSK	4.5	8.3	6.3	6.3	4.6	6.4	3.6	4.9	4.8	5.4	5.3	5.1

*Values reported as grams amino acid per 100 grams of protein.
Blank spaces represent incomplete analyses.

TABLE III

Effect of various fertilizer treatments on per cent crude protein of alfalfa stems*

Treatments	Number of Cutting (2)				(3)				(4)			
				Average				Average				Average
Check	13.1	13.4	15.0	13.8	13.6	14.0		13.8	16.4	14.8	13.6	14.9
K	11.5	13.6	14.1	13.1	12.7	12.5	13.4	12.9	14.1	15.8	14.2	14.7
R_1P_1B	11.8	12.3	11.4	11.8	11.6	11.2	12.2	11.7	14.4	15.7	14.6	14.9
R_1P_1BK	11.5	12.4	12.8	12.2	11.4	12.3	11.0	11.6	13.4	14.5	13.1	14.7
P_2	14.1	13.1	13.8	13.6	14.5	12.8	14.1	13.8	16.1	14.1	15.6	15.3
P_2K	14.3	12.5		13.4	13.7	12.9	13.2	13.3		14.4	13.5	14.0
P_1	14.2	13.8		14.0	14.2	12.9		13.6	16.1	14.4	14.4	15.0
P_1K	12.5	13.6	14.1	13.4	12.3	12.8	13.8	13.0		13.5	14.1	13.8
R_2	14.1	15.1	12.6	13.9	14.8	15.1	14.1	14.7	16.6	15.3	14.9	15.6
R_2K	12.5	13.8	12.7	13.0	12.8	12.3	12.9	12.7	14.4	12.6	13.4	13.5
R_1P_1S	14.6	13.3	14.3	14.1	13.9	13.4	14.1	13.8	14.5	14.4	13.5	14.2
R_1P_1SK	12.6	12.2	14.1	13.0	13.1	13.1	13.3	13.2	14.0	13.4	13.8	13.7
R_1	12.9	12.3	12.6	12.6	14.2	12.3	13.9	13.5	15.2	13.7	15.1	14.7
R_1K	12.9	14.1	12.1	13.1	13.2	12.3	12.1	12.5	13.4	12.9	13.9	13.4
R_1P_1	16.2	13.8	14.2	14.8	14.5	14.2	13.8	14.1		15.1	15.0	15.1
R_1P_1K	13.2	12.8	13.5	13.2	13.3	12.1	12.9	12.8	14.7	15.2		14.9
R_1P_1Mg	14.8	14.6	13.9	14.4	12.8	14.3	14.2	13.8	13.7	15.4	15.0	14.7
R_1P_1KMg	12.8	13.1	13.5	13.1	14.1	12.8	12.9	13.3	14.2	14.2	12.9	13.8
R_1P_1BMgS	11.1	11.8	13.6	12.1	12.5	12.6	12.3	12.5	14.4	14.3	14.8	14.6
R_1P_1BMgSK	11.9	11.1	11.6	11.5	12.0	11.4	11.9	11.8	14.5	14.6	14.6	14.6

*Values reported as per cent crude protein based on 16 per cent nitrogen.
Blank spaces represent incomplete analyses.

TABLE IV

Effect of various fertilizer treatments on the leaf-stem ratio of alfalfa

Treatments	Number of Cutting (2)				(3)				(4)			
				Average				Average				Average
Check	1.9	1.7	1.6	1.8	.8	.6	.6	.6	1.4	1.5	1.7	1.5
K	1.4	1.8	1.7	1.6	.7	.9	.7	.8	1.7	1.6	1.5	1.6
R_1P_1B	1.7	2.3	1.7	1.9	1.2	1.2	1.0	1.1	1.8	1.5	1.8	1.7
R_1P_1BK	1.8	1.5	1.7	1.7	.9	1.2	.9	1.0	1.5	1.8	1.8	1.7
P_2	2.0	1.7	2.1	1.7	1.0	.8	.6	.8	1.8	1.5	1.5	1.6
P_2K	1.6	1.4	2.1	1.7	.7	.6	.9	.7	1.8	1.2	1.7	1.5
P_1	1.6	1.2	2.1	1.6	1.4	.7	1.0	1.0	1.7	4.0	1.6	2.4
P_1K	1.5	1.9	1.4	1.6	.5	.9	.6	.7	1.5	1.7	1.6	1.6
R_2	2.4	1.8	1.9	2.0	.9	.9	.9	.9	1.6	1.4	1.7	1.6
R_2K	1.8	2.0	2.0	1.9	.7	.9	.7	.8	1.7	1.5	1.7	1.6
R_1P_1S	1.5	2.1	2.2	1.9	.7	.8	.6	.7	1.6	1.7	1.9	1.7
R_1P_1SK	1.8	1.2	1.8	1.6	.7	.6	1.0	.7	1.3	1.4	1.8	1.5
R_1	1.9	1.6	1.5	1.7	.9	.6	.8	.8	3.5	1.6	1.5	2.2
R_1K	1.5	1.5	1.4	1.5	.7	.7	.9	.8	1.9	1.7	1.7	1.8
R_1P_1	1.4	1.9	2.1	1.8	.7	.8	.6	.7	1.4	1.3	1.8	1.5
R_1P_1K	1.3	1.8	1.5	1.8	.7	.7	.6	.7	1.7	1.5	1.8	1.7
R_1P_1Mg	1.4	2.4	2.2	2.0	.8	.6	.7	.7	.7	1.5	1.8	1.3
R_1P_1MgK	1.6	1.4	1.4	1.5	.7	.6	.6	.6	1.4	1.7	1.6	1.6
R_1P_1BMgS	1.3	1.4	1.6	1.4	.8	1.1	1.0	1.0	1.5	1.9	1.2	1.5
R_1P_1BMgSK	1.7	1.4	1.6	1.6	1.0	.9	.7	.9	1.6	1.5	1.5	1.5

TABLE V

Effect of various fertilizer treatments on the aspartic acid content of alfalfa leaves

Treatments	Number of Cutting (2)			Average	(3)			Average	(4)			Average
Check	11.1		9.2	10.2	10.2	11.0	10.8	10.6				
K	8.3		9.1	8.7	13.2	10.8	10.8	11.6				
R_1P_1B	7.3			7.3	10.4	10.2		10.3				
R_1P_1BK	9.2	9.7		9.5	9.8		10.8	10.3				
P_2	9.9		12.4	11.2	13.0			13.0				
P_2K					10.1		10.6	10.4				
P_1	9.0	7.7		8.3			11.3	11.3	11.1			11.1
P_1K	9.8		10.5	10.1	11.5			11.5				
R_2												
R_2K	10.0		8.2	9.1	11.7	11.2		11.4		9.5		9.5
R_1P_1S			8.6	8.6								
R_1P_1SK	8.9			8.9								
R_1	8.8	7.4		8.1	11.9	10.2	11.0	11.0				
R_1K	9.8			9.8		10.1		10.1				
R_1P_1	10.0	12.8	12.5	11.7								
R_1P_1K	9.0		10.2	9.6	10.9	11.4	11.8	11.4	9.9		11.6	10.8
R_1P_1Mg		11.4		11.4	11.2	9.4		10.3				
R_1P_1KMg		9.0		9.0		11.6		11.6				
R_1P_1BMgS	9.9	10.4	11.5	10.6			9.2	9.2	12.3			12.3
R_1P_1BMgSK	10.0	10.1		10.0	10.3	12.4		11.4				

*Values reported as grams amino acid per 100 grams of protein.
Blank spaces represent incomplete analyses.

TABLE VI

Effect of various fertilizer treatments on the glutamic acid content of alfalfa leaves*

Treatments	Number of Cutting (2)				(3)				(4)			
				Average				Average				Average
Check	6.0	6.9	7.0	6.6	7.0	6.2	7.1	6.3				
K	6.2	4.8	6.1	5.7	6.9		5.7	6.3				
R_1P_1B	6.0	7.5		6.8	6.3	6.5		6.4				
R_1P_1BK	7.0	5.9		6.4	7.2	6.1	5.8	6.4				
P_2	5.3	7.3	4.7	5.7	5.8	5.3		5.6				
P_2K					4.9		5.2	5.0				
P_1	6.2	5.2		5.7	7.9			7.9	5.6			5.6
P_1K	5.6	6.1	5.8	5.8	6.4	6.1	4.8	5.8				
R_2		5.3		5.3	4.8	5.2		5.0				
R_2K	5.5		6.0	5.7							4.1	4.1
R_1P_1S	6.3		5.9	6.1								
R_1P_1SK		5.2		5.2			6.8	6.8				
R_1	5.9	5.6	7.5	6.3	6.6		7.8	7.2				
R_1K	7.2	6.3		6.7								
R_1P_1	5.8		4.7	5.2								
R_1P_1K	6.0		5.6	5.8	5.9		6.8	6.3	6.5	6.0		
R_1P_1Mg		6.3		6.3	5.4	6.0		5.8				
R_1P_1MgK	5.2	5.5		5.4								
R_1P_1BMgS	5.5	7.0	4.4	5.7	6.0	6.0	5.7					5.7
R_1P_1BMgSK	6.7	6.5		6.6	7.4	6.4	5.8	6.6				

*Values reported as grams amino acid per 100 grams of protein.
Blank spaces represent incomplete analyses.

TABLE VII

Effect of various fertilizer treatments on per cent crude protein of alfalfa leaves*

Treatments	Number of Cutting (2)				(3)				(4)			
				Average				Average				Average
Check	25.4	27.9	27.1	26.5	22.4	21.2	22.4	22.0	28.1	26.2	26.6	26.6
K	25.9	24.4	27.0	25.8	20.8	21.9	21.8	21.5	26.5	26.6	26.1	26.4
R_1P_1B	26.0	25.6	28.5	26.7	23.6	23.3	22.2	23.0	27.6	26.9	25.7	26.7
R_1P_1BK	25.9	26.9	25.7	26.2	21.6	22.4	23.1	22.4		26.9	24.4	25.6
P_2	27.1	23.8	28.5	26.5	22.9	21.9	21.6	22.2	28.1	25.0	27.3	26.8
P_2K	28.1	27.8	24.9	27.0	23.5	22.0	22.4	22.6	28.8	28.1	25.9	27.6
P_1	26.6	27.3	24.7	26.2	15.8	21.8	22.1	20.0	26.1	27.9	26.7	26.9
P_1K	26.0	26.3	24.8	25.7	21.2	21.8	22.2	21.7	27.7	25.5	27.2	26.8
R_2	26.4	25.5	25.1	25.7	24.3	21.8	21.6	22.5	27.8	26.9	26.3	27.0
R_2K	26.1	25.6	25.5	25.7	21.7	22.4	22.0	22.0	26.3	26.4	25.4	26.0
R_1P_1S	29.1	26.3	26.4	27.2	23.1	21.8	21.6	22.2	26.4	26.3	26.4	26.4
R_1P_1SK	25.8	26.4	26.4	26.2	22.3	21.1	21.4	21.6	27.0	27.1	25.7	26.6
R_1	26.8	28.7	27.3	27.6	21.7	23.1	22.3	22.3	26.4	26.9	27.1	26.8
R_1K	26.6	25.4	26.1	26.0	21.8	21.6	22.2	21.9	25.9	25.4	25.8	25.7
R_1P_1	29.4	25.8	27.8	27.6	23.2	22.8	22.8	22.8	28.1	27.9	26.9	27.6
R_1P_1K	26.6	28.3	26.9	27.3	22.4	22.0	21.5	22.0	26.4	27.4	27.2	27.0
R_1P_1Mg		26.4	25.9	26.2	22.7	22.2	22.4	22.4	26.2	28.9	27.3	27.5
R_1P_1MgK	26.6	27.3	26.8	26.9	21.4	22.1	21.3	21.6	27.7	26.9	26.7	27.1
R_1P_1BMgS	27.2	26.8	25.8	26.6	22.3	21.9	23.7	22.7	27.8	27.0	27.6	27.5
R_1P_1BMgSK	26.9	25.9	25.3	26.0	23.7	21.6	21.3	22.3	27.0	27.6	27.6	27.5

*Values reported as per cent crude protein based on 16 per cent nitrogen.
Blank spaces represent incomplete analyses.

FIGURE 1

Relationship between the glutamic acid content of the leaves and stems in the second cutting

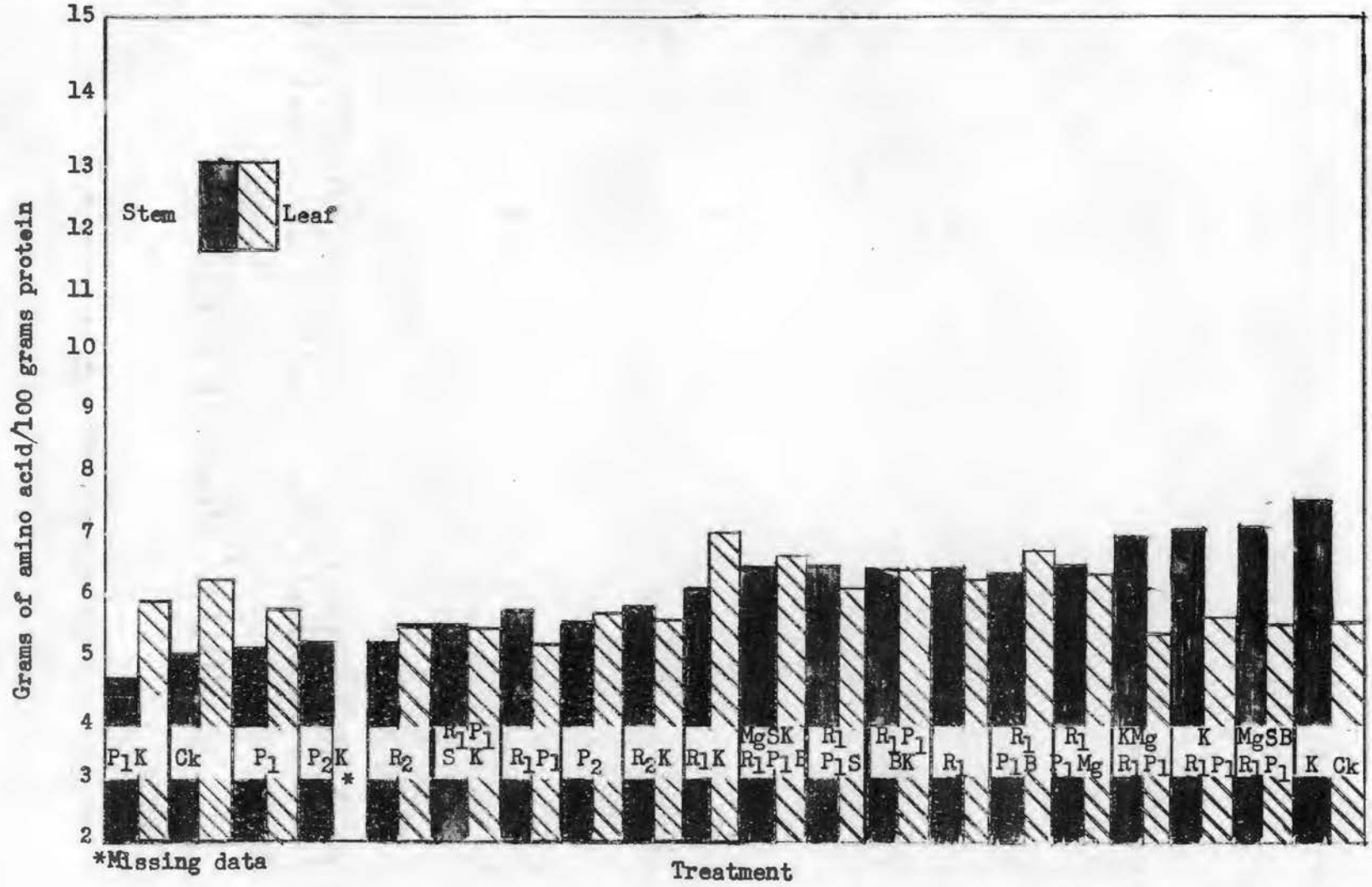
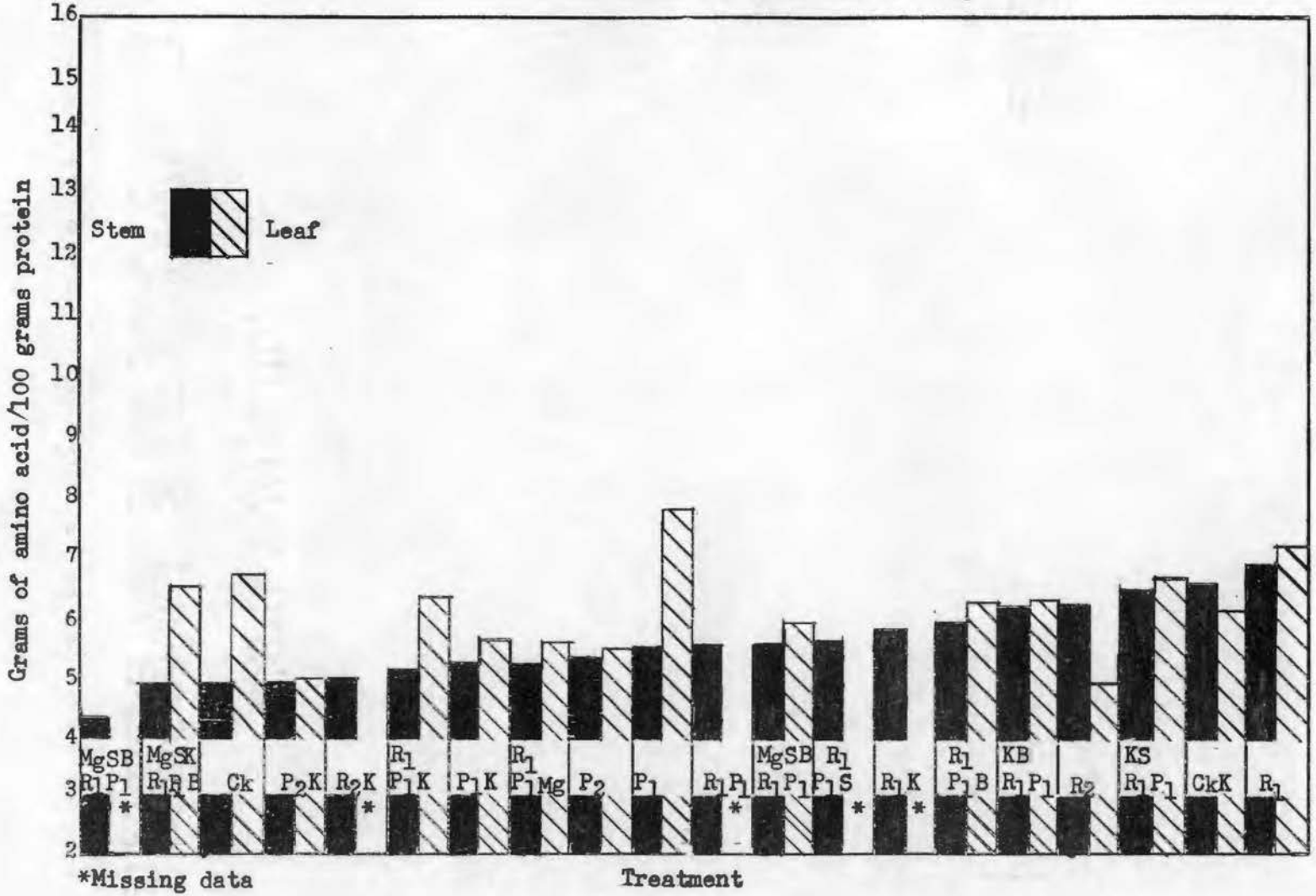


FIGURE 2

Relationship between the glutamic acid content of the leaves and stems in the third cutting



*Missing data

FIGURE 3

Relationship between the glutamic acid content of the leaves and stems in the fourth cutting

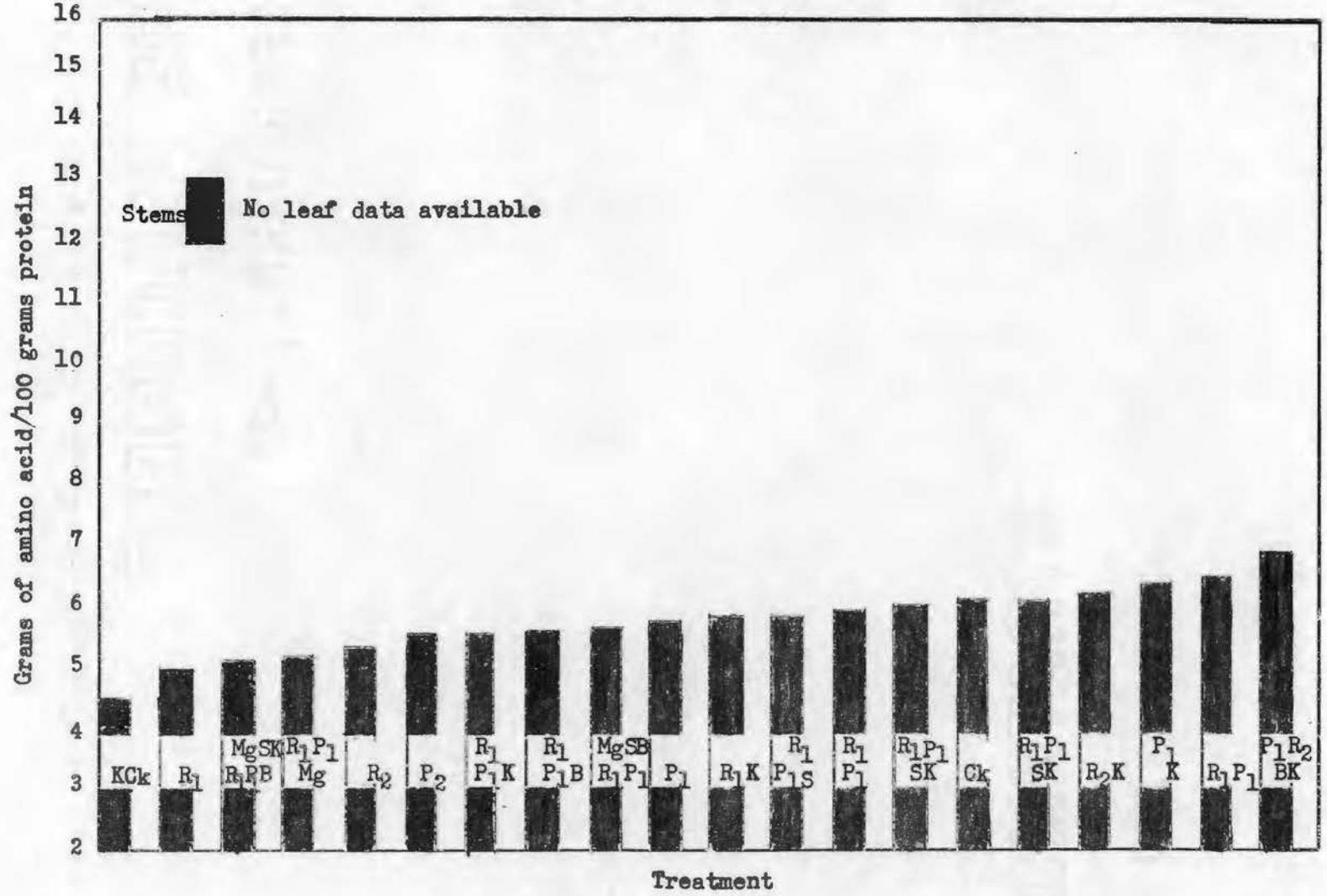
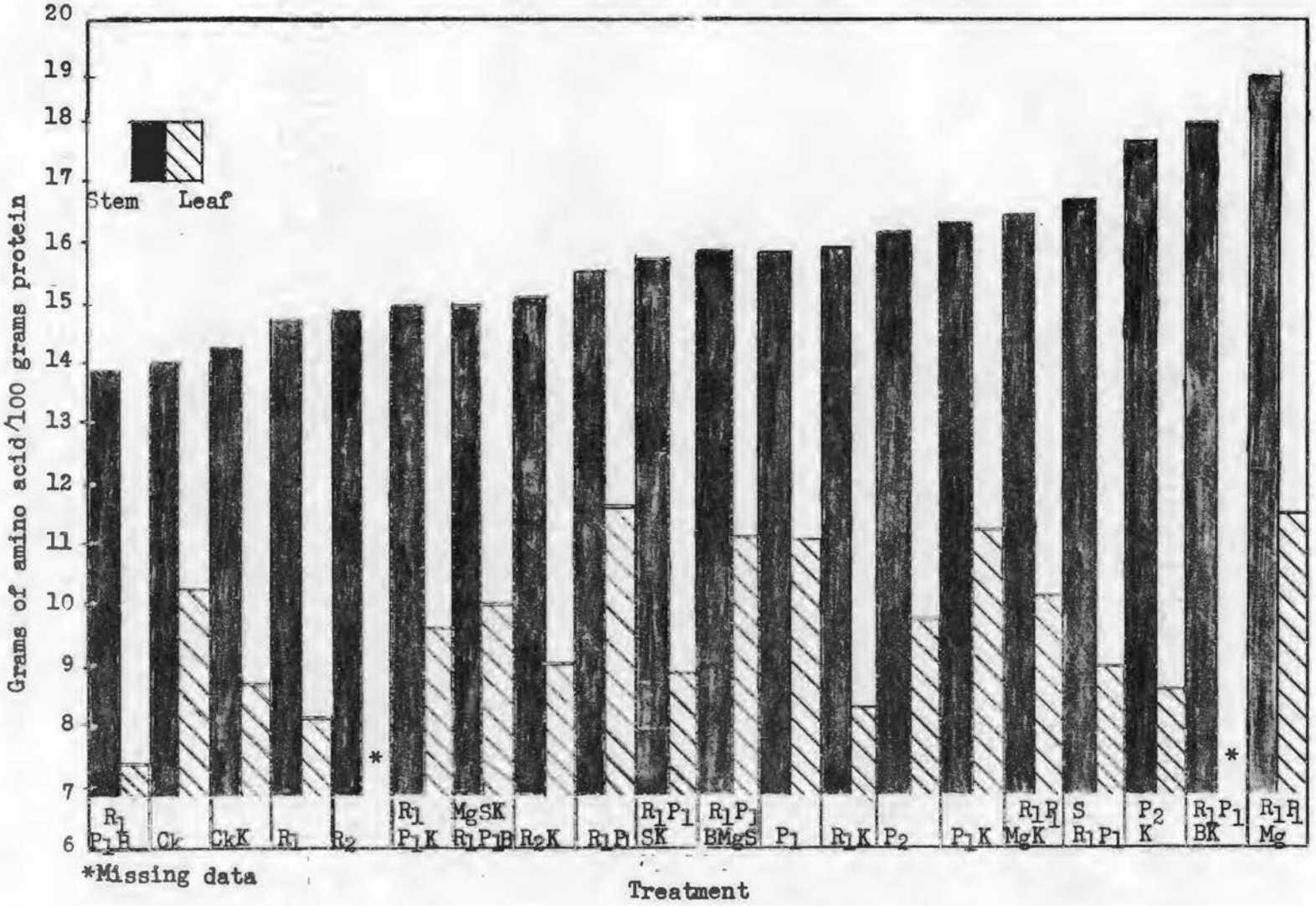


FIGURE 4

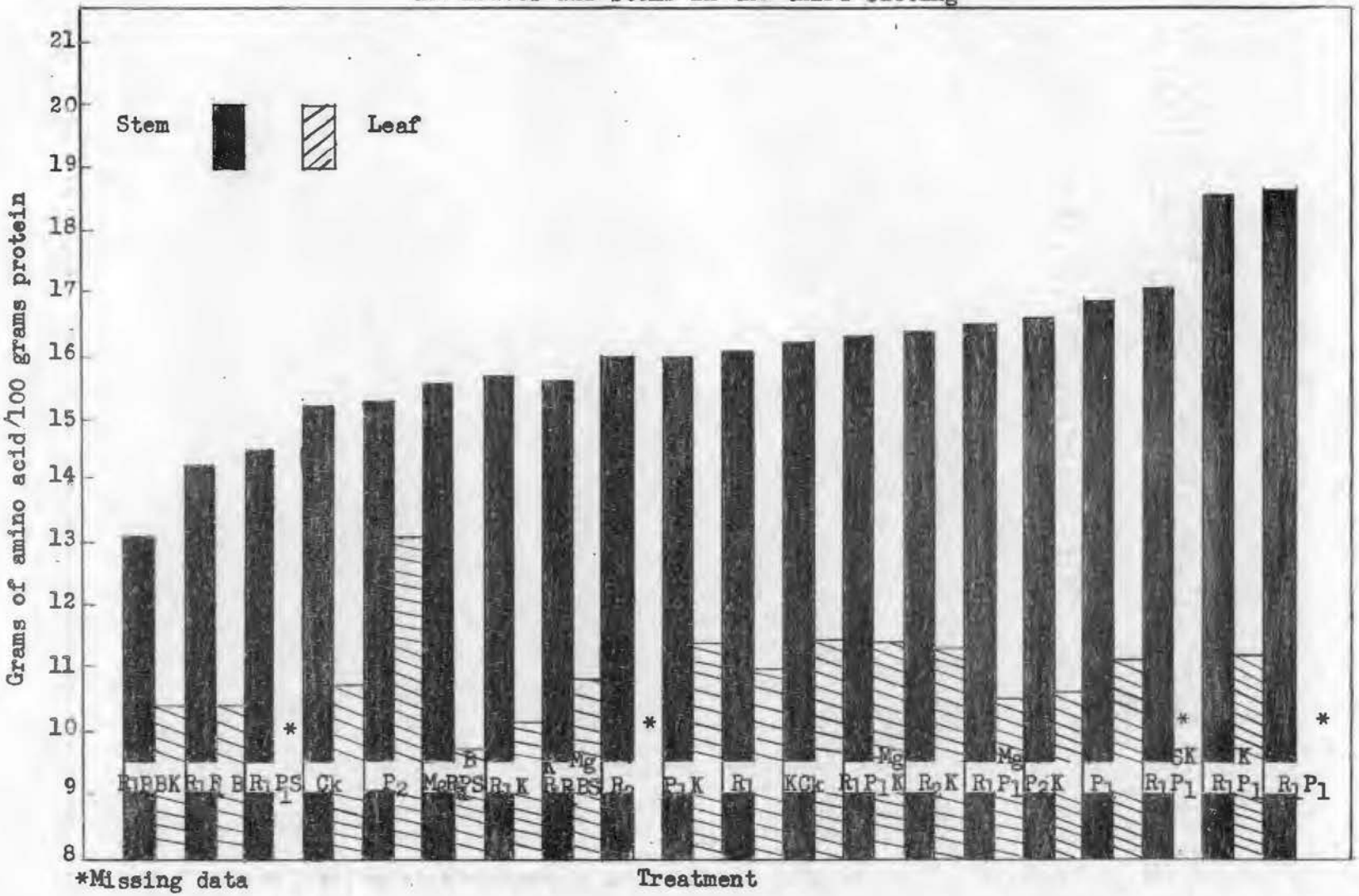
Relationship between the aspartic acid content of the leaves and stems in the second cutting



*Missing data

FIGURE 5

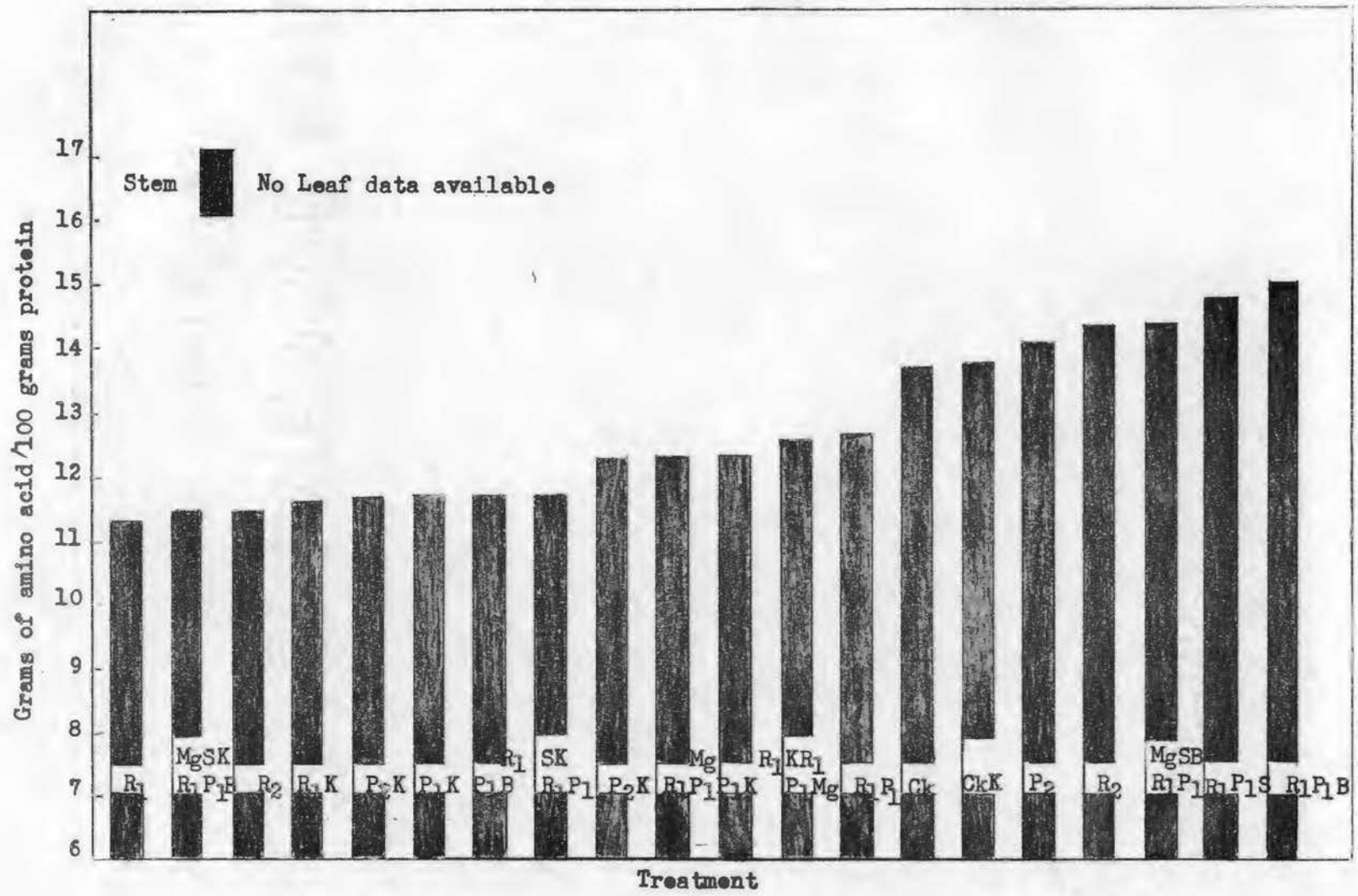
Relationship between the aspartic acid content of the leaves and stems in the third cutting



*Missing data

FIGURE 6

Relationship between the aspartic acid content of the leaves and stems in the fourth cutting



CHAPTER IV

STUDIES ON THE IMPROVEMENT OF THE MICROBIOLOGICAL ASSAY FOR GLUTAMIC ACID

Introduction and Literature Review

Micro-organisms are known to be specific in their nutritional requirements, their rate of growth being determined by the presence of certain nutritional factors. During the past years a procedure which makes use of micro-organisms for the determination of glutamic acid has been developed and adopted for routine use. This procedure was selected for routine use by many workers because chemical methods which had been employed for a quantitative determination of glutamic acid were tedious, inconvenient, or unreliable.

Several early investigators (11, 12, 14-16) reported that L. arabinosus required glutamic acid for sustaining normal growth. These early investigators described a quantitative method for the determination of L-glutamic acid by the use of L. arabinosus 17-5.

Lewis and Olcott (11) described a method for the microbiological assay of glutamic acid and studied some of the different factors which affect this assay. It has been

known that a change in the salt concentration of the medium affects the growth of L. arabinosus. Considerable amounts of sodium chloride are present in some protein hydrolysates as a result of the neutralization of the hydrochloric acid used in the hydrolysis. Lewis and Olcott (11) investigated the effect of sodium chloride and found that the addition of 40 milligrams and of 200 milligrams of sodium chloride per tube to the basal medium which already contained 40 milligrams per tube of sodium chloride stimulated acid production in the presence of small amounts of glutamic acid. Acid production was plotted against the concentration of glutamic acid added in graded amounts. Sigmoidal curves were obtained; the form and position of these curves to some extent depended on the time and temperature of incubation, the concentration of cells and the composition of the basal medium. A group of amino acids were tested individually by the addition of each to the basal medium of glutamic acid-free casein hydrolysate. The results indicated that some amino acids have a small stimulating effect. A further group of amino acids were tested which had an inhibitory effect. The inhibitory effects of aspartic acid and arginine were considered large enough to account for the initial plateau of the standard curves for glutamic acid. Asparagine was also observed to be inhibitory, but to a lesser degree. These investigators stated that if the aspartic acid content is high, the values for glutamic acid will be low. However, they suggested that if

glutamic acid is present in larger amounts than aspartic acid, the error may be negligible.

Hac *et al.* (16) observed a depression in the standard curve used for glutamic acid determination with *L. arabinosus*. The depression was thought to be due to the inability of the organism to convert low levels of glutamic acid to glutamine. Glutamine, instead of glutamic acid, was suggested as being the substance required for growth. They found that the depression of the standard curve was also a function of pH. As the pH of the medium was brought below 7.0, growth was initiated at lower concentrations of glutamic acid until a pH of 5.0 was obtained, at which the plateau of the growth curve was eliminated. They found in general that the activity of glutamic acid was increased by increasing the concentration of the inoculum, lengthening the incubation period, lowering the pH to 6.0 and adding ammonium salts to the medium. Lyman *et al.* (14) substantiated these findings and proposed that small amounts of glutamine be added to the basal medium used for the assay of glutamic acid.

Ayengar (29), in discussing the role of glutamine, reported that by varying the amount of glutamine in the glutamic acid standard, maximum stimulation was obtained with 20 micrograms of glutamine and 50 micrograms of glutamic acid per tube. In another experiment a constant amount of glutamine (15 micrograms) was added to varying amounts of glutamic acid. The stimulation increased with

increasing amounts of glutamic acid up to a level of 50 micrograms per tube. Results indicated that when glutamic acid was used by the bacteria, part of it must be converted to glutamine. They concluded that glutamine, once formed, can be used to form an intermediate which contains residues otherwise contributed by glutamic acid.

Baumgarten et al. (30) pointed out that glutamine should be present in the medium only in small amounts as it possesses glutamic acid activity. During autoclaving, glutamine was converted to the ammonium salt of pyrrolidone-carboxylic acid which has no activity. Their results indicated that smooth curves were always obtained when the medium contained asparagine. Because asparagine does not undergo any change during heat sterilization, its substitution for aspartic acid in assay media was considered a more preferable procedure when assaying for glutamic acid than the addition of glutamine.

Dunn et al. (15) were able to obtain reliable results in the determination of glutamic acid in casein and silk fibroin by the substitution of asparagine for aspartic acid and other modifications of the original medium. These modifications consisted of a ten-fold increase in the levels of adenine, guanine, uracil and vitamins in the medium.

The studies of Brickson et al. (12) indicated that, when the concentration of aspartic acid in the medium was reduced, the amount of depression was decreased and the lag was completely eliminated at levels of 40 micrograms per tube.

Aspartic acid was 10 to 20 times as inhibitory as asparagine. The addition of an inhibitory amino acid by way of the sample might result in a growth suppression in the sample tube which is not encountered in standard tubes. They recommended a procedure in which a moderately high concentration of all the constituents would be maintained, so that the effect of adding an inhibitory amino acid would be minimized. They stated that the competitive aspartic acid-glutamic acid growth inhibition found in L. arabinosus appears to be a function of the enzyme system which converts glutamic acid to glutamine. Aspartic acid may inhibit the reaction by competing for the enzyme site. Satisfactory results for the determination of glutamic acid were obtained by replacing the aspartic acid in the medium (22) by one-half as much L-or DL-asparagine.

In view of the several difficulties encountered in the determination of glutamic acid, improvement of assay conditions would be desirable. Poor reproducibility and low values have been characteristic of the results obtained in the determination of glutamic acid in alfalfa. The present study was designed to determine the optimum conditions for a glutamic acid assay in the presence of high levels of aspartic acid in the sample materials.

Specific Procedures

The general procedures used in this study such as sterilization, incubation, titration, preparation of tubes

and inoculation have already been described. Since the improvement of the glutamic acid assay is a study of certain specific constituents of the basal medium, only the preparation and dispensing of the medium and the glutamic acid standard solutions will be discussed here.

Basal Media and Standard Solutions

The medium used was essentially that recommended by Henderson and Snell (22) with proper modifications which have been described in the previous chapter. The basal medium was prepared free of asparagine, glutamic and aspartic acids.

The medium was prepared at such a concentration that 0.6 ml. delivered the same amount of all the growth factors (except the ones being studied) that were normally delivered at the 1 ml. level. Aspartic acid, asparagine and aspartic-asparagine combinations were prepared as separate solutions and at such concentrations that total volume of 1 ml. of the basal medium was delivered per tube. Standard solutions of glutamic acid at a concentration of 50 micrograms per ml. were also prepared. L-aspartic acid was added to each of two standard glutamic acid solutions at 50 and 100 microgram levels. The standard glutamic acid solutions containing the two different levels of aspartic acid were dispensed into the odd rows in separate racks. Water was added to each tube to make a total volume of 2 ml. Standard solutions with no added aspartic acid were dispensed into the even rows in a similar manner. The racks were sterilized,

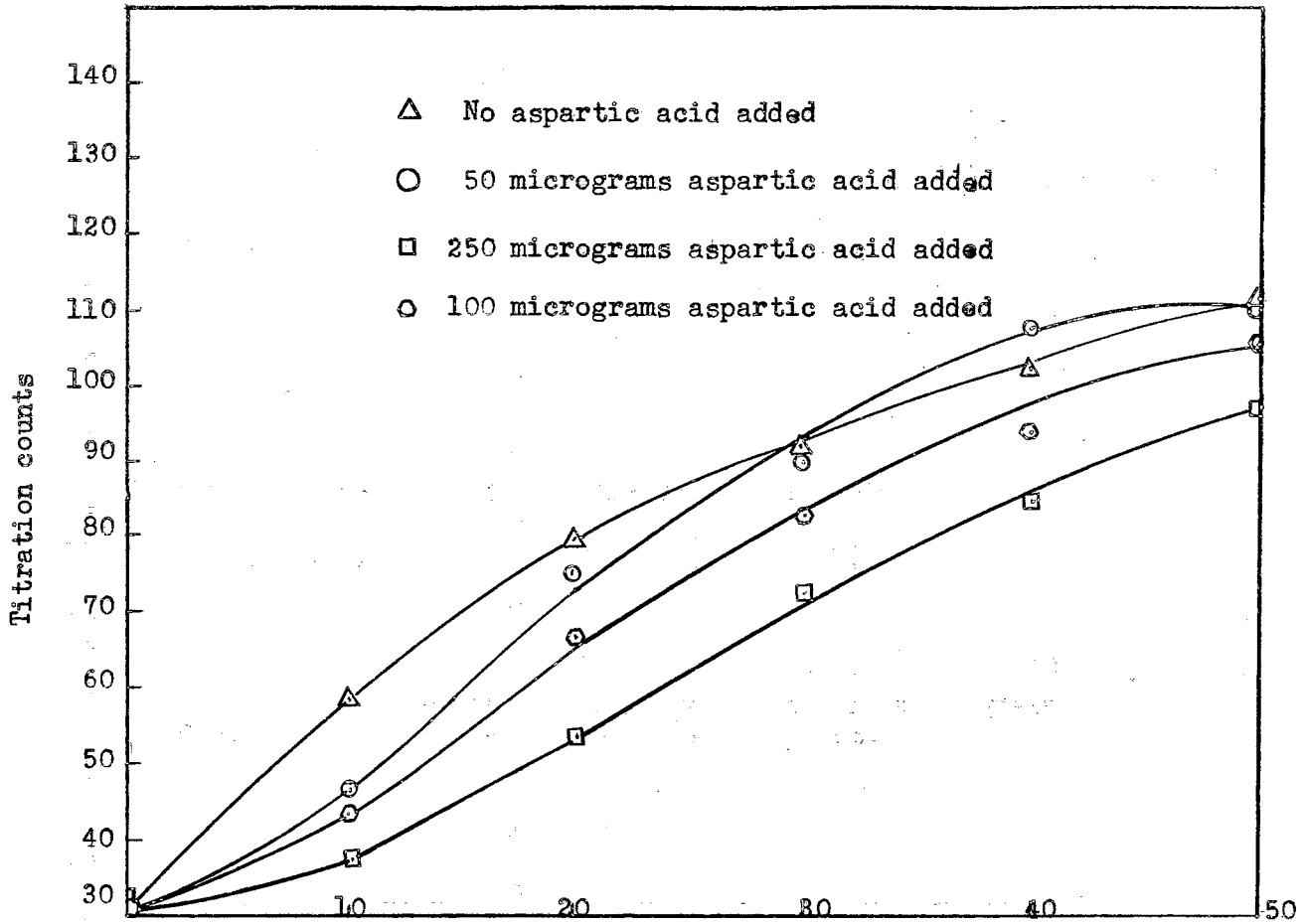
incubated and titrated according to the routine procedure previously described.

Results and Discussion

Figure 7 shows an inhibition of the utilization of glutamic acid by L. arabinosus when grown in the presence of 500 micrograms of asparagine per tube and varying concentrations of L-aspartic acid. The results indicate a varying degree of inhibition depending on the level of aspartic acid added. When assaying for glutamic acid in samples containing 50 micrograms or more of aspartic acid, it may be inferred from this figure that appreciable errors in the assay value for glutamic acid would be encountered. Experiments were designed to determine if the per cent inhibition caused by varying amounts of aspartic acid was constant from assay to assay. The results shown in Figure 7 were duplicated in a different assay at a different time. The inhibition was calculated in terms of per cent depression from the normal curve grown with a medium containing only 500 micrograms of asparagine. Results were plotted and are shown in Figure 8. The results indicate that there are significant differences in the degree of inhibition of glutamic acid utilization in the presence of varying concentrations of aspartic acid. These results indicate that poor reproducibility of assays may depend upon the varying degree of inhibition between repeated assays. A possible explanation of these latter results might be that a certain growth factor which affects the inhibition by aspartic acid of the

FIGURE 7

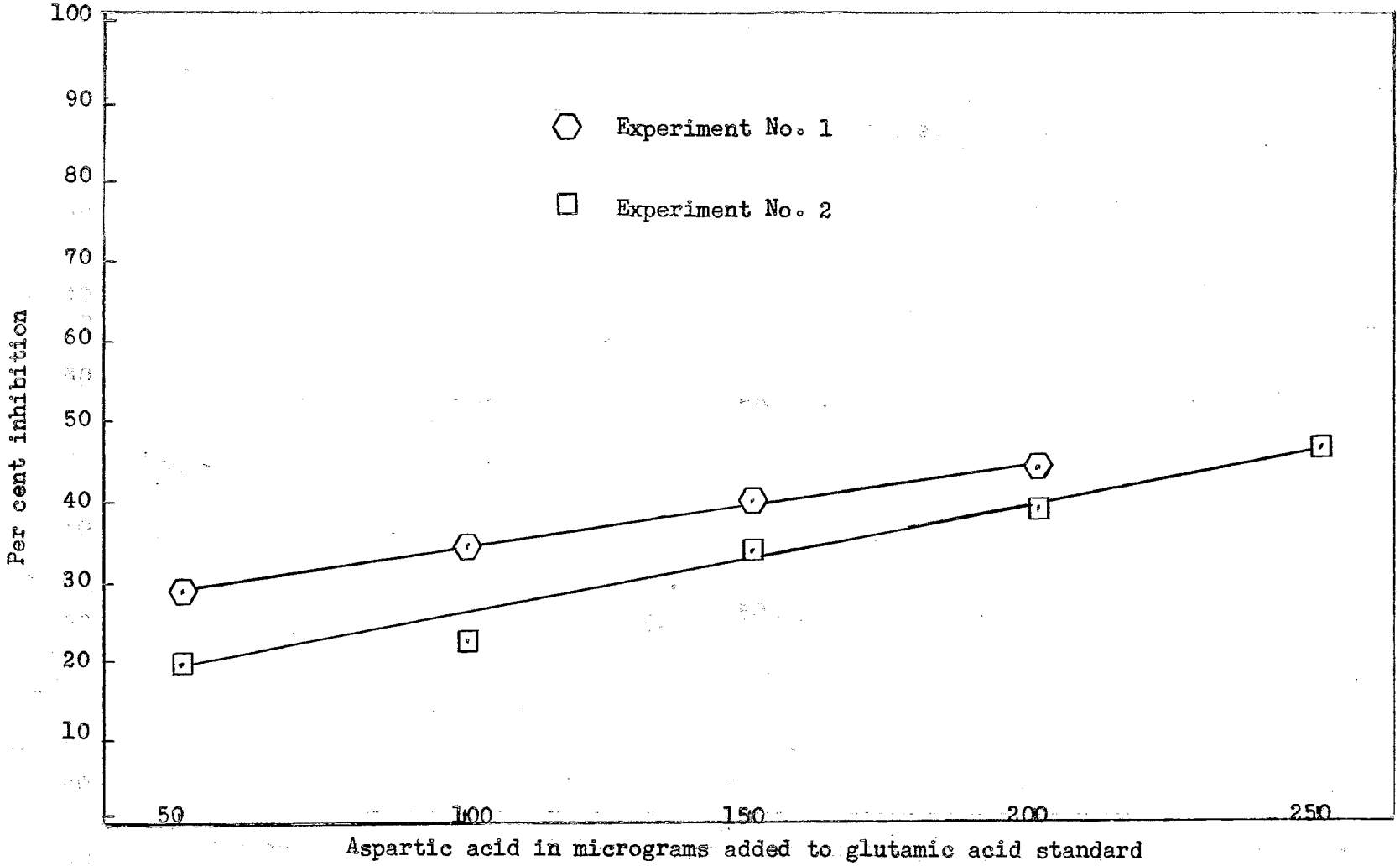
Study of the inhibition of the utilization of glutamic acid by L. arabinosus using varying amounts of aspartic acid*



*500 micrograms of L-asparagine added per tube

FIGURE 8

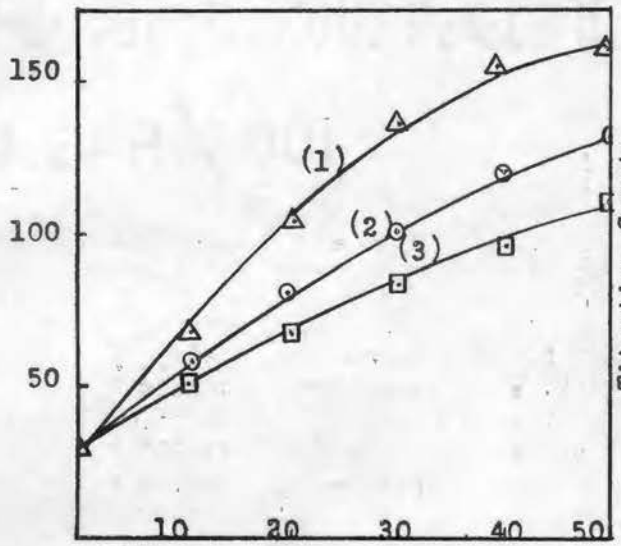
Per cent inhibition with varying amounts of aspartic acid of the utilization of glutamic acid by L. arabinosus



utilization of glutamic acid is at a critical concentration and difficult to control. Experiments were designed to determine optimum concentrations of aspartic acid and asparagine in the basal medium when assaying for glutamic acid in samples containing high levels of aspartic acid. Solutions were prepared which simulated samples containing 50 and 100 micrograms of aspartic acid per ml. These solutions were added to the medium containing asparagine or aspartic acid or combinations of both. Results are shown in Figures 9 and 10. These figures show the effects of adding 50 and 100 micrograms of L-aspartic acid to media containing varying levels of L-asparagine, DL-aspartic acid or combinations of both. Figure 9a shows the results of adding 50 micrograms and 100 micrograms of aspartic acid to a medium already containing 500 micrograms of L-asparagine. This figure demonstrates an inhibition which has already been discussed in connection with Figures 7 and 8. Figure 9b, curves 2 and 3 show the results of 1000 and 1050 micrograms, respectively, of DL-aspartic acid in a basal medium with no L-asparagine. These results indicate a large depression attributable to the competitive inhibition of very high levels of aspartic acid upon the utilization of glutamic acid by L. arabinosus. Curve 1 in each of Figures 9 and 10 was obtained on a medium which contained 500 micrograms of L-asparagine per ml. This curve may be considered the control growth curve for glutamic acid, and represents the conditions which have been used routinely

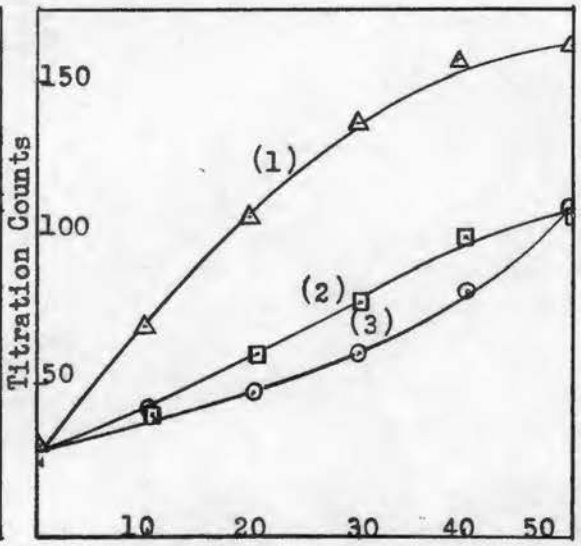
FIGURE 9

Effect of various concentrations of aspartic acid and asparagine upon the utilization of glutamic acid by L. arabinosus



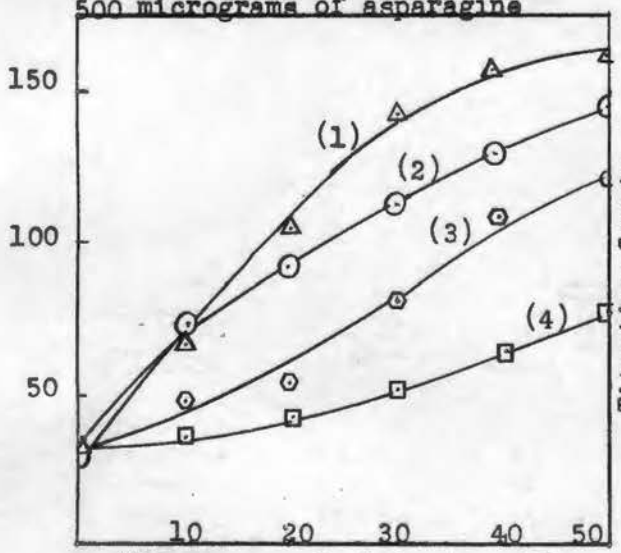
Micrograms of glutamic acid

FIGURE 9a (1) No aspartic acid 500 micrograms of asparagine (2) 50 micrograms of aspartic acid and 500 micrograms of asparagine (3)100 micrograms of aspartic 500 micrograms of asparagine



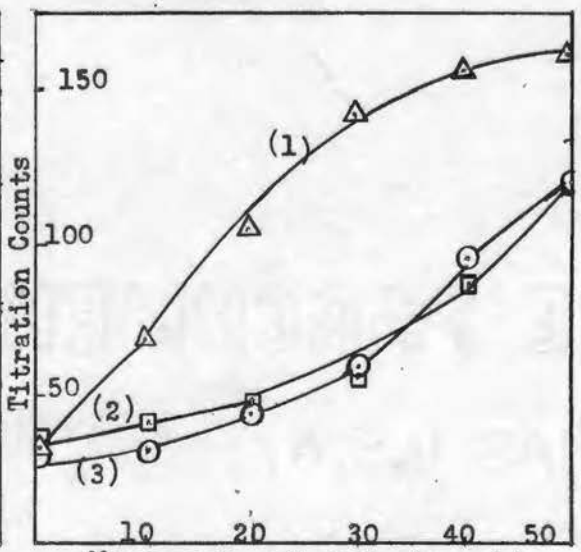
Micrograms of glutamic acid

FIGURE 9b (1) No aspartic acid 500 micrograms of asparagine (2) 1000 micrograms of aspartic acid no asparagine (3) 1050 micrograms of aspartic acid no asparagine



Micrograms of glutamic acid

FIGURE 9c (1) No aspartic acid 500 micrograms of asparagine (2) No aspartic acid 100 micrograms of asparagine (3) 50 micrograms of aspartic acid 1000 micrograms of asparagine (4) 500 aspartic acid 500 asparagine

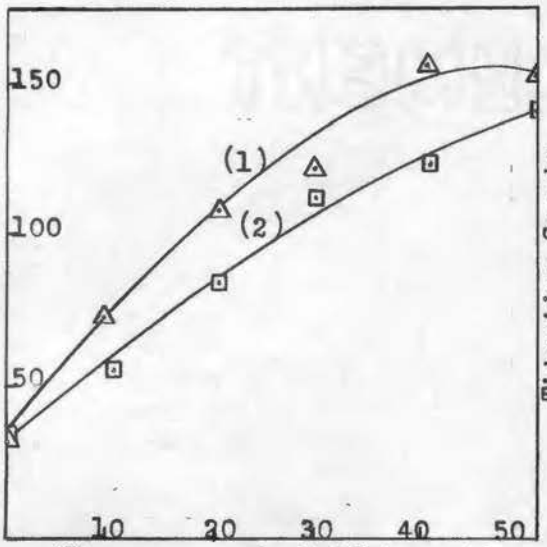


Micrograms of glutamic acid

FIGURE 9d (1) No aspartic acid 500 micrograms of asparagine (2) 500 micrograms of aspartic no asparagine (3) 550 micrograms of aspartic acid no asparagine

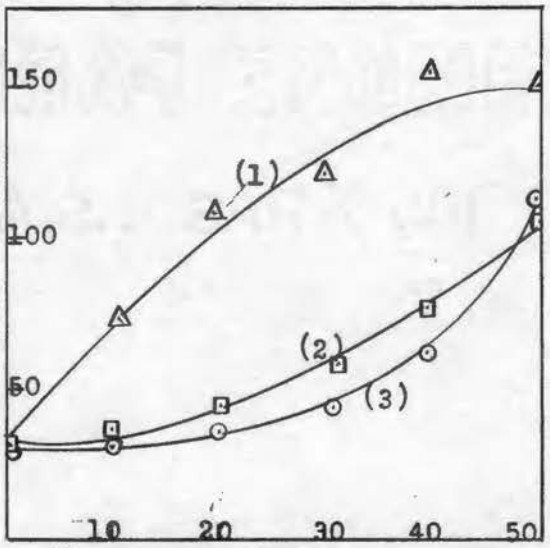
FIGURE 10

Effect of various concentrations of aspartic acid and asparagine upon the utilization of glutamic acid by L. arabinosus



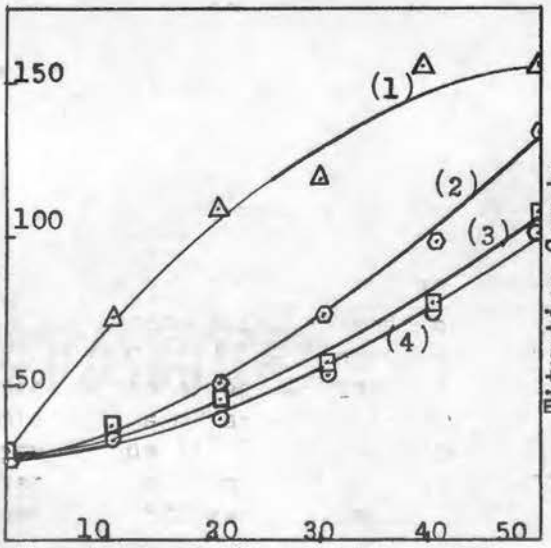
Micrograms of glutamic acid

FIGURE 10a (1) No aspartic acid 500 micrograms of asparagine (2) No aspartic acid 1000 micrograms of asparagine



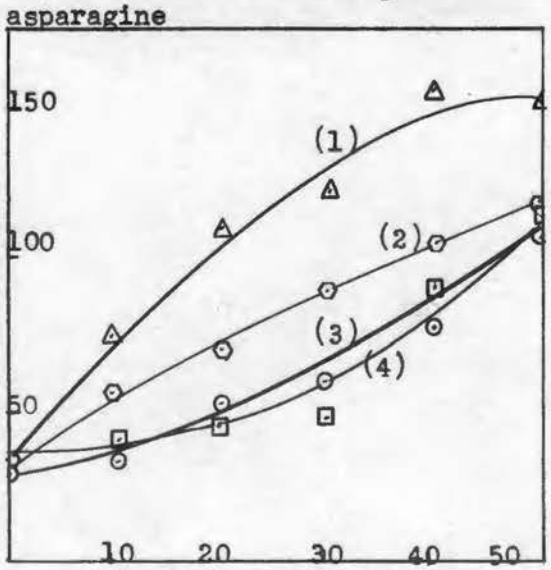
Micrograms of glutamic acid

FIGURE 10b (1) No aspartic acid 500 micrograms of asparagine (2) 1100 micrograms of aspartic acid no asparagine (3) 500 micrograms of aspartic acid 500 micrograms of asparagine



Micrograms of glutamic acid

FIGURE 10c (1) No aspartic acid 500 micrograms of asparagine (2) 500 micrograms of aspartic acid no asparagine (3) 1000 micrograms of aspartic acid no asparagine (4) 600 aspartic 500 asparagine



Micrograms of glutamic acid

FIGURE 10d (1) No aspartic acid 500 micrograms of asparagine (2) 100 micrograms of aspartic 500 micrograms of asparagine (3) 600 micrograms of aspartic acid no asparagine (4) 100 micrograms of aspartic 1000 micrograms of asparagine

in glutamic acid determinations. Growth at the 1000 microgram level of L-asparagine in the basal medium, as recommended by Brickson et al. (12), is shown in Figure 9c, curve 2. Figure 9c, curve 3 represents the results when 50 micrograms of L-aspartic acid are added to the Brickson medium. Errors in absolute values for glutamic acid assayed in the presence of high levels of aspartic acid would be obtained using either the Brickson medium or the present modification which has been used at this station. Figure 9d, curve 2 indicates results obtained while using aspartic acid in the medium at one-fourth the concentration recommended by Henderson and Snell (22). Curve 3 demonstrates the lack of depression from curve 2 when 50 micrograms of aspartic acid might be added by way of the sample. Figure 9d, curves 2 and 3 represent optimum conditions when using aspartic acid in the medium at one-fourth concentration recommended by Henderson and Snell (22). The results in this figure indicate that when the medium contains 500 micrograms of DL-aspartic acid per tube, any additions of L-aspartic acid by way of the sample give less depression. However, the growth curves obtained at this level of aspartic acid are in a state of serious lag and are not desirable for use. Figure 10 shows similar relationships with more depression severity than was obtained in Figure 9. Again the conditions which gave the least depression upon the addition of 100 micrograms of L-aspartic acid were 500 micrograms of aspartic acid or one-fourth the amount recommended by Henderson and Snell (22).

A procedure, described earlier, in which the same amount of L-aspartic acid present in the sample is added to the glutamic acid standard solution is considered a superior procedure. Results were obtained by this procedure showing no difference between the depression of the standard curve and sample curve. Figure 9a, curve 2 represents the standard or sample growth curve when 50 micrograms of L-aspartic acid is added by way of the sample to the medium already containing 500 micrograms of L-asparagine. The superiority with respect to reduced depression of Figure 9a, curve 2 and Figure 9d, curve 3 can readily be seen. A disadvantage of this procedure is that the values for aspartic acid contents of different materials may be difficult to obtain. It might, in fact, be desirable to determine the aspartic acid in a sample before the analysis for glutamic acid is initiated.

Figure 9c, curve 4 demonstrates a growth curve which possesses a marked depression. The conditions for this curve are 500 micrograms of DL-aspartic acid and 500 micrograms of asparagine or approximately equimolar concentrations. Figure 9c, curve 4 may be compared with Figure 9b, curves 2 and 3. From the comparison of these three curves, it can readily be seen that the curve with the approximately equimolar concentrations of 500 micrograms of asparagine and 500 micrograms of aspartic acid is more markedly depressed than the curve with 1000 micrograms of aspartic acid. Such a relationship would not be suspected, since it is known

that aspartic acid is 10 - 20 times more inhibitory than asparagine under many conditions. These results indicate that aspartic acid accentuates the depression in the presence of asparagine. This might indicate that aspartic acid and asparagine have a synergistic action in the inhibitory mechanism, and that at equal concentrations they exert a maximum depression of the glutamic acid growth curve.

A further modification of the glutamic acid assay was suggested by the improved results obtained when L-asparagine was added in dry form to the basal medium just prior to use. This procedure was thought necessary because of the suspected slow hydrolysis of asparagine to aspartic acid when stored in acid conditions for long periods of time. Improvements on the glutamic acid assay procedure found in this study along with improvements found by Hac *et al*. (16) should be employed for future glutamic assay determinations.

Conclusions

Results obtained in these studies show that serious depression from the normal glutamic acid growth curve are obtained when levels of aspartic acid in the medium are too high. Experiments were initiated to determine the optimum concentration of aspartic acid, asparagine or combination of both in the medium when assaying for glutamic acid in the presence of high levels of aspartic acid. Results indicate that if aspartic acid is used in the medium, the concentration should be 500 micrograms per tube. However,

this concentration of aspartic acid causes a serious depression of the normal growth curve and is not desirable for assay purposes. It is felt that a better procedure would be to add 500 micrograms of L-asparagine in the medium and add also the same amount of L-aspartic acid which is present in the sample to the standard glutamic acid solutions. By this procedure a lag difference between standard tubes and sample tubes is avoided. This procedure, in addition to yielding a good characteristic growth curve for L-glutamic acid, proves to be superior under assay conditions to other mentioned possible improvements.

The improvements found and suggested in this thesis for the determination of glutamic acid in the presence of high levels of aspartic acid may be summarized as follows: (1) Add the same amount of aspartic acid to the glutamic standard solution that occurs in the sample. (2) Add 500 micrograms of asparagine in the dry form to the basal media just prior to use. These along with using heavy inoculum and adjusting the pH to 6.0 as outlined by Hac *et al.* (16) serves to give reliable results for the glutamic acid assay.

CHAPTER V

A STATISTICAL ANALYSIS OF VARIANCE DUE TO THE POSITION OF TUBES IN THE AUTOCLAVE AND INCUBATOR DURING MICROBIOLOGICAL ASSAY PROCEDURES

Introduction and Literature Review

It has been observed in this laboratory, as well as others, that certain procedural conditions in the microbiological assay may be appreciable sources of variation. Of these, differences in the positions of the tubes inside the autoclave and incubator during the sterilization and incubation periods were particularly suspected. Several investigators (17, 18, 19) have found such variation in the procedures of the sterilization and incubation processes. Brownless and Lapedes (17) observed that when a number of racks were analyzed with all tubes containing the same amount of Vitamin B_{12}, there were systematic drifts in growth from one part of the rack to another, possibly caused by position in the autoclave during sterilization. This procedure was repeated on different autoclaves, and in every experiment significant variations were found. In one series of 10 racks the variations from one end of the rack to the other averaged 3.2 per cent ranging from 1.0 to 5 percent. Campbell et al. (18) prepared 100 tubes which

were handled in sets of 25. The tubes were arranged in four blocks which conformed to a 5 by 5 Hyper-Greco-Latin Square design. The design was arranged so that the effects of the position of tubes, rows, and columns in the autoclave and rows and columns in the incubators were all balanced within each of four blocks. An analysis of variance was made on each block. The results showed that the positions in the autoclave and in the incubator both had significant effects on the values obtained. A change in the position in the incubator caused the greatest difference. The variance between rows and columns within blocks exceeded the error variance by 2.6 times in the autoclave and by 4.3 times in the incubator. These results were later substantiated by Bliss (19).

It was thought desirable to study the effects of the position of the tubes in the autoclave and in the incubator upon the values obtained with the microbiological assay procedure used at this station. With the aid of the Oklahoma State University Statistical Department, a statistical study was designed to check these effects.

Procedure for the Statistical Analysis

Statistical Design

Morrison[1] designed a statistical approach to determine the source of variation due to position differences in the

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[1]Assistant Professor, Mathematics Department, Oklahoma State University.

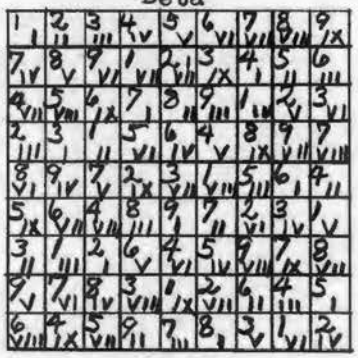
incubator and in the autoclave in a microbiological assay. The orthogonal Latin squares were selected from the publication, "Experimental Design," by Fisher and Yates (31). The arrangement of the Latin squares is shown in Figure 11. Four Latin squares were designated alpha, beta, delta and gamma. The design was arranged so that the effects being studied were all balanced within each of four squares. Consequently, each square was a unit within itself. Once a tube was assigned, it remained within that square, with only position changes, throughout the experiment. During the sterilization period each square assumed a predetermined position inside the autoclave as shown in Figure 12a. Each tube within a square assumed a predetermined random position in its respective square, and its position was recorded. The Latin squares, after randomized inoculation, were reassigned their location within the incubator as shown in Figure 12b. Tubes were rearranged within the squares according to the Arabic numbers and Roman numerals of each Latin square. An example of the tube rearrangement is as follows: A tube which occupies the position of Column 5, Row V in the alpha square during sterilization would be reassigned according to appropriate Arabic numbers and Roman numerals to Column 9, Row I within the same square during the incubation period. With this type of design, variation due to position in the autoclave and position in the incubator, along with variation in columns and rows of tubes within the squares, can be observed.

FIGURE 11

Nine x nine orthogonal Greco-Latin squares

Alpha

Beta

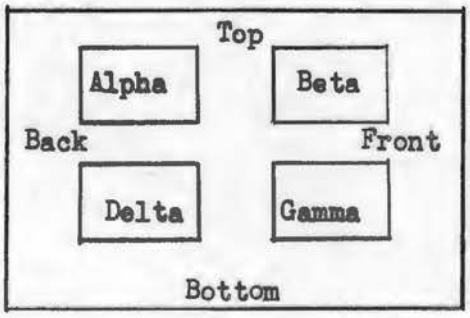


Gamma

Delta

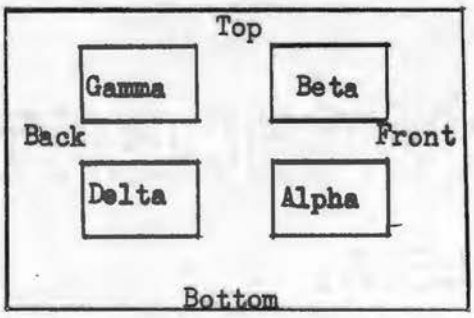
FIGURE 12

Arrangement of squares in the autoclave and incubator



Autoclave

FIGURE 12a



Incubator

FIGURE 12b

Preparation and Randomization of Tubes

Three hundred and twenty-four tubes were numbered according to the Latin square design. The tubes were completely randomized for preparation. A glutamic acid standard solution at a concentration of 50 micrograms per ml. was dispensed into each tube at a 0.5 ml. level by the procedure previously described. The basal medium was added at a level of 1 ml., along with 0.5 ml. water, to make a total volume of 2 ml. of solution per tube. The tubes were fitted with cotton plugs to prevent contamination while handling during randomization procedure. The tubes were placed in a preheated autoclave according to the statistical design which was described earlier, and were sterilized by a standardized procedure for 5 minutes at 120°C. Following sterilization the tubes were again completely randomized for inoculation with L. arabinosus. The procedure followed for the preparation of the inoculum was the same as that described under General Procedure. After autoclaving and inoculating, the tubes were placed in their assigned position in the incubator and incubated for 65 hours. A Cenco forced air circulation Model 803 constant temperature ($\pm$ 1°C.) incubator was used in all of the studies reported in this thesis. When incubation was completed, the tubes of all four squares were completely randomized again and titrated. Values were tabulated and prepared for statistical analysis.

Results and Discussion

Results of the analysis of variance are shown in Table VIII. Large variations due to rack positions on the shelves in the autoclave and incubator were indicated. Smaller but still significant variations due to tube position within the squares were also observed.

TABLE VIII

Analysis of Variance

Studies on Position of Tube During Sterilization and Incubation

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F Value
TOTAL	323	50372.75		
Latin Squares	3	19691.02	6563.67	83.074**
Shelves in Autoclave Squares ($\alpha+\beta$) vs ($\gamma+\delta$)	(1)	774.69	774.69	9.805**
Shelves in Incubator Squares ($\beta+\gamma$) vs ($\alpha+\delta$)	(1)	15964.08	15964.04	202.051**
Remainder	(1)	2970.25	2970.25	37.593**
Rows in Autoclave	32	4336.40	1135.51	1.715*
Columns in Autoclave	32	3105.73	97.05	1.228
Columns in Incubator	32	2735.72	85.49	1.082
Rows in Incubator	32	5336.63	166.77	2.111*
Error	192	15167.27	79.01	

*Significant at the 5 per cent level
**Significant at the 1 per cent level
Coefficient of Variation - 6.46 per cent

The large variations among squares, shelves in the autoclave, shelves in the incubator and rows in the incubator are indicated by the significant "F" values in this table. The largest indicated differences according to the "F" values were due to the positions of the squares between shelves in the incubator. Smaller but still significant differences were due to the position of squares in the autoclave. The analysis of variance shows that differences among rows in the autoclave and among rows in the incubator were significant. The coefficient of variation is 6.46 per cent. This value, obtained by dividing the overall mean into the standard error, is a measure of the uncontrolled variation. After the statistics of an experiment are summarized, one may judge its success partly by the magnitude of the coefficient variation.

The variations found in this study due to the sterilizing and incubating processes upon the position of tubes may be caused by several different factors. It is known that by sterilizing the medium in an autoclave, some effects which are stimulatory to growth and some effects which are deleterious to growth are produced. These effects may be a function of the temperature. A difference in temperature within the autoclave might produce one or the other or both effects in some of the tubes without affecting the other tubes. The constancy of the temperature within the autoclave depends upon the circulation of the steam throughout the autoclave. The circulation would be impaired to a greater

degree in the autoclave if it were filled to its maximum capacity. This test was not conducted at maximum capacity of the autoclave; therefore, a larger variation than found in this study might be expected when the autoclave would be operated at a maximum rack capacity. The largest difference within the autoclave during sterilization was between shelf positions. This indicates that whenever possible, assay racks should be kept as a unit on the same shelf in both the incubator and autoclave during microbiological procedure. It is imperative, also, that a standardized method of sterilization, such as the one already described, should be used in the sterilization procedure.

Borek and Waelsch (32) reported that a temperature increase of 2° (from 35° to 37°) changed the status of phenylalanine from that of a nonessential nutrient to an essential nutrient. Such a change in temperature might be involved in the cause of variation in this study due to the incubator.

With respect to the effect of incubation, the difference according to the "F" test was between shelves. This difference may be due to evaporation, heat gradients or other unknown causes. Evaporation might be caused by an improper fit of the lids which cover the tubes within the racks. Heat gradients would be due to improper circulation of warm air within the incubator. This effect would be expected to be most severe at maximum rack capacity of the incubator because of less circulation. It is important, therefore, to note

that the results reported here were obtained under conditions which were not at maximum capacity. However, under actual assay conditions operating at maximum capacity, greater variation might well be expected.

Improvements in incubation and in the procedure of analysis should be employed to reduce the amount of variation between racks and assays. One improvement which might aid in reducing the variations would be a different construction of the rack so as to allow greater circulation of air. Experiments might be conducted to see if a more optimal length of incubation period might be found. A different type of incubator such as the water incubator might be employed. A block randomized design as outlined by Campbell (18) might be adopted for routine use. A proper design, along with keeping the assay as a unit on the same shelf in the incubator and autoclave whenever possible, should reduce the error of position of tubes to a minimum.

Along with all these possible improvements which have been suggested, it is felt that the greatest improvement would be obtained by the incorporation of a proper statistical design which would account for some of the sources of variation in the assay procedure.

Conclusion

A statistical approach was designed to analyze the variations due to assay tube position during the sterilization and incubation steps in the microbiological procedure. Results show significant differences between shelves within

the incubator and the autoclave during the microbiological procedure. Differences between rows within the square on the same shelves in the incubator were seen to be significant. When tubes are arranged in a systematic order within the racks, their position effects would be expected to exert a large influence upon the values obtained, and in many cases, cause serious errors in assay results. Procedures which would minimize the possible effects upon tube position would be desirable. It is felt that, along with the improvements in sterilization and incubation procedures, rack construction, etc., a method involving block randomization or complete randomization of the tubes within the assay should be developed and adopted for routine use.

CHAPTER VI

GENERAL SUMMARY

Aspartic and glutamic acids were determined in alfalfa which had been variously-fertilized. The results indicated that no systematic trends due to fertilizer treatment were evident in the variations found in the amino acid content of alfalfa protein. Potassium, phosphorus and sulfur seemed to exert the largest effect upon the amino acid composition of leaf and stem protein. Poor reproducibility and low values were obtained on the glutamic acid values for alfalfa. These variations were attributed to the aspartic-glutamic acid inhibitions. It was deemed necessary to determine optimal conditions for the determination of glutamic acid in materials containing high levels of aspartic acid.

Results indicated that the normal growth curve was depressed considerably with as little as 50 micrograms of aspartic acid per ml. of sample material. By using varying amounts of aspartic acid and/or asparagine, optimal conditions were determined for the determination of glutamic acid. The amount of aspartic acid in the medium which was least susceptible to further depression caused by 50 or 100 micrograms of additional aspartic acid was 500 micrograms of

aspartic acid per tube. However, the resulting growth curve is so severely depressed that it is not suitable for assay purposes. Improvements found and suggested in this thesis for the determination of glutamic acid in the presence of high levels of aspartic acid are: (1) Add the same amount of aspartic acid to the glutamic standard solution that occurs in the sample. (2) Add 500 micrograms of asparagine in the dry form to the basal media just prior to use. These along with using heavy inoculum and adjusting the pH to 6.0 as outlined by other workers serve to give reliable results for the glutamic acid assay.

A statistical approach was designed to analyze the variation due to assay tube position during the sterilization and incubation steps in the microbiological procedure. Results show significant differences between shelves within the incubator and the autoclave during the microbiological procedure. Differences between rows within the squares on the same shelves in the incubator were seen to be significant. Procedures were outlined which would minimize their position effects. Such procedures were: (1) Block randomization of tubes on the same shelves of the incubator and autoclave. (2) Improved circulation of warm air within the incubator or other improvements in incubation. (3) Different rack construction to allow more freedom in circulation of warm air.

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APPENDIX

A. Media for Storage and Transfer of Organisms

Agar Medium

Yeast extract	10.0 gm.
Glucose	2.5 gm.
Agar	15.0 gm.
K-acetate	5.0 gm.
Water to 1000 ml.	

Liquid Transfer Medium:

Glucose	1.0%
K-citrate	1.0%
K-acetate	0.1%
K_2HPO_4	0.5%
NH_4Cl	0.3%
Tryptone	0.5%
Yeast extract	0.5%
Salts C soln.*	1.0%
Vitamin soln.*	0.5%

Dissolved in water, and pH adjusted at 6.0

The media were sterilized and stored in the refrigerator.

B. Basal Media for Microbiological Assays

Amino acid mix** (for 100 tubes at 2 ml. final assay volume):

DL-Alanine	200 mg.	DL-Threonine	40 mg.
DL-Aspartic acid	200 mg.	DL-Tryptophan	40 mg.
L-Glutamic acid	200 mg.	DL-Valine	40 mg.
L-Arginine HCl	40 mg.	Glycine	20 mg.
DL-Isoleucine	40 mg.	L-Cystine	20 mg.
L-Lysine HCl	40 mg.	L-Histidine HCl	40 mg.
DL-Methionine	40 mg.	L-Leucine	20 mg.
DL-Phenylalanine	40 mg.	L-Proline	20 mg.
DL-Serine	40 mg.	L-Tyrosine	20 mg.
		Made up to 50 ml. with acid and heat.	

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*Composition given in Appendix B.
**Amino acid being assayed is omitted.

B (Continued)

Sugar mix (for 100 tubes at 2 ml. final assay volume):*

Glucose	4.0 gm.
K-citrate H_2O	4.4 gm.
K-acetate (anhydr.)	0.2 gm.
NH_4Cl	0.6 gm.
K_2HPO_4	1.0 gm.
Salts C soln.	4.0 ml.
AGU-soln.	2.0 ml.
X-soln.	2.0 ml.
Vitamin soln.	2.0 ml.

50 ml. of amino acid mix is added, and the total made up to 100 ml. pH adjusted to the desired value.

Solutions for the above sugar mix:

Salts C

$FeSO_4 \cdot 7H_2O$	0.5 gm.
$MnSO_4 \cdot 7H_2O$	2.0 gm.
$MgSO_4 \cdot 7H_2O$	10.0 gm.

Dissolved with the aid of HCl, and made up to 250 ml.

AGU-Soln.

Adenine-sulphate	250 mg.
Guanine HCl	250 mg.
Uracil	250 mg.

Dissolved with the aid of HCl and made up to 250 ml.

Vitamin Soln.

Thiamin	25.0 mg.
Niacin	25.0 mg.
Ca-panto-thenate	25.0 mg.
Pyridoxal	5.0 mg.
Riboflavin	25.0 mg.
PABA	5.0 mg.
Biotin**	0.25 mg.
Folic acid***	0.25 mg.

Riboflavin dissolved first with hot water and acid, then the rest of the vitamins added and volume made up to 250 ml.

X-Soln.

Xanthine	250 mg.

Dissolved in dilute KOH and made up to 250 ml.

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*Na-acetate was substituted for K-acetate for L. arabinosus.
**Biotin stored in soln. in 50% EtOH.
***Folic acid stored in soln. in dil. KOH or NaOH in 50% EtOH.

VITA

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