THE INDEPENDENT AND MULTIPLE CONTRIBUTION OF CERTAIN ENVIRONMENTAL FACTORS ON THE SEASONAL VARIATION IN AMIDE NITROGEN FRACTIONS OF GRASSES

A Thesis

Submitted to the Faculty of Purdue University by James Bashore Beard

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ABSTRACT


The purpose of this study was to evaluate the seasonal variations in nitrogen fractions of grass leaf tissue and to determine the environmental factors influencing this variation. Initial studies were conducted under controlled climate conditions with the variable being four temperature treatments of 50, 60, 70, and 80°F. Six nitrogen fractions of bentgrass and bermudagrass were quantitatively analyzed. The total nitrogen and non-protein nitrogen were measured by standard Kjeldahl procedures while the total free ammonia, glutamine, asparagine, and total amide fractions were analyzed by a modification of a method described by Pucher, Vickery and Leavenworth.

This controlled climate study showed the total nitrogen content of bentgrass and bermudagrass to increase with temperature up to 70°F and then decrease slightly at 80°F. The non-protein nitrogen content increased as temperature increased in bentgrass while the opposite was true for bermudagrass. The asparagine fraction was more responsive to temperature changes than glutamine in both bentgrass and bermudagrass. In bentgrass the asparagine content declined with increases in temperature while in bermudagrass the asparagine decreased as temperature was
increased up to 70°F. and then began to increase at 80°F. The glutamine fraction increased slightly as temperature increased, with the increase being greater in bentgrass than bermudagrass.

More intensive studies were conducted on closely mowed bentgrass under natural environmental conditions utilizing a multiple factor statistical approach to evaluate the influence of environmental parameters including air temperature, soil temperature, soil moisture, light intensity, wind activity, evaporation rate and air pressure. All the plant and environmental data was entered on punch cards and statistical analyses conducted by means of high speed computers.

Employing the multiple factor approach, the amide responses observed under the more variable natural environment directly contradicted the results obtained under the limited controlled climate conditions. Thus, conclusions based solely on controlled climate chamber data would not have been valid if generalized to dynamic natural situations. This emphasizes the importance of verifying controlled climate results with studies under natural conditions.

Six linear multiple regression-correlation analyses indicated temperature to be the major environmental factor influencing the seasonal variations in all six nitrogen fractions measured. Temperature accounted for one-half to two-thirds of the predicted variation in the total nitrogen, asparagine, glutamine, and total amide fractions. The coefficients of determination for the total free ammonia and non-protein nitrogen fractions were low, indicating a minimum response to direct environmental influences. The $R^2$ was not increased when the analyses were computed with the independent environmental factors based on the average of the four days prior to the plant observation instead of on a one day previous
basis. This suggests that temperature influences on the nitrogen fractions are expressed rapidly within the plant.

All six nitrogen fractions decreased in quantity when the soil temperatures exceeded 80°F, thus implying an overall slowdown in the nitrogen metabolism processes of the bentgrass plant. The total nitrogen content increased as soil temperature advanced up to 80°F, with total nitrogen contents above 5% being associated with soil temperatures above 70°F.

The glutamine fraction was more responsive to temperature changes than asparagine thus suggesting a more immediate role in nitrogen metabolism. The glutamine content decreased markedly as temperatures advanced, declining from 0.06 to 0.01% expressed on a dry weight basis. This critically low amide level during the higher temperature periods could become detrimental to normal nitrogen metabolism within the plant should adverse stress situations occur. The asparagine fraction decreased to a lesser extent than glutamine and showed a delayed response thus suggesting it to be a secondary supplier of nitrogen.
THE INDEPENDENT AND MULTIPLE CONTRIBUTION OF CERTAIN ENVIRONMENTAL FACTORS ON THE SEASONAL VARIATION IN AMIDE NITROGEN FRACTIONS OF GRASSES

INTRODUCTION

Two bentgrass species are commonly used on golf putting greens. Agrostis palustris Huds. or creeping bentgrass is most frequently used, but Agrostis tenuis Silth. or colonial bentgrass is sometimes seeded. These species are the preferred grasses for putting greens in the northern, humid regions of the United States ranging as far south as North Carolina and Georgia. Within this region there are approximately 3,775 golf courses with 6,057 acres of bentgrass under putting green management. The management of this grass represents one of the most highly specialized uses of grass presently known.

The game of golf demands that the bentgrass be maintained at a cutting height of three-sixteenths to one-fourth of an inch, which limits the leaf area available for the production of photosynthate and imposes an abnormal stress on the plant. The turf surface must be smooth, dense, without grain, and firm to avoid deflection of the ball, yet it must have resilencey. To achieve these standards, current practices require daily mowing, high levels of fertilization, irrigation to insure constant water availability and the use of fungicides, insecticides, and herbicides to prevent turf damage or to remove undersirable plants which disrupt the turf uniformity.
The high temperature periods of mid-summer are especially detrimental to proper growth and maintenance of closely mowed bentgrass turf. Considerable knowledge has accumulated concerning bentgrass response to high temperatures in terms of root growth and dieback, leaf production and general turf appearance. However, much less is known about the actual temperature effects on the physiological functions and biochemical reactions within the plant. Exploratory investigations indicated that certain water soluble nitrogen fractions vary greatly with temperature changes. The group of nitrogen compounds that were particularly responsive to temperature variations were the amides.

Asparagine and, in particular, glutamine, among the amides, play a central role in protein metabolism. Glutamine has the ability to donate its amino nitrogen to protein synthesis processes as well as being able to store its amide group as a labile source of nitrogen for amino acid and protein synthesis. It is also significant that glutamine is a key intermediate in the routing of soluble nitrogen reserves to protein synthesis within the plant. In addition, it has been suggested that nitrogen is transported to the active centers of protein synthesis in the form of glutamine.

Proper nitrogen fertilization is an important key in a good turfgrass management program. Most nitrogen studies within the plant have involved measurement of the total nitrogen levels of the tissue. Practically no literature could be found concerning investigations of the seasonal variations in amide nitrogen fractions within plant tissue. Thus, the purpose of this investigation was to evaluate the seasonal variation in amide levels and to determine environmental factors which influence this variation.
Nitrogen fertilization is a vital segment in a grass management program. Nitrogen promotes growth through its incorporation into the protein constituents of plants. It also functions in the maintenance of green color through its key position in the chlorophyl molecule. In reviewing past research one finds extensive literature on soil-plant nitrogen relationships, particularly in terms of total nitrogen levels, but only limited work on the influence of external factors on various plant nitrogen fractions. Even less has been reported concerning the influence of environment on various nitrogen fractions, especially on a seasonal basis.

**Role of the Amides**

Included in the soluble nitrogen fraction of plant tissue are the amides which play a significant role in nitrogen metabolism. Extensive research of the past fifteen years has clarified the traditional process whereby inorganic nitrogen is combined with organic compounds to form amino acids or their amides. Oxaloacetic acid, α-ketoglutaric acid and pyruvic acid are the favored nitrogen acceptors with glutamine and asparagine being the two principal amides formed. An exhaustive coverage of research in the area of nitrogen metabolism will not be made since recent reviews (24, 29, 30, 31) have given excellent coverage. Ketic studies on the conversion of C\(^{14}\) labeled acetate into amino acids by the leaves and roots of bean seedlings were made by Rogers (22). The labeled
acetate was rapidly incorporated into glutamate and aspartate and more slowly into other amino acids. Also, the dicarboxylic acids, α-ketoglutarate and oxaloacetate, were labeled at rates closest to those of glutamate and aspartate. Thus, it has been established that, α-ketoglutaric acid is one of the main ports of entry for nitrogen into organic compounds of plants.

The conversion of amino acids into amides has received considerable attention from researchers. Glutamine synthesis in a cell free plant system was demonstrated by Elliot (7) in 1951 and later confirmed by Webster (28). They found that the synthesis requires ATP as an energy source plus magnesium ions. A similar synthesis reaction has been shown (32) for asparagine when ammonia, ATP, magnesium ions, and labeled aspartate are supplied. Once formed these two amides, particularly glutamine, are capable of readily supplying nitrogen for amino acid synthesis. Also, by combining with ammonia molecules the amides prevent accumulations of toxic ammonia levels within the plant tissue. Not only does glutamine participate in the metabolism of ammonia, but it is also a specific nitrogen donor for nitrogen atoms 3 and 9 in the purine ring; and for the amino group in guanosine-5-phosphate and glucosamine-6-phosphate as well as a direct precursor of glutamine residues in proteins. Thus, these amides serve as a key nitrogen pool in nitrogen metabolism.

**Environmental Influences on Amides**

Many papers can be found where the levels of glutamate, aspartate, glutamine, and asparagine have been determined on a particular species and a given set of environmental conditions. However, few investigations have dealt with the direct influence of environment on amide levels
as is exemplified by the brevity of this literature review.

Crane and Steward (4, 5) studied the influence of daylength on the amide levels of mint plant, Mentha piperita. They found the storage of soluble nitrogen in leaves and stems of plants grown under short days to far exceed the storage level of plants under long days as expressed on a dry weight basis. This was attributed to possible balancing effects of light and dark periods upon protein synthesis and breakdown, long days favoring synthesis more than breakdown and vice versa. Crane and Steward also presented evidence that glutamine and asparagine act reciprocally. Glutamine represented a larger percentage of the soluble nitrogen in short day plants by day and asparagine by night. They do not suggest that glutamine and asparagine are directly convertible, but that during the long night in which protein breakdown predominates over synthesis, asparagine increases; while during the day when synthesis predominates over breakdown, glutamine increases. Thus, amide variations from short day influences are due to long nights or darkness, while long day influences are due to the predominance of light effects. In addition, long days resulted in the accumulation of α-ketoglutarate while short days depleted the α-ketoglutarate pool.

The influence of night temperature on amide levels was investigated by Rabson and Steward (20, 21). They found low night temperatures of 50°F. to result in accumulations of soluble nitrogen, particularly amides; while high night temperatures of 80°F. resulted in a great reduction in glutamine and some increase in asparagine. This they attributed to the low night temperatures restricting protein breakdown and turnover, thus the amide remains predominately glutamine which is formed in the light; while high night temperatures promote protein breakdown.
and turnover, thus the asparagine which is formed in the dark accumulates from the products of breakdown. Low night temperatures favored α-ketoglutarate accumulation, while high night temperatures depleted the α-ketoglutarate levels. These results indicated that night temperature interacts or overrides the effect of daylength on the amide levels in mint. A low night temperature was largely the determining factor in the asparagine levels of plants grown under short days. Mint plant amide levels responded to as little as one night's exposure at low temperatures. These results prompted Steward and Pollard (24) to regard the point of contact between keto acid and amino acid metabolism as a focal point for the effects of environment on nitrogen metabolism.

**Related Studies**

Greenhill (11) reported that the protein fraction of the total nitrogen in grass tissue was more or less constant under all conditions of grazing by livestock. The proportion of amide nitrogen and ammonia nitrogen showed no relationship to the total nitrogen content of the herbage or to one another. However, the higher proportions of non-protein nitrogen and nitrate nitrogen were associated with higher total nitrogen contents. In general, the non-protein nitrogen was less than 20% of the total nitrogen; the total nitrogen level ranged from 2.8 to 5.2% of the dry matter; and the non-protein-nitrogen values, expressed as percentages of the total nitrogen, ranged from 12 to 23%, the amide nitrogen from 2.7 to 81%, the ammonia nitrogen from 1.0 to 2.0% and the nitrate nitrogen from 0.1 to 4.0%.

Burton, et al. (3) found high nitrogen fertilization to increase the protein fraction of bermudagrass leaves. Wilson (33) reported the
soluble nitrogen content of grasses to usually be low but to vary from 15 to 30% of the total nitrogen. He found high values in the spring and fall with lower values occurring between June and August. Sullivan et al. (26) found the protein level of forage grasses to remain at a uniform level during spring and summer with an increase in the fall period. Ferguson and Terry (9) observed seasonal variations in amide levels of herbage but did not amplify the statement. Recently, Fauconneau (8) reported glutamine to be the most important amide in grasses. No report of an attempt to study the seasonal fluctuation in the amide content of grasses was found.

**Method of Approach**

Basic studies concerning plant responses to certain environmental factors can be performed under controlled conditions where only one or a few of the influencing factors are allowed to vary while all other factors are held constant. Until recently most researchers have employed this method. The weakness in this method is that under natural field conditions the environmental factors are not constant but are part of a dynamic system. The plant response to this system will vary depending upon the extent to which each factor contributes to the whole.

Ideally, the best approach to the problem is to measure the plant responses under natural field conditions and at the same time measure the environmental factors influencing plant response in one complete study. Then the relationships between plant responses and environmental factors can be determined by statistical analysis. Only with the recent development and availability of constant recording devices and high speed electronic computing machines has this type of approach become feasible.
This biostatistical approach has been used in a general way by several investigators (6, 12, 13) utilizing gross weather data. Norman (25) strongly endorsed the biochemical approach to ascertain the relationships between plant and ecological factors. In this investigation the multiple factor approach is to be utilized with the plant response being a biochemical measurement.
SECTION I
SECTION I

THE INFLUENCE OF TEMPERATURE ON THE AMIDE CONTENT OF BENTGRASS AND BERMUDAGRASS UNDER CONTROLLED CLIMATE CONDITIONS

The purpose of this study was to ascertain the influence of temperature on the total nitrogen, non-protein nitrogen, free ammonia, glutamine, asparagine, and total amide levels of bentgrass and bermudagrass leaf tissue under controlled climate conditions. Exploratory investigations employing chromatography techniques, indicated large variations in amide levels between the two species and that variations within species also occurred due to temperature changes.

Materials and Methods

Plant Culture Methods

This study was conducted in the controlled climate chambers at Purdue University. The two grass species included in this investigation were the Old Orchard (C-52) variety of creeping bentgrass, Agrostis palustris, and an unnamed selection of bermudagrass, Cynodon dactylon. These two grasses were chosen because the former is a common species of the cool, humid North and the latter is a common species of the warm, humid South; thus permitting comparisons to be made between the temperature responses of cool and warm season grasses.

Sod strips, one-half inch in thickness, were cut from four year old sod and brought into the greenhouse for a two day adjustment period on
September 20, 1960. Then, the sod was trimmed and placed in wood flats 18 by 9 by 9 inches which had been filled with a mixture of five parts loam soil, one part sand, and one part ground peat. Care was taken so that the surface of the sod was level with the top of the flat to facilitate sampling.

Two flats of each species were placed in each of four controlled climate chambers where constant temperatures were maintained at 50, 60, 70, and 80°F. This gave a total of 2.3 square feet of sod for each grass under each of the four temperature treatments. A sixteen hour day-length was maintained during the study. Light intensity at the surface of the sod was 800 foot candles using a fluorescent light source supplemented with unfrosted incandescent bulbs. The chambers were maintained at a relative humidity of 55 percent and were provided with a constant exchange of air.

The treatments were watered daily with deionized water to maintain optimum moisture conditions. Winthrops "A" nutrient solution was applied at seven day intervals. Both the bentgrass and bermudagrass were clipped every four days at a height of one inch. No disease or insect activity was observed on any of the treatments during the interim of the experiment.

The treatments were maintained under the conditions described above for seventy days to permit the grass to reach an equilibrium with the environment. Three samplings of leaf material were obtained on December 1, 1960, December 24, 1960, and January 16, 1961. The leaf samples were harvested with hand clippers which had a special pan attached to one blade to catch the leaf clippings. All vegetative material was harvested above the one inch height, placed in plastic bags, sealed, labeled, and
immediately stored in a -20º F. chamber to minimize bacterial and enzymatic action.

Analytical Methods

Tissue samples obtained from the frozen material were prepared by lyophilizing two 35 gram samples from each treatment. Following lyophilization the samples were immediately ground through a forty mesh sieve and stored in an airtight container.

Six nitrogen fractions of the leaf tissue were quantitatively measured and evaluated to ascertain how these fractions were influenced by the four temperature conditions. The six nitrogen fractions determined were: total nitrogen, non-protein nitrogen, free ammonia, glutamine, asparagine and total amide content of bentgrass and bermudagrass leaf tissue. The analytical procedures employed in these determinations are as follows:

A. Total Nitrogen Analysis

The total nitrogen content of the plant tissue was determined by means of a modified Kjeldahl procedure.

Reagents:

(a) Concentrated sulfuric acid.
(b) Catalyst: a finely ground mixture of 10 parts Na₂SO₄ and 1 part CuSO₄·5H₂O.
(c) 40% sodium hydroxide.
(d) Mossy zinc.
(e) 4% boric acid.
(f) Standardized 0.02 N hydrochloric acid.
(g) Indicator: 1 gm brom cresol green and 0.4 g. methyl red dissolved in 200 ml of ethyl alcohol.
Determination:

One gram of plant tissue and five grams of catalyst were placed in a 800 ml digestion flask to which was added 30 ml of concentrated sulfuric acid. The digestion was carried out for two hours, the samples cooled, and 110 ml of water added to the digestion mixture. Distillation was performed by adding 100 ml of 40% sodium hydroxide and a small quantity of mossy zinc to the digestion flask. The ammonia distillate was collected in flasks containing 25 ml of 4% boric acid solution until 175 ml of solution was collected. The ammonium-borate complex was titrated with 0.02N standardized hydrochloric acid solution using a methyl red-brom cresol green indicator. A blank determination was made with each run of twenty samples. After the titration values were corrected for the blank, calculations of the percent nitrogen were made.

B. Amide Analysis

Many methods are available for the quantitative determination of the amides but few of these methods are practical where large numbers of samples are to be analyzed. A modification of the method described by Pucher et al. (19) and improved by both Vickery et al. (27) and Steward et al. (25) was chosen for use in this study. This procedure is based on the observation that the amide group of glutamine is completely hydrolyzed in two hours at 100° C. within the range of pH 6 to 7, while the amide group of asparagine is completely hydrolyzed only under dilute acid conditions. Vickery et al. (27) showed that the interference from asparagine is negligible in a glutamine determination when the hydrolysis is carried out at pH 6.5 for two hours. Thus, under the controlled conditions of partial hydrolysis, glutamine and asparagine can be separated.
Following digestion, distillation of a sample was performed in a simple vacuum apparatus at 60° C. for thirty minutes. The ammonia resulting from the distillation was absorbed in standardized 0.02 N hydrochloric acid and determined by back-titrating with 0.01 N sodium hydroxide.

Reagents:
(a) Phosphate-borate buffer, pH 6.5: a mixture of 750 ml of 0.1 M potassium dihydrogen phosphate (13.6 grams per liter) and 250 ml of 0.05 M borax (19.1 grams per liter).
(b) Borax-sodium hydroxide mixture: dissolve 5 grams of borax in 100 ml of 0.5 N sodium hydroxide (reagent grade, ammonia free).
(c) Standardized 0.02 N hydrochloric acid.
(d) Standardized 0.01 N sodium hydroxide: ammonia free.
(e) Sulfuric acid, 6 N: prepared from special grade low in nitrogen.
(f) Sodium hydroxide, 1 N: prepared from high grade ammonia free stock.
(g) Indicator: mix 100 ml of 0.2 percent methyl red in fifty percent alcohol plus 15 ml of 0.1 percent methylene blue.

Apparatus:

The distillation apparatus is shown in figure 1. It was constructed by sealing standard tapered, ground glass joints to a 500 ml Pyrex ring-neck flask, to serve as a still, and to a 28 x 200 mm Pyrex test tube to serve as a combined receiver and condenser. The condensing device or receiver was supported in a pint milk bottle furnished with a sponge rubber cushion at the bottom and filled with ice water. The distilling flask was loosely clamped to a stand, so arranged, to permit the entire flask to immersed in a water bath during distillation. The air inlet
Figure 1. Amide Distillation Apparatus.

TO WASH BOTTLE AND AIR INLET

TO VACUUM

BALL & SOCKET JOINT

KJELDAHL CONNECTING BULB

WATER BATH

28 x 200 mm PYREX TEST TUBE IMMERSED IN COLD WATER TO ACT AS CONDENSER
tube admits air that has bubbled through a small wash bottle containing diluted hydrochloric acid to remove any ammonia from the air flow.

Extraction:

An aqueous extract was prepared by heating five grams of dry, ground plant tissue at 80°C with 80 ml of water for ten minutes in a 100 ml volumetric flask with constant stirring. The flask was then rapidly cooled in ice water and made to 100 ml volume. The extract was decanted from the solids, centrifuged at 4,500 rpm for fifteen minutes, and filtered under vacuum with a buckner funnel. This aqueous extract was then used in making non-protein-N, ammonia-N, total amide-N, asparagine-N, and glutamine-N determinations and will hereafter be referred to as "solution A".

Free Ammonia-N Determination:

A 20 ml aliquot of solution A and 10 ml of phosphate-borate buffer were pipetted into the distillation flask. Three ml of 0.02 N hydrochloric acid was delivered to the receiver, the ground glass joints lubricated with a little vaseline, and the apparatus assembled and immersed in the water bath with 3 ml of sodium hydroxide-borate solution being added just before inserting the air inlet tube. The apparatus was evacuated and the air inlet adjusted to permit a flow of 2 to 3 bubbles per second. The distillation was allowed to proceed for thirty minutes with the water-bath being maintained at 60°C. At the end of the distillation period the flask was raised, air admitted through the air inlet, the connection on the vapor tube opened, and the vacuum disconnected. The vapor tube was rinsed inside and out into the receiver with a few ml of water, the distillate transferred by rinsing to a beaker and back titrated with 0.01 N sodium hydroxide using a two millileter buret having
0.01 ml subdivisions. The end point was obtained by titrating to a pH of 5.0 as measured on a Leeds and Northrup pH meter and verified with a methyl red-methylene blue indicator.

**Glutamine-N Determination:**

A 5 ml aliquot of solution A was mixed with 10 ml of phosphate-borate buffer solution in a 25 x 200 cm test-tube which was closed by a rubber stopper fitted with 20 cm of 1 mm bore, heavy walled, glass tubing. The lower surface of the stopper and the orifice of the tube were previously moistened with a few drops of water. The test-tube was placed in a constant level, boiling water-bath for exactly two hours, then removed, and placed in cold water until analyzed. A few drops of water are washed down the orifice of the stopper tube to wash any volatilized ammonia back into the test-tube. The contents of the test-tube were then transferred to the distillation apparatus with 20 ml of water, 3 ml of borax-sodium hydroxide solution added, and the apparatus assembled. The procedures followed from this step on were the same as previously described for the free ammonia determination.

A 5 ml aliquot of solution A was mixed with 1 ml of 6 N sulfuric acid in a 25 x 200 mm test-tube closed by a stopper fitted with 20 cm of 1 mm bore glass tubing described previously. The test-tube was heated at 100° C. in a constant level water-bath for exactly three hours, then removed, and placed in cold water until analyzed. The contents of the test-tube were then transferred to the distillation flask as described previously and 10 ml of 1 N sodium hydroxide were added to neutralize the hydrolizing acid. Then 15 ml of borax-sodium hydroxide mixture were added and the apparatus assembled. The procedures followed from this step on were the same as described for the two previous determinations.
Two distillation blanks were run at the beginning of each analysis period to clear the system of ammonia plus a blank distillation at the end of the day which was used as a correction factor for the apparatus and reagents. This procedure gives a determination of the glutamine-N and total amide-N when corrected for the apparatus blank and for the free ammonia-N. The asparagine-N is obtained by subtracting the glutamine-N from the total amide-N. A standard containing a known amount of glutamine and asparagine was used to check the above procedure. Results showed an average recovery for four replications of 95.6% for glutamine and 90.7% for asparagine with a maximum variation of 1.8% between replications. The recovery percentages were similar to the values reported by Vickery and Purcher.

C. Non-Protein Nitrogen Analysis

The non-protein nitrogen analysis of the tissue was made from an aliquot of solution A obtained from the hot water extraction described previously in the amide analysis methods. The analytical procedure employed was a modified micro-kjeldahl method recommended by the AOAC (1). Reagents:

(a) Sodium hydroxide-sodium thiosulfate solution: 50 grams NaOH and 5 grams Na$_2$S$_2$O$_3$·5H$_2$O dissolved in 100 ml H$_2$O.

(b) Boric acid solution: 4 grams H$_3$BO$_3$ dissolved in 100 ml H$_2$O.

(c) Mercuric sulfate solution: dilute 12 ml conc. H$_2$SO$_4$ to 100 ml with H$_2$O and dissolve 10 g. red mercuric oxide.

(d) Methyl red-methylene blue indicator: 2 parts of 0.02% alcoholic methyl red solution mixed with 1 part of 0.2% alcoholic methylene blue solution.
(e) Standardized 0.02 N hydrochloric acid.

Apparatus:
(a) Digestion rack: electric heaters with a rheostat control.
(b) Distillation apparatus: a one piece Kemmerer-Hallet type.
(c) Digestion flasks: 100 ml flasks.

Determination:
A 3 ml aliquot of the plant extract was placed in a 100 ml kjeldahl flask with 1.5 g. of potassium sulfate, 1.5 ml of mercuric sulfate solution and two glass beads. Then 3 ml of conc. sulfuric acid was added and the digestion carried out on the digestion rack until 30 minutes after the solution cleared. Next, the solution was cooled ten minutes and 15 ml of water added. Then 15 ml of NaOH-Na₂S₂O₃ reagent was added and the flask transferred to the steam distillation apparatus. The distillate was collected in a 50 ml erlenmeyer containing 10 ml of boric acid solution and four drops of methyl red-methylene blue indicator. Following distillation the ammonia captured by the boric acid is directly titrated with 0.02 N hydrochloric acid using a 5.0 ml buret having 0.01 ml subdivisions. A standard containing a known amount of nitrogen was used to check the distillation and digestion procedures. On four replications 100% of the nitrogen was recovered from the standard plus or minus one percent.
Results and Discussion

The effect of 50, 60, 70, and 80°F. constant temperatures on the total nitrogen content of bentgrass and bermudagrass leaf tissue as expressed on a dry weight basis is shown in figure 2. When temperature was increased from 50 to 60°F., the total nitrogen level rose sharply in both species; from 3.3 to 4.3% in bentgrass and from 2.6 to 3.6% in bermudagrass. In bentgrass the total nitrogen level continued to increase as temperature was increased, with a peak at 70°F. then a slight drop at 80°F. This peak at 70°F. coincides with the temperature at which optimum growth of bent occurs. Actually, there is little difference between the total nitrogen content under the 60, 70, and 80°F. treatments. After the sharp rise in total nitrogen level from 50 to 60°F. in bermudagrass, there was a general leveling off under the 60, 70, and 80°F. treatments with a slight drop at the 70°F. temperature. Note that the total nitrogen content of bermudagrass leaf tissue averaged approximately one percent less than bentgrass leaf tissue at all four temperatures. Figure 3 shows that the non-protein nitrogen content of bentgrass, as expressed on a dry weight basis, increased as temperature was increased from 50 to 80°F. However, the rate of increase was not nearly as great in going from 70 to 80°F. as from 50 to 70°F. The non-protein nitrogen level increased from 0.42 to 0.68% in increasing temperature from 50 to 70°F. In bentgrass leaf tissue the non-protein fraction remains a relatively constant percentage of the total nitrogen
Figure 2. The Influence of Four Temperatures on the Total Nitrogen Fractions of Bentgrass and Bermudagrass.

Figure 3. The Influence of Four Temperatures on the Non-Protein Nitrogen Fractions of Bentgrass and Bermudagrass.
content as temperature is increased from 50 to 70°F and then increases as the temperature is increased to 80°F.

The non-protein nitrogen level in bermudagrass responded the opposite to bentgrass as temperature was increased (Figure 3). The non-protein nitrogen content decreased slightly as temperature was increased from 50 to 60°F, and then showed a markedly decrease from 0.75 to 0.55% as temperature was increased from 60 to 80°F. Thus, as temperature increases from 50 to 80°F, the non-protein nitrogen fraction becomes a smaller fraction of the total nitrogen content in the plant.

Appendix table 1 shows the free ammonia level responses to the four temperature conditions to be similar for both bentgrass and bermudagrass, with the levels in bermuda being slightly less. In both species the free ammonia level increases with temperature until a peak is reached at 70°F, followed by a decided decreased at 80°F.

Figure 4 shows the influence of four constant temperature treatments on the amide levels of bentgrass leaf tissue expressed on a dry weight basis. The glutamine content increased as temperature was increased from 50 to 60°F, dropped slightly at 70°F and increased again at 80°F. In general, there was an increase in the glutamine level as temperature increased although the increase from 6.07 to 0.11% was not large.

The asparagine level appears much more responsive to temperature than glutamine under these controlled climate conditions. The asparagine content decreased markedly as temperature increased from 50 to 80°F, although the rate of decrease was less in the 60 to 70°F range. The asparagine fraction decreased from 0.13% at 50°F to 0.04% at 80°F, a two-thirds reduction. Note that the range of asparagine levels was
Figure 4. The Influence of Four Temperatures on the Amide Nitrogen Fractions of Bentgrass.

Figure 5. The Influence of Four Temperatures on the Amide Nitrogen Fractions of Bermudagrass.
much greater than for glutamine. When expressed on a non-protein nitrogen basis the asparagine nitrogen fraction decreased from 6.54 to 1.34% as temperature increased while glutamine decreased slightly from 3.24 to 3.07% (Appendix table 3). The total amide content for bentgrass is obtained by summing the glutamine and asparagine values. The total amide content expressed on a dry weight basis increases only slightly from 50 to 60°F. and then decreases as temperature increases from 60 to 80°F. This drop in the total amide is primarily due to the asparagine fraction. Expressed on a non-protein nitrogen basis, the total amide nitrogen content decreased from 9.78 to 4.41% as temperature increased from 50 to 80°F. (Appendix table 3).

The influence of four constant temperature treatments on the amide content of bermudagrass, expressed on a dry weight basis, is shown in figure 5. The glutamine fraction shows practically no response to increases in temperature within the 50 to 80°F. range. However, if glutamine nitrogen is expressed on a non-protein nitrogen basis there is an increase with temperature from 1.80 to 2.96% (Appendix table 3). In general, the glutamine content is lower in bermudagrass than in bentgrass.

Asparagine appears to be the amide fraction in bermudagrass which is most responsive to temperature. When temperature was increased from 50 to 70°F. the asparagine content was reduced by more than one-half or from 0.37 to 0.14% expressed on a dry weight basis. Then as temperature was increased to 80°F. the asparagine content increased to 0.22%. Note that the asparagine fractions are approximately three times larger in bermudagrass than in bentgrass. The variation in total amide content of bermudagrass within the four temperature treatments is primarily an
an expression of a change in the asparagine fraction.

These results show that under the controlled climate conditions of this experiment, the total nitrogen level increases sharply as temperature is increased from 50 to 60° F. in both bentgrass and bermudagrass. Then, when temperature is increased to 80° F. there is a general leveling off of the total nitrogen content in both species. The non-protein nitrogen fraction increases as temperature is increased in bentgrass tissue while exactly the opposite occurs in bermudagrass leaf tissue.

The glutamine fraction remained relatively constant in both species when subjected to the four temperature treatments, with a slight increase occurring in the case of bentgrass. Asparagine appeared to be the amide that was most responsive to temperature. In bentgrass leaf tissue the asparagine content decreased as temperature was increased. In bermudagrass leaf tissue asparagine decreased as temperature increased to 70° F. then increased at the 80° F. temperature treatment.