Amino Acid and Protein Metabolism in Bermuda Grass During Water Stress[1, 2]

N. M. Barnett[3] and A. W. Naylor

Department of Botany, Duke University, Durham, North Carolina

Received May 31, 1966.

Summary. The ability of Arizona Common and Coastal Bermuda grass [*Cynodon dactylon* (L.) Pers.] to synthesize amino acids and proteins during water stress was investigated. Amino acids were continually synthesized during the water stress treatments, but protein synthesis was inhibited and protein levels decreased.

Water stress induced a 10- to 100-fold accumulation of free proline in shoots and a 2- to 6-fold accumulation of free asparagine, both of which are characteristic responses of water-stressed plants. Valine levels increased, and glutamic acid and alanine levels decreased.

^{14}C labeling experiments showed that free proline turns over more slowly than any other free amino acid during water stress. This proline is readily synthesized and accumulated from glutamic acid. It is suggested that during water stress free proline functions as a storage compound.

No significant differences were found in the amino acid and protein metabolism of the 2 varieties of Bermuda grass.

In the study of biochemical changes in plants under water stress conditions, increasing attention has been paid to changes in nitrogen compounds. Proteolysis and interruption of protein synthesis are generally found to be results of water stress (6, 11, 20), although both increases and decreases of protein have been found to follow each other (3). Radioisotopes have been used to show the effects of water stress on RNA synthesis and degradation (4). The study presented here reports the effects of water stress on levels and turnover of both free and protein-bound amino acids as shown by ^{14}C labeling.

Water stress induces a characteristic change in the levels of free amino acids, especially a great increase in free proline (3, 6, 12) and amides (3, 9). The accumulation of amides is thought to be the result of incorporation of free ammonia released by deamination of amino acids, which were in turn released by proteolysis induced by water stress (9). Few attempts have been made to explain the accumulation of free proline. The origin and function of this proline is considered in this paper.

Two varieties of Bermuda grass have been used in the present study. These varieties differ somewhat in their general response to water stress, and it was desired to see if under water stress conditions differences also exist in their nitrogen metabolism. An extensive study by Ratnam (13) showed these differences in drought response of Arizona Common and Coastal varieties: Water content and cuticular transpiration are higher in Common Bermuda; Common Bermuda leaves develop a lower (more negative) water potential in a given time without water than do Coastal Bermuda leaves. Leaf damage is generally greater and appears sooner in Common than in Coastal leaves. In general, Ratnam's experimental results tend to support the conclusion that leaves of Coastal are slightly superior to Common in drought avoidance.

Materials and Methods

Plant Material. Clonal material of Arizona Common and Coastal Bermuda grass [*Cynodon dactylon* (L.) Pers.] was propagated in a 2:1 mixture of sandy loam and sand in 7 inch clay pots. Plants were grown in the greenhouse and were fertilized periodically with commercial fertilizer. The grasses were transplanted into new soil-sand mixture when growth ceased to be vigorous. Tops were cut off periodically. Experiments were conducted in fall or winter, when growth was slow and there was no flowering.

Water Potential Measurement. Water potential was measured with the thermocouple psychrometer device described by Boyer (2).

Labeling of Plants with $^{14}CO_2$. Labeling ex-

[1] Research supported by NSF GB-1879 and the Herman Frasch Foundation. It represents part of a dissertation submitted to the Graduate School of Arts and Sciences, Duke University, in partial fulfillment of the requirements for the Ph.D. degree.

[2] Present address: Department of Botany and Plant Pathology, Purdue University, Lafayette, Indiana.

periments were conducted in an isotope hood. Two adjacent rows of plants in clay pots were illuminated from opposite sides with 150-w reflector spot lamps so that shading was minimized. Light was filtered through 9 cm of 0.5 % copper sulfate solution in glass tanks cooled by tap water flowing through copper coils. Light intensity was 1000 ft-c at the leaf surface. Plants were left in place throughout the labeling and sampling periods. Daylength was 8 hours.

For incubation with $^{14}CO_2$, a 15.8 liter bell jar was placed over a plant. Bell jar and plant rested on a glass plate, to which the bell jar was sealed with silicone grease. A 10 ml beaker containing 200 μc of $NaH^{14}CO_3$ (specific activity 25 μc μmole^{-1}) was suspended on a wire inside the top of the bell jar. The bell jar top was sealed with polyvinyl chloride film. To generate $^{14}CO_2$, 0.1 ml of 20 % lactic acid was injected through the film into the beaker; the film was immediately sealed with Scotch tape. After one-half hour, 0.5 ml of concentrated NaOH was injected into the beaker. The bell jar was removed five minutes later. The maximum concentration of CO_2 generated was 0.0012 %, which is small compared to the normal concentration of CO_2 in air. No artifacts due to high CO_2 concentration were likely to have been induced.

Extraction of Free Amino Acids. Plant tissue was killed by boiling it for 3 minutes in 80 % (v/v) ethanol. Tissue and ethanol were stored at −20°. The ethanol was subsequently decanted and saved, and the tissue was ground with mortar and pestle with acid-washed sand and fresh 80 % ethanol. The homogenized sample was refluxed 15 minutes on a steam bath. The sample was centrifuged 15 minutes at 27,000 g. The supernatant fraction was added to the original ethanol in which the tissue was killed. The pellet was refluxed again in 40 % ethanol. This procedure of refluxing and centrifuging was done 4 times in all, once with 80 % ethanol, twice with 40 % ethanol, and once with water. All supernatant fractions were pooled. Four extractions yielded 94 % of the free amino nitrogen obtained in 6 extractions (80 % ethanol, twice in 40 % ethanol, 3 times in water). Pooled extracts were further purified by evaporation almost to dryness at 45° under reduced pressure, taking up the residue in 2 ml of 0.1 N HCl, and centrifuging the suspension 10 minutes at 1° at 27,000 g. This procedure was repeated once or twice; the pellet was discarded each time. Extracts were then purified by the cation exchange method of Wang (18). Recovery of free amino nitrogen in this method was 91 %. This solution was reduced to dryness and the residue was taken up in a small amount of 0.1 N HCl.

Analysis of Amino Acids. Amino acids were measured on an automatic amino acid analyzer using the 1-column technique and buffer sequence of Piez and Morris (10). The analyzer was calibrated with standard mixtures of amino acids. At the column temperature of 60°, glutamine is cyclized to pyrrolidone carboxylic acid, which does not react with ninhydrin. Consequently sample glutamine was not measured. Radioactivity of the analyzer stream was monitored continuously with a Packard 317 scintillation detector and 320E pulse height analyzer. One channel of the amino acid analyzer recorder was used to record radioactivity.

Extraction of Soluble Protein. One grass shoot (up to 15 cm high and 0.5 g dry weight) was cut into 1.5 cm segments and ground with 2 ml water and acid-washed sand with mortar and pestle at 1°. The homogenate was centrifuged at 27,000 g at 1° for 10 minutes. The supernatant fraction was saved. The pellet was ground again with water and sand at 1° and recentrifuged. The water soluble protein in the combined supernatant fraction was precipitated by adding an equal volume of 20 % trichloroacetic acid (TCA) and allowing to settle at least 10 minutes. The protein was centrifuged at 27,000 g for 10 minutes, the pellet was resuspended in 10 % TCA and recentrifuged; then the supernatant was discarded. The pellet was decolorized by twice incubating at one-half hour at 37° with 2 ml of a 2:2:1 (v/v/v) mixture of ethanol, ether, and chloroform, and centrifuging each time. The protein precipitate was dissolved overnight in 1 ml of 1 N NaOH.

Protein Measurement. Protein was measured both by the method of Lowry et al. (8) and by summing the amino acids in protein hydrolysates as measured on the analyzer.

Hydrolysis of Protein. Protein solutions were made to 6 N HCl in 2-piece hydrolysis tubes. After evacuation of air, the solutions were hydrolyzed at 110° for 20 hours. The small amount of humic acid formed was removed by filtration. Hydrolysates were dried on a flash evaporator at 45°. Water was added to the residue and the sample was redried repeatedly to remove excess HCl. The hydrolysate was dissolved in 1 ml 3 M citric acid for analysis on the amino acid analyzer.

Results

In addition to the water soluble protein amino acids, several other amino acids were detected by column chromatographic analysis of ethanolic extracts of Bermuda grass tops. On the basis of elution time and comparative color yield (blue/yellow absorption) the non-protein amino acids α-aminobutyric acid, β-alanine, and pipecolic acid were tentatively identified. The last 2 were present in minute amounts. Extracts of large samples of whole tops revealed minute quantities of still more unidentified ninhydrin-positive compounds. This is illustrated in figure 1 where many small unidentified peaks are shown. Two such compounds were present, however, in amounts large enough to measure. One, labeled N throughout

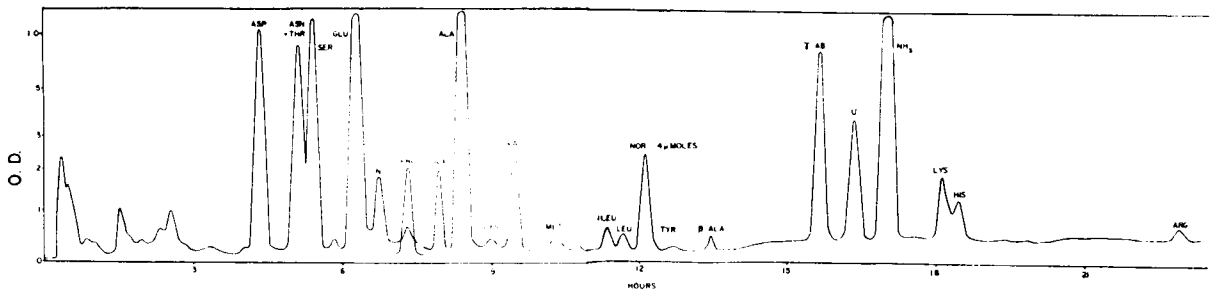

FIG. 1. Chromatogram trace of free amino acids of Bermuda grass produced by the automatic amino acid analyzer. Norleucine (4 μmoles) was added to the sample of fresh tops as an internal standard.

this study, was eluted between glutamic acid and proline. It emerged from the column about 15 minutes after standard citrulline, and disappeared upon hydrolysis with acid.

The other unknown, labeled U, was eluted between α-amino-butyric acid and ammonia. It emerged from the column at the same time as standard ethanolamine and δ-*allo*-hydroxylysine. It was stable to acid hydrolysis. In an attempt to identify this unknown, 2.4 ml fractions were collected during an amino acid analysis of an extract of 136 g fresh Coastal Bermuda grass tops. The solutions in the 3 tubes containing the unknown U were pooled and deionized on a 0.9 × 10 cm column of Dowex 50 × 8, NH_4 form (7). The ammonia solution of the unknown was dried at 40° under reduced pressure. The residue was dissolved in 1 ml 0.1 N HCl. Two 1-dimensional chromatograms were run, using 50 μl of unknown, plus standards, on Whatman No. 1 chromatography paper. Solvent systems were 71% phenol, and *n*-butanol-propionic acid-water [45.3:22.5:32.2, v/v/v (1)]. The unknown chromatographed the same distance as ethanolamine in both systems (phenol-water: unknown R_F .70, ethanolamine R_F .68; *n*-butanol-propionic acid-water: unknown R_F .55, ethanolamine R_F .52). The unknown U was therefore tentatively identified as ethanolamine.

An experiment was conducted to determine the effects of water stress on the composition and turnover of both free and protein-bound amino acids. Duplicate sets of both Arizona Common

Table I. *Effect of Water Stress on Fresh Weight, Dry Weight, Total Free Amino Acids, and Water-soluble Protein in Bermuda Grass Shoots*

Treatment	Water potential range, bars	Fr wt, mg*	Dry wt, mg*	Total free amino acids, μmoles per shoot*	Water-soluble protein: μmoles hydrolyzed amino acids per shoot*	mg/shoot	Total amino N, μmoles per shoot
Common							
Control	−4.1 to −7.9	228.6 ± 102.9	46.3 ± 23.2	8.72 ± 3.49	18.9** ± 3.5	3.06	27.6
Moderate stress	−10 to < −37	174.4 ± 69.2	67.6 ± 25.9	13.9 ± 5.71	12.6 ± 4.7	2.11	26.5
Severe stress	< −37	84.6 ± 36.1	61.3 ± 28.6	16.7 ± 4.3	9.31 ± 2.41	0.95	25.0
Coastal							
Control	−4.1 to −4.7	341.3 ± 116.3	69.4 ± 24.2	9.26 ± 2.68	23.7 ± 11.4	3.24	33.0
Moderate stress	−18 to < −37	179.6 ± 48.6	52.4 ± 13.9	20.4 ± 7.8	13.5 ± 5.3	2.07	33.9
Severe stress	−33 to < −37	128.9 ± 61.7	82.6 ± 29.7	28.0 ± 9.8	9.31 ± 4.13	1.07	37.3

* Average and standard deviation of 5 determinations made in a 77-hour period.
** Four determinations only.

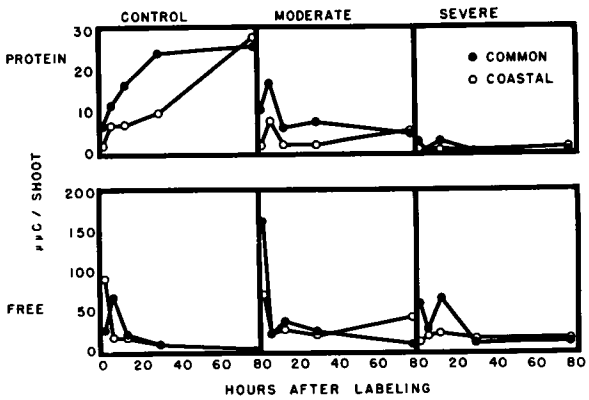

FIG. 2. Time course of change in radioactivity of the free amino acid fraction and soluble protein fraction in Bermuda grass with increasing water stress.

and Coastal Bermuda grass plants in pots were placed under lights in the isotope hood at the beginning of the water stress treatment. One-half of the plants were to be labeled with $^{14}CO_2$. The other half were given the same water stress treatments as the labeled plants but were used for fresh and dry weight measurements and water potential measurements. All stolons and branched shoots were removed. Each plant consisted of 15 to 20 upright shoots 10 to 15 cm high. Water was withheld from treated plants, and controls were watered daily. Water potential was measured daily using as a sample 1 shoot from an unlabeled duplicate plant. It was desired to label plants at each of 2 stress levels which were arbitrarily set at approximately −15 bars (moderate stress) and −30 bars (severe stress) respectively. These stress levels were

Table II. *Changes in Amounts of Free Amino Acids in Bermuda Grass Shoots with Increasing Water Stress*

Amino acid	Control	Moderate stress	Severe stress
	μ moles/gram dry weight**		
Common			
Aspartic acid	11.8 ± 8.9	4.5 ± 1.8	8.4 ± 3.5
Asparagine; threonine	24.6 ± 4.1	29.8 ± 13.7	64.2 ± 17.1
Serine	9.9 ± 2.3	8.3 ± 2.7	11.0 ± 4.5
Glutamic acid	28.7 ± 9.2	10.5 ± 4.8	4.7 ± 1.9
N	...	...	0.8 ± 0.3
Proline	< 2.7	30.5 ± 23.9	69.3 ± 33.0
Glycine	1.8 ± 1.3	1.7 ± 1.1	1.2 ± 0.7
Alanine	31.9 ± 12.3	15.2 ± 3.8	11.6 ± 4.2
1/2-Cystine	...	...	0.6** ± 0.1
Valine	2.1 ± 0.7	3.5 ± 1.6	7.0 ± 2.1
Isoleucine	...	0.9** ± 0.4	1.2 ± 0.4
γ-Aminobutyric acid	3.2 ± 2.1	7.0 ± 4.1	4.3 ± 1.4
U	...	0.8 ± 0.2	1.5 ± 0.9
Ammonia	94.3 ± 36.6	78.0 ± 54.0	55.4 ± 26.4
Lysine	0.5 ± 0.4	0.7 ± 0.1	1.0 ± 0.4
Histidine	...	0.5 ± 0.1	1.4 ± 0.3
Arginine	...	0.8 ± 0.3	2.5 ± 0.1
Totals	211.5	192.9	246.5
Coastal			
Aspartic acid	7.0 ± 5.9	9.0 ± 4.2	9.7 ± 1.7
Asparagine; threonine	9.4 ± 3.3	60.1 ± 21.5	62.5 ± 29.9
Serine	7.9 ± 1.3	18.5 ± 7.9	13.4 ± 6.3
Glutamic acid	22.3 ± 7.7	17.7 ± 6.5	5.4 ± 2.3
N	0.9 ± 0.1	1.3 ± 0.3	0.7 ± 0.3
Proline	< 1.1	138.0 64.0	126.0 34.0
Glycine	0.8 ± 0.2	2.7 ± 1.3	1.8 ± 0.4
Alanine	21.4 ± 6.9	17.3 ± 4.9	13.1 ± 6.3
1/2-Cystine	...	0.8**	0.5** ± 0.1
Valine	1.3 ± 0.2	12.1 ± 3.7	8.4 ± 3.2
Isoleucine	...	2.5 ± 1.9	1.6 ± 0.7
γ-Aminobutyric acid	4.5 ± 3.7	8.4 ± 3.2	4.7 ± 0.9
U	...	1.8 ± 0.4	1.4 ± 0.5
Ammonia	50.8 ± 17.3	81.5 ± 25.6	48.5 ± 6.2
Lysine	0.4	2.2 ± 0.8	1.3 ± 0.4
Histidine	...	1.7 ± 0.1	1.3 ± 0.2
Arginine	...	1.7 ± 0.5	1.8 ± 0.7
Totals	128.0	377.4	302.5

* Average and standard deviation of 4 analyses of common control shoots, 5 of all others. The shoots were each up to 15 cm long and weighed approximately 0.5 g.

** Three or 4 determinations only.

reached at 5 and 7 days without water, respectively. On these days separate plants of each variety were labeled with 200 μc $^{14}CO_2$ as described above. Separate shoots were excised from each of the labeled plants for measurement of free amino acids and of protein-bound amino acids at 1, 5, 12, 29, and 77 hours after the end of the labeling period. Fresh weight, dry weight, and water potential were measured on shoots from unlabeled duplicate plants every day during the 77-hour sampling period after labeling.

A summary of the measurements made is given in table I. During the 77-hour sampling period, water stress was increasing. The highest water potential measurement given was made at the time of labeling, and it decreased thereafter. Therefore all averages of measurements made during the sampling period apply to the entire period when water potential was changing, and not to any average water potential.

Total free amino acids doubled in Common and tripled in Coastal shoots (table I) at maximum water stress employed. At the same time, water-soluble protein decreased in stressed shoots to less than half that of controls. The sum of free and protein-bound amino acids for each variety remained almost constant among all treatments.

The time course of changes in ^{14}C labeling of the total free and water-soluble protein-bound amino acids is shown in figure 2. Initial incorporation of ^{14}C was greatest into, and the most label was retained in, the free amino acid fraction from moderately stressed shoots. Incorporation of label into the free amino acid fraction of severely stressed shoots was not as great as for moderately stressed shoots, but again more label was retained than in controls. Greater treatment differences were found in the incorporation of label into protein. Controls accumulated label into protein continuously during the sampling period. Label in protein from moderately stressed shoots reached a maximum after 5 hours and declined thereafter. At the 77th hour the amount of label in protein from moderately-stressed shoots was only 20 % that

Table III. *Effect of Water Stress on Protein Composition*

Average and standard deviation of 4 measurements for Common control, 5 for all others. Cystine and tryptophan not determined and not included in calculations. Amide N not determined.

Amino acid	Mole percent Control	Moderate stress	Severe stress
Common			
Aspartic acid	10.1 ± 1.5	9.0 ± 1.2	9.1 ± 1.1
Threonine	4.9 ± 0.5	4.0 ± 0.3	4.4 ± 0.4
Serine	4.9 ± 0.2	4.7 ± 0.3	5.1 ± 0.6
Glutamic acid	11.0 ± 0.6	11.5 ± 0.4	11.5 ± 0.8
Proline	5.5 ± 0.3	6.0 ± 0.4	5.5 ± 0.7
Glycine	9.7 ± 0.4	11.0 ± 0.7	11.0 ± 0.7
Alanine	9.9 ± 0.5	10.9 ± 0.4	10.9 ± 0.3
Valine	8.3 ± 0.5	8.5 ± 0.4	8.4 ± 0.6
Methionine	1.4 ± 0.6	1.6 ± 0.6	1.7 ± 0.3
Isoleucine	5.5 ± 0.6	6.2 ± 1.0	5.4 ± 0.2
Leucine	9.1 ± 0.6	9.6 ± 0.4	9.3 ± 0.5
Tyrosine	3.1 ± 0.2	2.9 ± 0.3	3.1 ± 0.4
Phenylalanine	4.1 ± 0.2	4.2 ± 0.2	4.1 ± 0.3
Lysine	6.3 ± 0.5	5.7 ± 0.3	6.2 ± 0.8
Histidine	1.8 ± 0.2	1.6 ± 0.3	1.7 ± 0.3
Arginine	4.5 ± 0.5	2.7 ± 0.6	3.1 ± 0.5
Coastal			
Aspartic acid	9.2 ± 0.5	9.2 ± 0.9	9.7 ± 0.5
Threonine	4.6 ± 0.3	4.1 ± 0.3	4.2 ± 0.4
Serine	5.0 ± 0.2	4.8 ± 0.3	5.4 ± 1.0
Glutamic acid	11.1 ± 0.6	11.6 ± 0.3	11.4 ± 0.9
Proline	5.9 ± 0.7	5.8 ± 0.5	5.4 ± 0.9
Glycine	9.9 ± 0.4	11.1 ± 0.5	10.7 ± 0.5
Alanine	10.2 ± 0.3	10.6 ± 0.5	10.9 ± 0.5
Valine	8.2 ± 0.2	8.5 ± 0.3	8.7 ± 0.2
Methionine	1.7 ± 0.4	1.9 ± 0.1	1.6 ± 0.5
Isoleucine	5.8 ± 0.2	5.5 ± 0.3	5.3 ± 0.3
Leucine	9.3 ± 0.2	9.6 ± 0.3	9.1 ± 0.7
Tyrosine	3.1 ± 0.3	2.9 ± 0.2	2.7 ± 0.3
Phenylalanine	4.4 ± 0.7	4.1 ± 0.2	3.9 ± 0.3
Lysine	6.0 ± 0.8	5.6 ± 0.5	6.2 ± 0.9
Histidine	1.8 ± 0.2	1.8 ± 0.3	1.7 ± 0.2
Arginine	3.9 ± 0.6	2.9 ± 0.5	3.1 ± 0.5

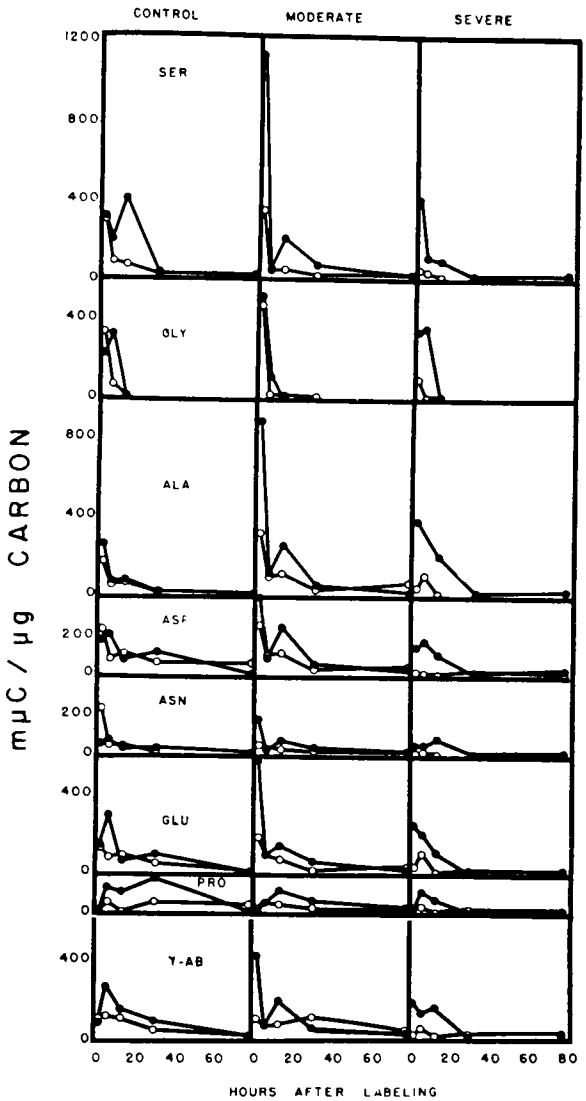

FIG. 3. Changes in specific activity of individual free amino acids from $^{14}CO_2$-labeled Bermuda grass shoots.

of controls. Very little label was incorporated into protein from severely stressed shoots. These data support the conclusion that free amino acids are readily synthesized during water stress, but are not as readily incorporated into protein as in unstressed controls.

Changes in amounts of individual free amino acids with increasing water stress are shown in table II. The free amino acid concentrations in control shoots of both varieties were similar except that Common shoots contained more than twice as much free asparagine as Coastal shoots. From comparison of the blue-yellow light absorption ratios for the ninhydrin reaction products of asparagine and threonine, and from preliminary analyses of mild acid hydrolysates of purified free amino acid extracts, it was found that the asparagine content of shoots is much greater than the threonine content. Therefore the combined asparagine-threonine peak was calculated as asparagine. The increase in this peak during water stress was also due to increased asparagine content. Free proline concentration increased dramatically during water stress to 10 to 125 times its control value. At the same time, valine content tripled, and glutamic acid and alanine concentrations decreased.

Serine, glycine, and alanine were the free amino acids that became most highly labeled (fig 3). Aspartic acid, glutamic acid, γ-aminobutyric acid, and asparagine all incorporated somewhat less label. All of the free amino acids found in the controls except proline became more highly labeled in the moderately stressed shoots than in any other treatment or the controls. Proline seemed to become labeled slowly. The low specific activity of proline obscures the fact that the great amount of proline present in stressed plants contained more than half the activity remaining in the free amino acid fraction after 77 hours. The irregularities in changes in specific activities of the individual amino acids occur for all the amino acids of a specific sample. This indicates that the differences reside in the level of labeling of individual shoots, and not that specific activities changed irregularly within any 1 shoot.

Changes in the amino acid composition of water-soluble protein with increasing water stress are given in table III. Conditions of hydrolysis did not permit preservation of cystine or tryptophan; consequently these had to be omitted from the calculations. The largest change in protein composition in stressed shoots was the 20 to 40% decrease in arginine content. There was also a small decrease in threonine content.

Changes in the specific activity of individual protein-bound amino acids are shown in figure 4. The higher specific activity in Common shoots is attributed to the smaller size of Common shoots, which results in a concentration of label when plants were exposed to equal amounts of label. The specific activity curves for most protein amino acids from control or moderately stressed shoots level off after 5 or 12 hours. Specific activity of protein amino acids from severely stressed shoots was considerably lower than in the other treatments. Generally maximum specific activity was achieved after 1 to 12 hours, followed by a sharp decline. The specific activity curves, together with the protein data of table I, are interpreted to mean that there is a net loss of protein during water stress although some synthesis occurred.

To test whether or not proline could be synthesized from glutamic acid (17) in stressed plants, control and stressed shoots were incubated with glutamic acid-U-^{14}C. Two well-watered control shoots and 2 shoots from a Coastal plant not watered for 6 days were excised and quickly placed in 0.05 ml of water contained in 2 cm conical centrifuge tube tips. The shoots were illuminated as before.

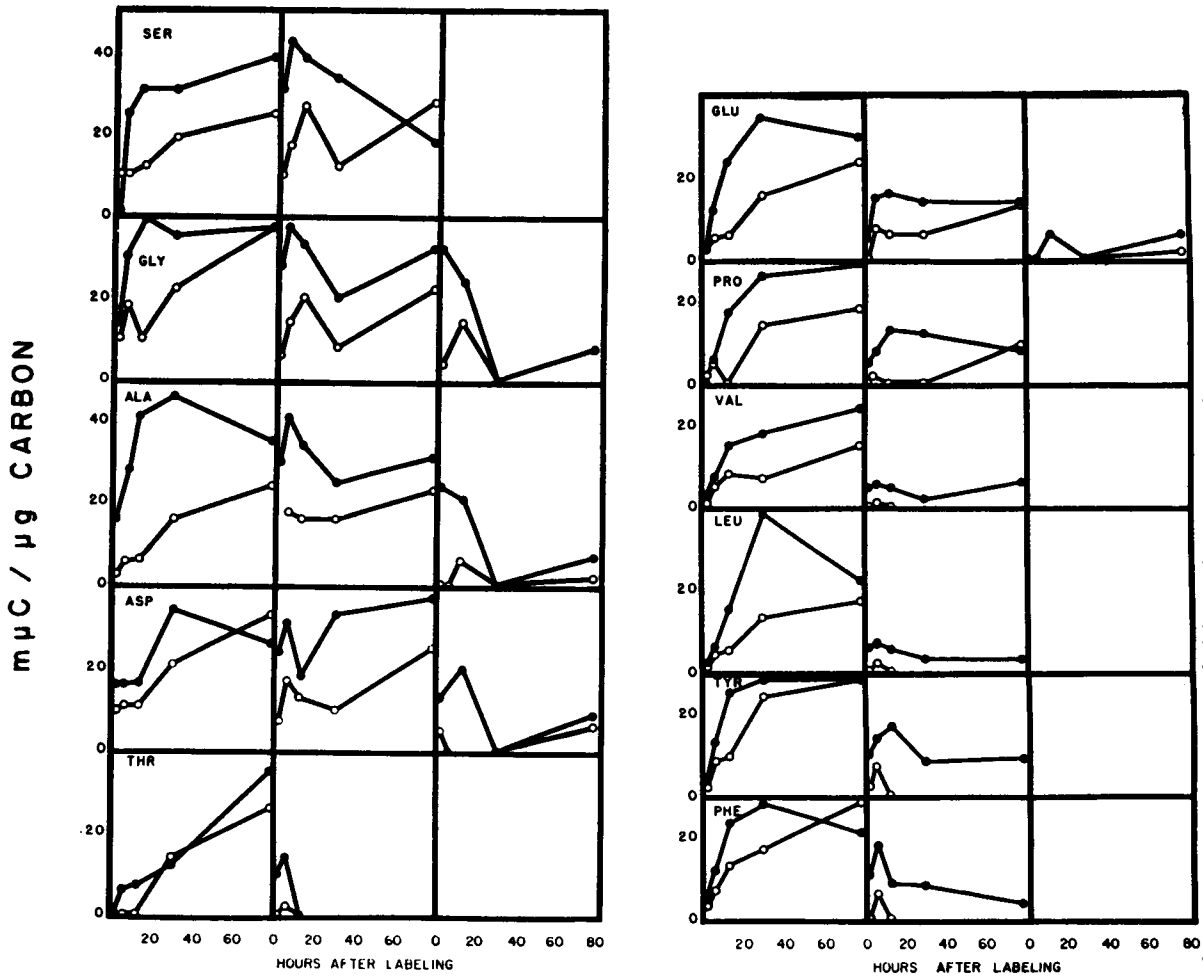

FIG. 4. Changes in specific activity of individual soluble protein amino acids from $^{14}CO_2$-labeled Bermuda grass shoots. Specific activities were calculated from average protein composition values for Common Bermuda controls and the measured radioactivity figures.

To the water was added 3.0 μc of randomly labeled ^{14}C-glutamic acid, monoammonium salt, specific activity 10 mc mmole^{-1}. Water was added to the vessels in 0.02 ml increments to replace that taken up by the shoots. One control and 1 stressed shoot were killed in boiling 80 % ethanol after 1 hour; the other 2 shoots were killed after 3 hours. Amino acid extracts were made in the usual manner, except that the ion exchange purification step was omitted. Radioactivity and ninhydrin-positive compounds were measured on the analyzer.

Results are shown in table IV. About half of the amino acids, plus at least 11 ninhydrin-negative compounds, became labeled both in control and stressed shoots. Proline was very slightly labeled in controls, but specific activity was fairly high because of the low amount present. The proportion of recovered label in proline from controls was less than 1 % at both sampling times, whereas this proportion in stressed plants was 6.0 % at 1 hour and somewhat less than 8.6 % at 3 hours. The actual activity in proline was 25 and 16 times greater in stressed plants than in controls at 1 and 3 hours respectively. It is concluded that water-stressed Bermuda grass shoots convert glutamic acid to proline, and accumulate the newly synthesized proline, much more readily than do well-watered shoots. Turnover of new proline was also very slow; labeled proline was still being accumulated 3 hours after labeling.

The data of table IV also show that glutamic acid-U-^{14}C disappears at about equal rates from stressed and control shoots. The largest amounts of label were recovered in several ninhydrin-negative peaks eluted before aspartic acid. These peaks represent sugars and organic acids which would ordinarily have been lost if the sample had been purified by the ion exchange method.

Table IV. *Distribution of Radioactivity in Soluble Compounds of Coastal Bermuda Grass Shoots Incubated with Glutamic Acid-U-^{14}C*

Compound	Control (−3.5 bars) 1	Control (−3.5 bars) 3	Stressed (−25.2 bars) 1	Stressed (−25.2 bars) 3
1*	...	...	...	67
2	71	...	...	194
3	53	170	...	
4	83	250	...	173
5	83	...	...	
6	120	53	...	101
7	77	53	...	80
8	11	...	...	...
9	12	...	...	14
10	6.5	2.4	...	...
11	10	3	...	5
Aspartic acid	75.5	31	...	67
Asparagine and threonine	19	7.1	14	34
Serine	15	16	12	15
Glutamic acid	268	188	410	192
Proline	2	6.5	50	101
Glycine	...	...	2	...
Alanine	77	28	47	81
12	8	5	...	1
13	40	11	35	28
Valine	1	2	...	...
Isoleucine	1	2	1	1
14	4	...	...	1
Leucine	3	...	2	1
15	2	...	1	2
γ-Aminobutyric acid	91.5	24	63	12
Totals	1130	854	790	1170

* Ninhydrin-negative compounds, numbered in order of elution from the analyzer column, were detected as radioactivity peaks only.

Discussion

Soluble protein levels in Bermuda grass were found to decrease with increasing water stress. Chen et al. (3) have reported successive increase, decrease, and a second increase in protein levels with increasing stress in citrus seedlings. These changes parallel Stocker's (15) activation, reaction, and restitution phases of drought response. The data presented here do not fit this pattern. However, the Bermuda grass data represents certain water stress levels, while the citrus data were taken at strict time intervals after withholding water.

The marked loss of protein-bound arginine in stressed Bermuda grass shoots has not been reported for other plants. This loss may reflect a preferential hydrolysis of arginine-rich protein. Such proteins are found in nuclei (5) and in ribosomes (16). Water stress can induce either an increase (19) or a decrease (14) in ribosomal RNA, but ribosomal proteins and nuclear proteins have not been investigated in connection with water stress. However, basic nuclear and ribosomal proteins as a whole are rich in lysine as well as in arginine, and no such loss in protein bound lysine was detected as a result of water stress. This could be interpreted to mean the loss of protein arginine involves the loss of some arginine-rich but lysine-poor protein. Furthermore, the loss in protein arginine may account for the observed very slight rise in free arginine.

During severe water stress, photosynthesis, starch accumulation, and protein synthesis are all inhibited to some degree. In stressed Bermuda grass shoots enough $^{14}CO_2$ was fixed to label free proline that turned over very slowly. The ^{14}C-glutamic acid labeling data clearly show that stressed shoots readily accumulated much more proline newly synthesized from glutamic acid than do control shoots. The slow turnover of labeled proline may also reflect an inhibition of proline catabolism. Free proline may be acting as a storage compound for both carbon and nitrogen during water stress, when both starch and protein synthesis are inhibited. Such a storage compound might be utilized for growth upon rewatering.

The changes in levels of free amino acids accompanying water stress in Bermuda grass are similar to those found in water stressed citrus

seedlings (3), pumpkin roots (20), and cut ryegrass (6).

Throughout this study, possible differences in nitrogen metabolism between Common and Coastal varieties were sought. During water stress, free proline accumulated to the highest levels in Coastal shoots. Under well watered conditions Common shoots contained the largest amounts of free asparagine. Aside from these minor observations, no differences were detected that might serve as a basis for explanation of the known differences in drought response. Such differences are still best explained on anatomical and morphological grounds (13).

Acknowledgments

We thank Dr. G. W. Burton, Tifton, Georgia, for clonal material of grasses, and Dr. P. J. Kramer for valuable advice and discussion.

Literature Cited

1. Benson, A. A., J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas, and W. Stepka. 1950. The path of carbon in photosynthesis. V. Paper chromatography and radioautography of the products. J. Am. Chem. Soc. 72: 1710–18.
2. Boyer, J. S. 1965. Effects of osmotic water stress on metabolic rates of cotton plants with open stomates. Plant Physiol. 40: 229–34.
3. Chen, D., B. Kessler, and S. P. Monselise. 1964. Studies on water regime and nitrogen metabolism of citrus seedlings grown under water stress. Plant Physiol. 39: 379–86.
4. Gates, C. T. and J. Bonner. 1959. The response of the tomato plant to a brief period of water shortage. IV. Effects of water stress on the ribonucleic acid metabolism of tomato leaves. Plant Physiol. 34: 49–55.
5. Johns, E. W. and J. A. V. Butler. 1962. Studies on histones. IV. The histones of wheat germ. Biochem. J. 84: 436–39.
6. Kemble, A. R. and H. T. Macpherson. 1954. Liberation of amino acids in perennial ryegrass during wilting. Biochem. J. 58: 46–50.
7. Lawrence, J. M. and D. R. Grant. 1963. Nitrogen mobilization in pea seedlings. II. Free amino acids. Plant Physiol. 38: 561–66.
8. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–75.
9. Mothes, K. 1956. Der Einfluss des Wasserzustandes auf Fermentprozesse und Stoffumsatz. In: Encyc. of Plant Physiol., v. 3, W. Ruhland, ed., p 656–64.
10. Piez, K. A. and L. Morris. 1960. A modified procedure for the automatic analysis of amino acids. Anal. Biochem. 1: 187–201.
11. Petrie, A. H. K. and J. G. Wood. 1938. Studies on the nitrogen metabolism of plants. III. On the effect of water content on the relationship between proteins and amino acids. Ann. Botany N. S. 2: 887–98.
12. Prusakova, L. D. 1960. Influence of water relations on tryptophan synthesis and leaf growth in wheat. Fiziol. Rast. 7: 139–48.
13. Ratnam, B. V. 1961. A study of some aspects of drought resistance of grasses. Ph.D. dissertation, Duke University, 1961.
14. Shah, C. B. and R. S. Loomis. 1965. Ribonucleic acid and protein metabolism in sugar beet during drought. Physiol. Plantarum 18: 240–54.
15. Stocker, O. 1960. Physiological and morphological changes in plants due to water deficiency. In: Plant Water Relationships in Arid and Semi-arid Conditions. UNESCO, Paris. p 63–104.
16. Ts'o, P. O. P., J. Bonner, and H. Dintzis. 1958. On the similarity of amino acid composition of microsomal nucleoprotein particles. Arch. Biochem. Biophys. 76: 225–26.
17. Vogel, H. J. and D. M. Bonner. 1954. On the glutamic-proline-ornithine interrelation in *Neurospora crassa*. Natl. Acad. Sci., Wash., Proc. 40: 688–94.
18. Wang, D. 1960. An ion-exchange resin method for the fractionation of alcoholic plant extracts. 186: 326–27.
19. West, S. H. 1962. Protein, nucleotide, and ribonucleic acid metabolism in corn during germination under water stress. Plant Physiol. 37: 565–71.
20. Zholkevitch, V. N. and T. F. Koretskaya. 1959. Metabolism of pumpkin roots during soil drought. Fiziol. Rast. 6: 690–700.