THE EVALUATION OF RESERVE CARBOHYDRATES IN MIDLAND BERMUDAGRASS (*Cynodon dactylon* L.)

by

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INTRODUCTION

As more is learned about plant energy relationships the importance of an accurate determination of the reserve energy (reserve carbohydrates) becomes greater. The nebulous term Total Available Carbohydrate (TAC) has been used for more than 50 years to indicate those carbohydrates available to a plant as energy. But as chemistry and biochemistry have advanced the use of such an all-encompassing term becomes more questionable.

In those plants storing fructosans or similar materials the evaluation of reserve carbohydrates is simplified due to their solubility in hot water, while structural materials are not. But in plants storing starch the reserve carbohydrate determination becomes difficult due to the lack of starch solubility in solvents that will not attack structural materials. Thus starch must be degraded before measurements can be made. It is the evaluation of the methods of degradation that is the basis of this research.

LITERATURE REVIEW

Since the early 1900's crop physiologists have tried to interrelate reserve carbohydrate status with crop responses to various management practices. Various methods have been used to estimate reserves, many based on the ability of diastatic enzymes to solubilize the native water insoluble starch. Others are based on acid hydrolysis to simple sugars, followed in either case by measurement as reducing sugars. Davis and Daish (1914) found taka-diastase to give more reproducible results than salivary or malt-diastase extractions. Davis and Sawyer (1915) considered the enzymes in taka-diastase similar to those in plants; thus they continued screening plant materials to which this total available carbohydrate (TAC) method was applicable. Horton (1921) using the methods of Davis and Daish was unable to obtain results with small experimental errors. The method was further evaluated by Shriner (1932) on grape starch, and by Poe and Judkola (1944) who studied the effects of preservatives on the taka-diastase extraction.

Along with early use of taka-diastase, various workers studied saliva extractions. Loomis (1935) recommended saliva extraction as reliable for starch, and further suggested that total acid hydrolyzable material not be reported as starch. Heinze and Murneek (1940) found saliva extractions

satisfactory for starch following an 80% ethanol extraction. The reference by Loomis to acid methods applies to the ethanolic-nitric acid extraction by Niemann et.al. (1935) which also extracted structural plant materials. Denny (1934) compared extraction procedures and found none of the acid methods reliable. Hoffpauir (1949) in an A.O.A.C. summary reported several satisfactory methods for large quantities of starch, but amounts less than 5% required a procedure utilizing the determination of starch as an iodine complex. McCready et.al. (1950), early workers with perchloric acid extractions of starch, solubilized the starch with perchloric acid and then determined it as the iodine complex. A similar method was used by Carter and Neubert (1954) on apples and by Adams and Emerson (1961) on Ponderosa pine needles.

Weinmann (1946) used saliva as a source of alpha-amylase to extract starch and also outlined the widely used taka-diastase method (1947), that was later modified by Lindahl et.al. (1948).

Smith (1962), using acid extractions, found that 2% v/v H_2SO_4 gave only slightly higher results than taka-diastase, and was satisfactory in an alfalfa regrowth study by Wolf et.al. (1962). In comparing various acid concentrations with taka-diastase as a standard, Smith et.al. (1964) found 0.2 $\underline{N}$ H_2SO_4 most nearly duplicated taka-diastase

extractions. Hassid and Neufeld (1964) standardized the perchloric acid extraction of starch in plant materials. The 9th edition of the A.O.A.C. Methods recommends a malt-diastase extraction for starch. Thus even today the extraction methods are nearly as numerous as the workers using them.

METHODS AND MATERIALS

Experiment I. Growth Chamber Study

Midland Bermudagrass (*Cynodon dactylon* L.) was chosen for this study because it stores starch as a reserve carbohydrate (Hawkins et.al. 1964). On April 16, 1965 rhizomes were obtained from a dormant stand of Midland on the Agronomy farm at Blacksburg. The rhizomes were washed and planted directly on a 1-inch soil bed in 20 by 14 inch greenhouse flats. Approximately 1/8 pound of rhizomes gave uniform coverage of the flat. The rhizomes were then covered with two inches of fine washed sand. Three weeks were allowed for establishment in a greenhouse where temperatures fluctuated between 60 ± 10 F at night and 90 ± 10 F during the day.

The flats were then placed in growth chambers with 14-hr. days at 75 F with 10-hr. nights at 65 F for five days. After cutting back all plants to three inches the following treatments were imposed: at day-0, NH_4NO_3 and lime at 30 and 300 lb N per acre with two temperature regimes (95-85 F and 60-50 F day-night temperatures respectively, at the same daylength used during the equilibration period). Four replications were used, but two were combined for chemical analysis. Due to the failure of the cooler chamber in the third week, results are given only for the first 14 days in that chamber. At 21 days all grass at the high temperature

was cut to the surface, the lights were turned off, and the temperature was set at 95 F for 14 days. Water was uniformly supplied every two days.

Rhizome samples were taken every seven days starting with day-0 by removing a section of the soil sand mixture starting from the end of the flat nearest the wall of the chamber. The rhizomes were washed free from sand, cut to about 3-inch lengths and dried at 80 C in a forced draft oven for 20 hours. The samples were ground to pass 40 mesh in a Wiley Mill and stored in a vacuum desicator until analysis.

Experiment II. Carbohydrate Depletion Study

In July rhizomes were removed from the same area as for Experiment I, brought to the laboratory, washed, cut to 2-inch lengths and placed on paper towels on large baking sheets. The towels were kept moist with distilled water with .1 gm thymol added per liter to retard pathogens. The rhizomes were then incubated at 37 C in the dark for 21 days. Samples were taken every seven days and handled as those from Experiment I.

Carbohydrate Analysis

Five methods were used to evaluate the total available carbohydrates. A 2% v/v H_2SO_4 and a 0.2 N H_2SO_4 extraction was used as outlined by Smith (1962, 1964). Taka-diastase

extraction was as outlined by Weinmann (1947) except that the clearing step was deleted. The other two methods are based on the assumption that starch is the major reserve carbohydrate in bermudagrass. The first is a perchloric acid extraction of starch as outlined by Hassid and Neufeld (1964) with the starch value combined with 80% ethanol soluble sugars to yield TAC. For the final method the ethanol soluble sugars were extracted by blending with 80% ethanol at 16,000 rpm for ten minutes followed by infiltration and heating for 20 minutes at 80 C. The material was centrifuged, decanted and washed four times with 80% ethanol with washings combined in a 100 ml volumetric. The starch was determined on the residue by adding 5 ml of .01 M phosphate buffer at pH 7.0 and heating 30 minutes in a boiling water bath. After cooling to room temperature 5 ml of alpha-amylase solution was added. The alpha-amylase contained 250 micrograms of protein per ml (Worthington Biochemical Corp.) and had a minimum activity of 790 Units/mg as measured by the method of Bernfield (1951). The sample was infiltrated and incubated for 30 minutes at 25 C; the solution was centrifuged and decanted into a 50 ml volumetric. The residue was extracted again using the same procedure, followed by two washes with warm water. This method was developed by the author with the aid of Dr. K. W. King, Professor of Biochemistry, Virginia Polytechnic Institute. Starch in the perchloric acid and alpha-amylase

extractions was determined by the phenol-sulfuric acid method (Dubois et.al. 1956), as were the 80% ethanol soluble sugars. The reducing sugars after hydrolysis of the two sulfuric acid extractions as well as the taka-diastase extraction were by the method of Nelson (1944)-Somogyi (1952). The glucose determination was by the notatin method using the Glucostat from Worthington Biochemical Corp. with their recommended method. All methods were based on a glucose standard and reported as percent dry weight.

PROCEDURE AND RESULTS OF PRELIMINARY STUDY

Weinmann (1947), Denny (1934), and Loomis (1935) encountered difficulties with acid extractions of reserve carbohydrates. Alexander (1960) reported that many species of the genera Aspergillus degraded cellulose (Aspergillus oryzae is the source of taka-diastase). Thus, the reliability of the acid extractions outlined by Smith (1962, 1964) or the taka-diastase method of Weinmann (1947) for accurately determining reserve carbohydrates is questionable.

A preliminary experiment was undertaken to evaluate the effects of two acid concentrations (2% and 0.2 N) and taka-diastase on hemicellulose extracted from rye according to Myhre and Smith (1960), and on washed lint cotton (cellulose). The acid and taka-diastase extractions were carried out as described earlier. These were compared with a hot water extraction where a 100 mg sample was heated with 30 ml of water for 45 minutes in a boiling water bath. The mixture was filtered and reducing power determined on the acid hydrolyzed filtrate. The results (Table 1) show that taka-diastase had little more effect on the hemicellulose than heating with water. Acid concentrations of 0.2 N and 2% dissolved 39 and 170% more hemicellulose than water.

Cellulose was slightly attacked by taka-diastase (1.14% dissolved), with the acids having no apparent effect. Due to the possibility of a cellulase system in the taka-diastase,

TABLE 1. Percent carbohydrate dissolved by four extraction procedures.

Material	Procedure			
	Taka-diastase %	2% H_2SO_4 %	.2N H_2SO_4 %	Hot Water %
Hemicellulose	31.52	79.50	40.75	29.50
Cellulose	1.14	0.30	0.05	0.05

a viscosity study was conducted. A 10% solution of carboxy-methyl-cellulose (Hercules Powder Co. Lot No. 40556) was used. A comparison of the taka-diastase at 50 mg per 20 ml and a crude cellulase (Meiji Seika Kaisha L.T.D. Rop-Mix) at 50 mg per ml was made. The viscosities were measured by the method of Spalding et.al. (1961) at 36.5 C. Decreases in viscosity of the CMC solution indicate breakdown of the compound. Results given in Figure 1 indicate a beta-glucosidase present in taka-diastase.

A reproducible unknown variable was possibly responsible for the second apparent increase from 20 to 30 minutes. The possibility of spurious results due to the hydrolytic effect of the acids on hemicellulose and the presence of a cellulase system in taka-diastase should be kept in mind when considering the results of experiments I and II.

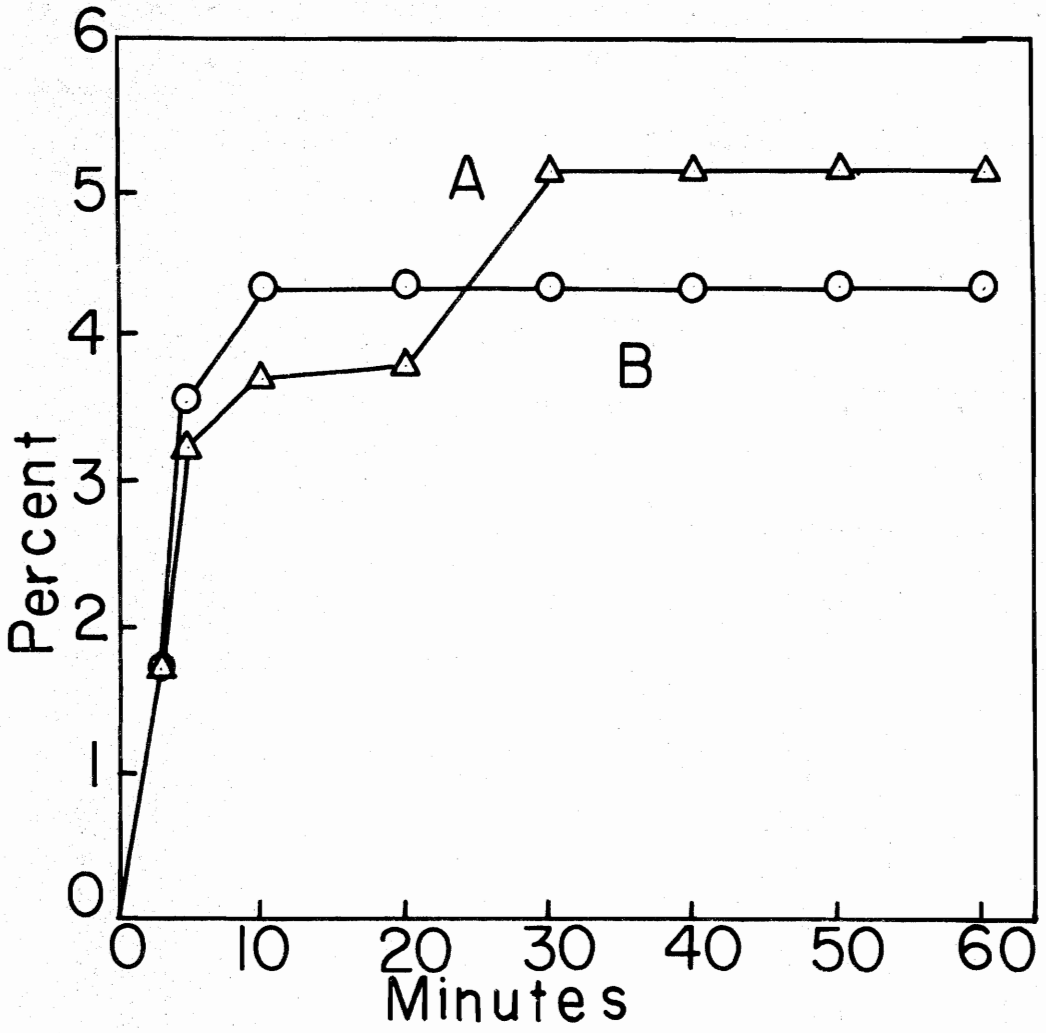

Figure 1. Percent reduction in viscosity of carboxy-methyl-cellulose by A-Taka-diastase and B-crude cellulase, at 36.5 C.

RESULTS

In Experiment I Midland bermudagrass was grown at 95 F (14:10-hr, light: dark periods); the TAC's declined during 14 days and then increased during the last 7 days in the light (Figure 2). With 30 lb N there was a decline in carbohydrates for 7 days and then an increase for the last 14 days. After 21 days, when all plants were grown in total darkness, there was a steady decline in available carbohydrates. At 65 F, there was a gradual TAC decline with both N levels for the 14 day experiment (Figure 3).

Although the carbohydrate curve trends for the treatment effects are similar, the magnitude of the values show about 4-fold differences among some of the analytical methods (Figs. 2 and 3 and Table 2). The 0.2 N H_2SO_4 was 2-4 percentage units higher than either enzyme method and the 2% H_2SO_4 13-14 units higher.

The percent glucose extracted (Figs. 4 and 5) follows essentially the same trends as the % TAC but at a lower level. The effects of nitrogen noted in % TAC occur with glucose. Taka-diastase, alpha-amylase, and 0.2 N H_2SO_4 extracted statistically similar amounts of glucose but significantly more than 2% H_2SO_4 (Table 3). The glucose results tend to minimize the differences found between the acid and taka-diastase extractions. Thus when TAC is determined as glucose plus ethanol soluble sugars; one can achieve statistical

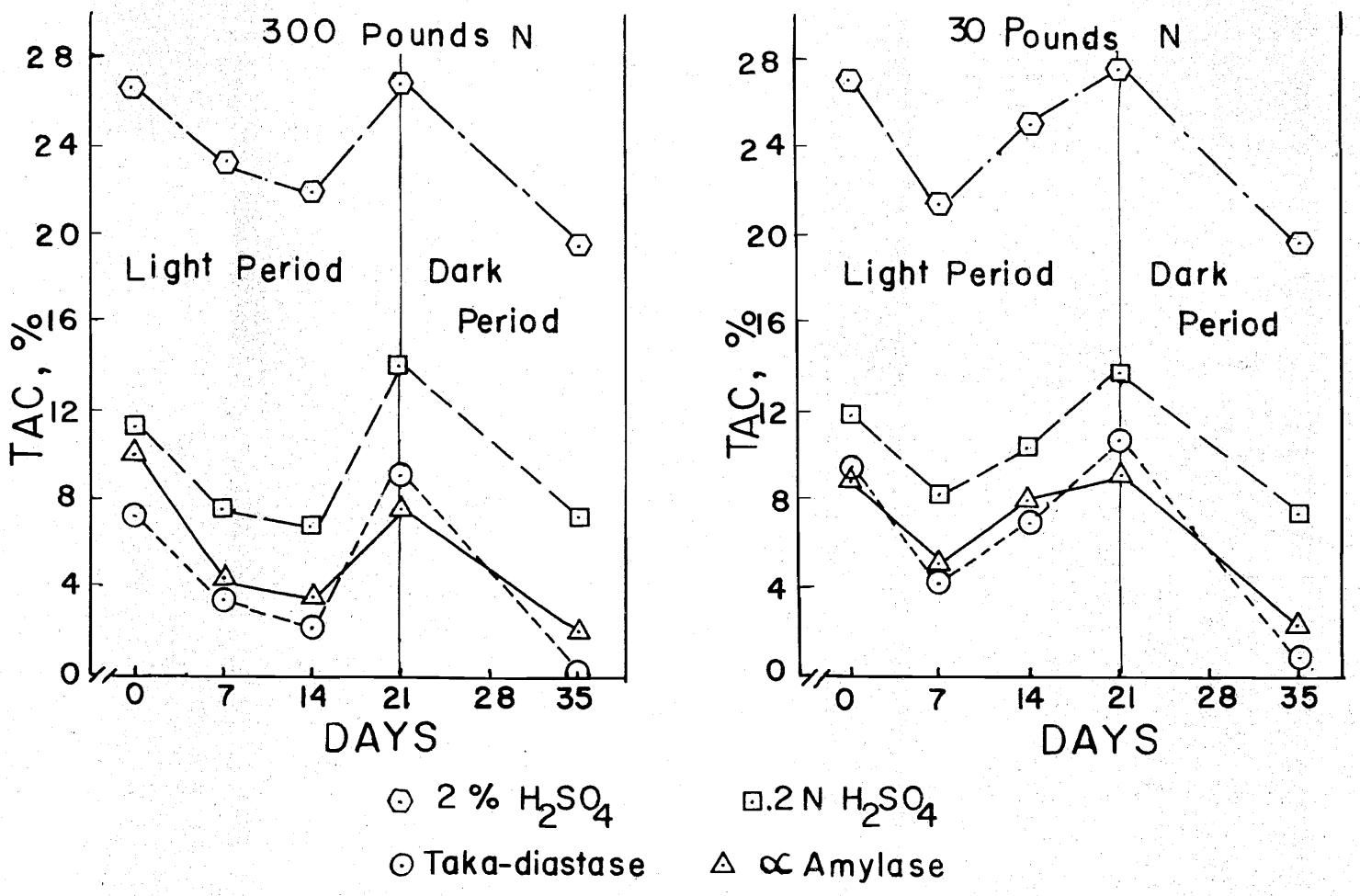

Figure 2. TAC determined by four analytical methods in Midland bermudagrass rhizomes grown at 95 F with two nitrogen rates and two light conditions.

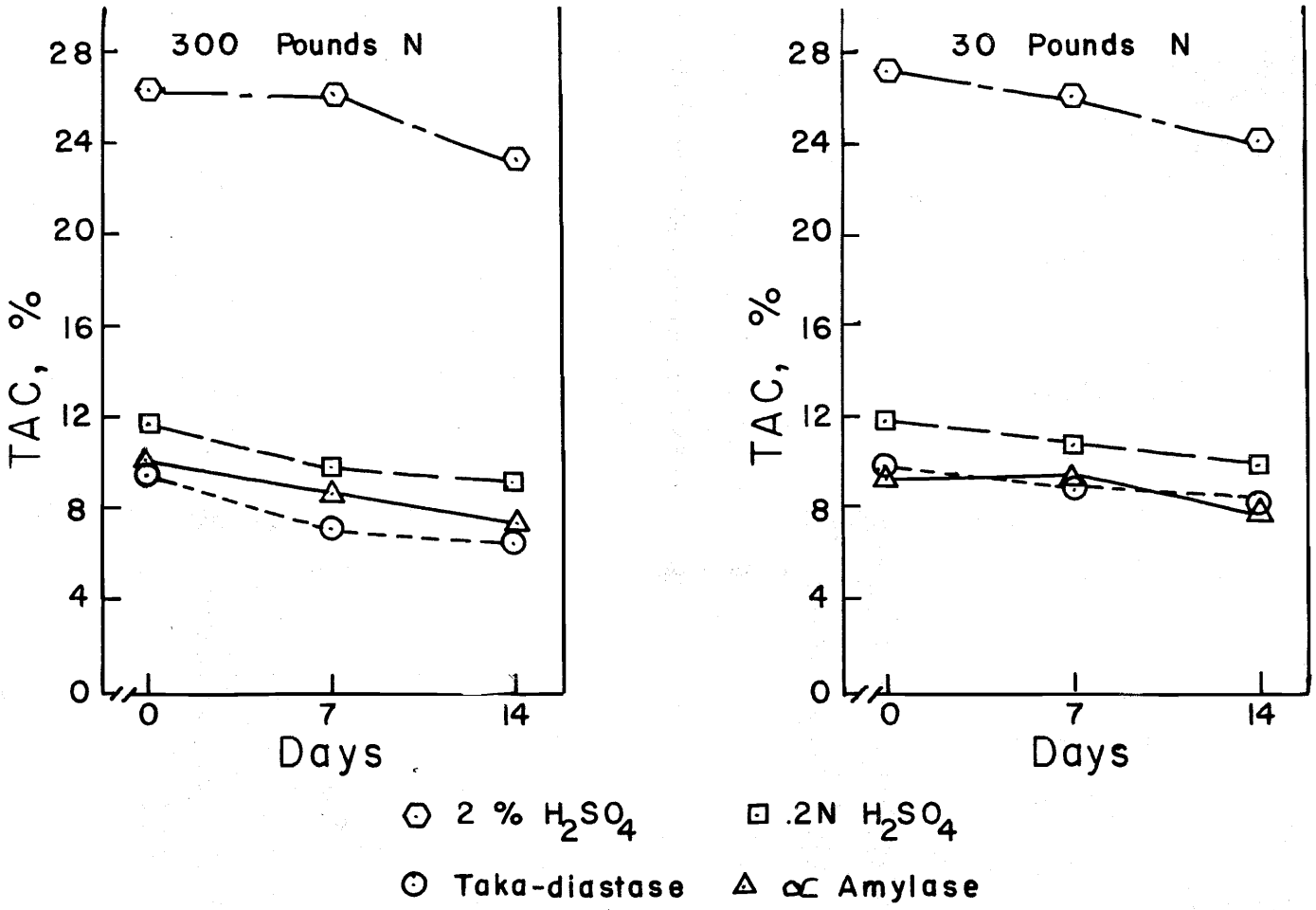

Figure 3. TAC determined by four analytical methods in Midland bermudagrass rhizomes grown at 60 F with two nitrogen rates.

TABLE 2. The mean TAC values for five methods of analysis for Midland bermudagrass rhizomes at two temperatures.[1]

Method	7-Day Samplings with temperatures		
	0-35 Days	0-14 Days	
	95 F	95 F	60 F
Taka-diastase	5.68 a	5.93 a	8.28 a
.2 N H_2SO_4	9.84 b	9.37 b	10.38 b
2% H_2SO_4	23.86 c	24.21 c	25.58 c
Alpha-Amylase	4.34 d	5.02 d	6.35 d
Perchloric acid	4.14 d	4.50 d	4.13 e

1 The means include both nitrogen levels; the values in a given column with different letters differ at the 5% level.

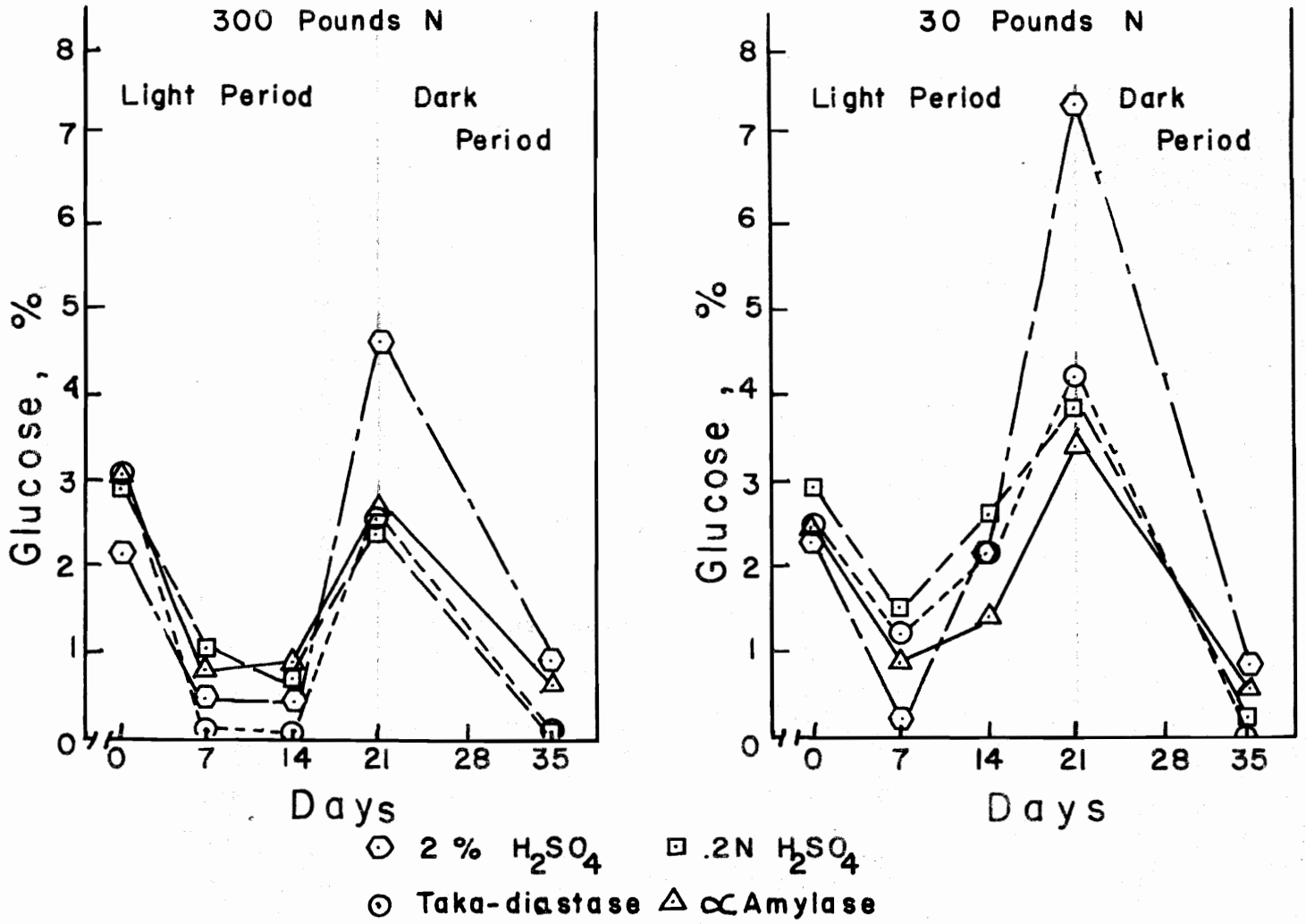

Figure 4. Glucose (starch by alpha-amylase) extracted by four methods in Midland bermudagrass rhizomes grown at 95 F with two nitrogen rates and two light conditions.

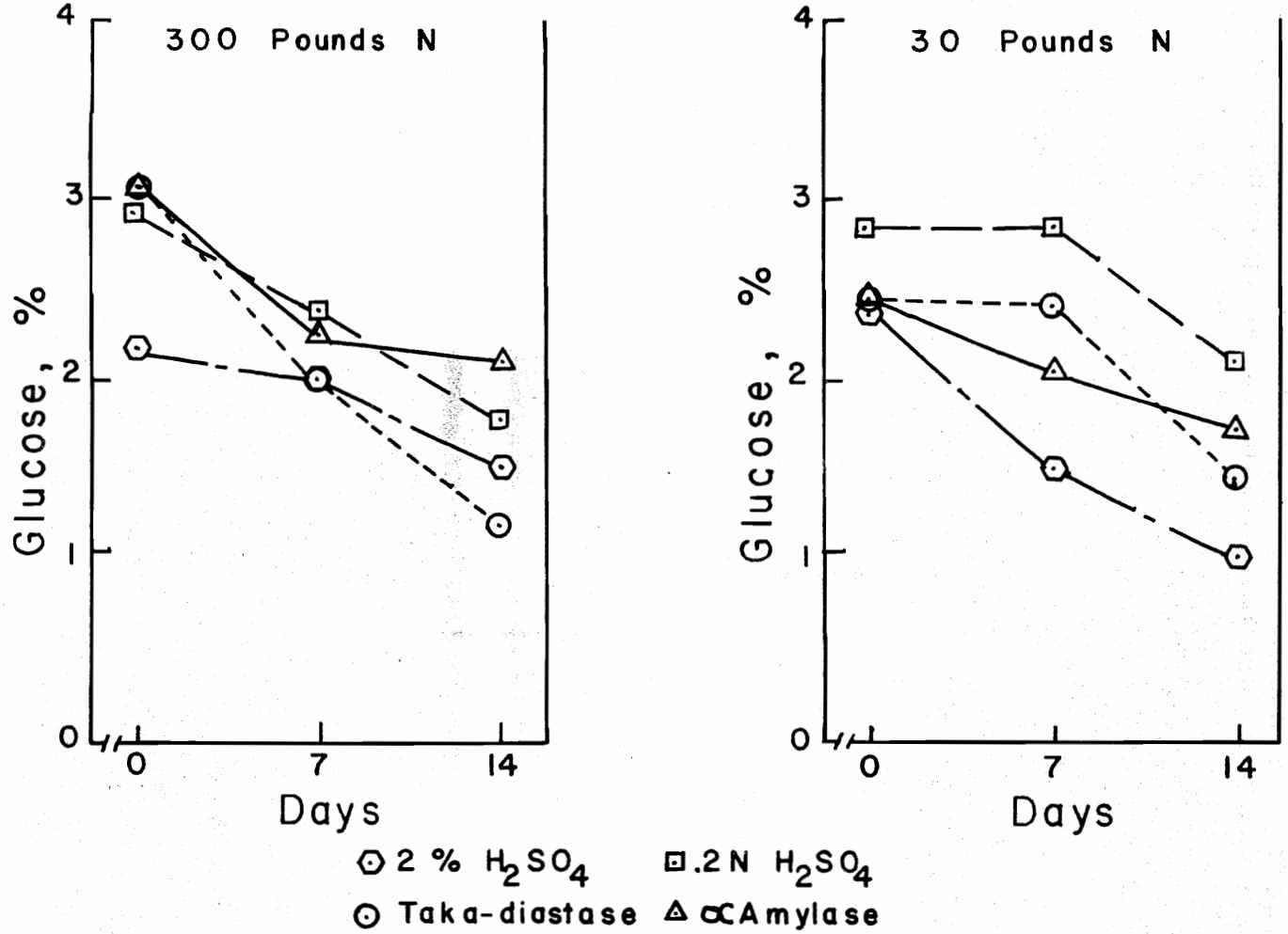

Figure 5. Glucose (starch by alpha-amylase) extracted by four methods in Midland bermudagrass rhizomes grown at 60 F with two nitrogen rates.

TABLE 3. The mean glucose values for four methods of analysis for Midland bermudagrass rhizomes at two temperatures.[1]

Method	7-Day Samplings with temperatures		
	0-35 Days	0-14 Days	
	95 F	95 F	60 F
Taka-diastase	1.57 a	1.49 a	2.08 a
.2 N H_2SO_4	1.80 a	1.91 ab	2.48 a
Alpha-Amylase	1.68 a	1.57 ab	2.25 a
2% H_2SO_4	2.14 b	1.30 b	1.78 b

1 The means include both nitrogen levels; the values in a given column with different letters differ at the 5% level.

sameness with four methods instead of two, ie. perchloric acid, alpha-amylase, 0.2 N H_2SO_4, and taka-diastase, instead of only perchloric acid and alpha-amylase.

Results of experiment II are given in Figure 6. Statistically all methods for % TAC are different, with the 0.2 N H_2SO_4 approximately 2 percentage units higher than either enzyme method and the 2 % H_2SO_4 12-16 units higher. The trends in % glucose, although erratic, show either acid extracting more than taka-diastase or the alpha-amylase.

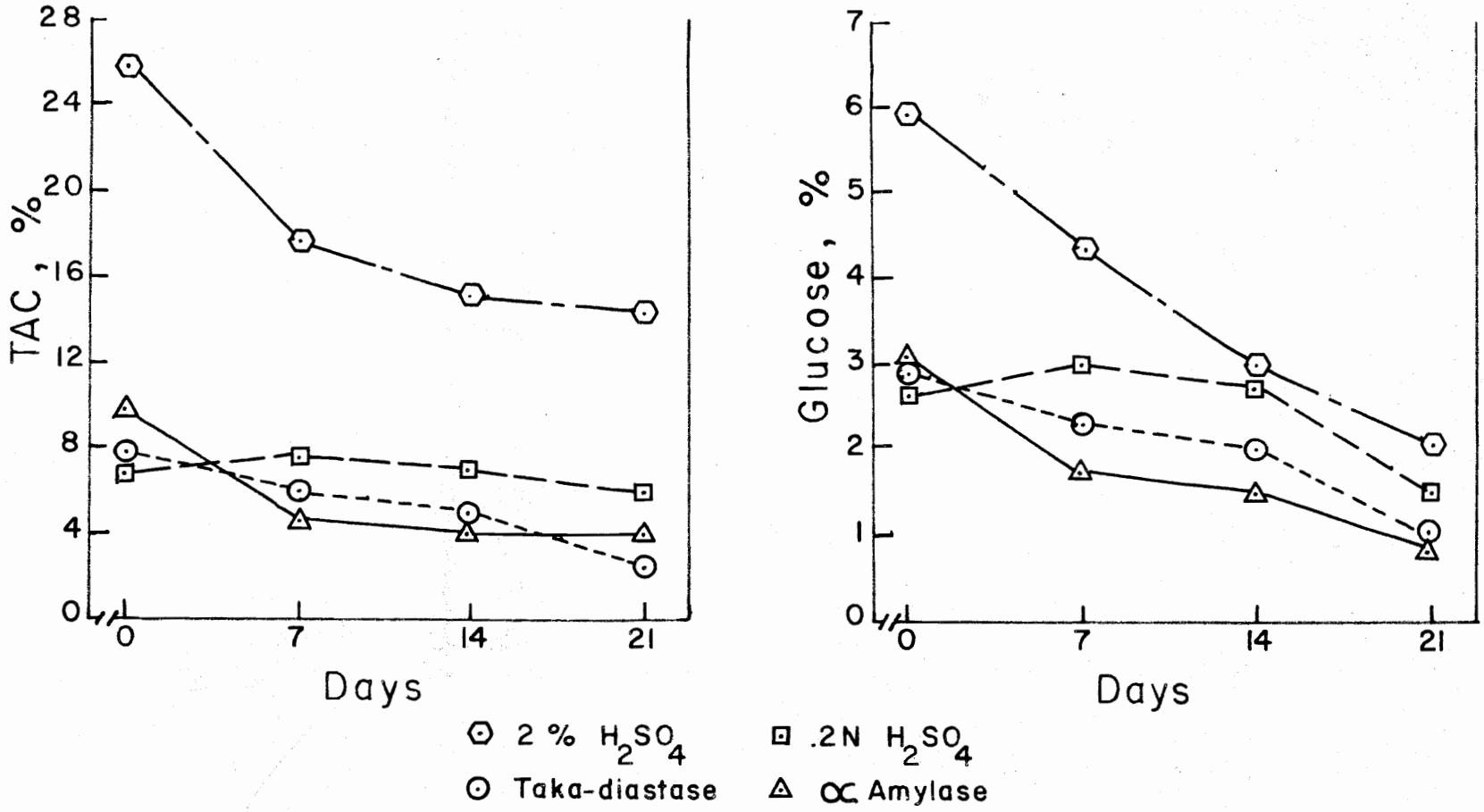

Figure 6. TAC and glucose determined by four methods in Midland bermudagrass rhizomes grown in the dark at 37 C for 21 days.

DISCUSSION

Reference by Alexander to the cellulase capability of *Aspergillus sp.* was substantiated by the viscosity study (Fig. 1). A beta-glucosidase system was found in taka-diastase, but the activity on plant materials may not be substantial. The second variable encountered, indicating a bimodal system, was reproducible.

The hydrolytic capacity of the acids on hemicellulose is not conclusive, since the hemicellulose used is probably modified by the extraction procedure. But the trends (Table 1) are noteworthy, in that acids seem to dissolve more hemicellulose as concentrations are increased.

The inherent limitation of taka-diastase is not readily apparent in either experiment. When the % TAC with taka-diastase and acid extractions are compared, the latter removed uniformly more reducing material (Figs. 2 and 3). This uniformity does not occur with glucose (Figs. 4 and 5). The drop in glucose extracted by 2% H_2SO_4 at 7 and 14 days (Fig. 4) is thought to be due to destruction of glucose by H_2SO_4 which may be greater than 10% by actual measure. This destruction may not be apparent at the other dates due to the increase in total glucose. The chances of getting erroneous data with acids are apparently greatest when the plants are actively growing i.e. at the 21 day sampling. The wide discrepancy between .2 N and 2% H_2SO_4 at this date

(Fig. 4) may be attributed to hemicellulose degradation yielding glucose as found by Phillips et.al. (1960); or degradation of cellulose in the amphorus state, or both. The susceptibility of native hemicellulose or cellulose to acid degradation probably increases in young, fast growing cells where structural materials are less stable and less lignified. The degree of error due to these degradation products would probably be greatest in stolons, stems or leaves where structural materials are in a greater state of flux than in the rhizomes.

The afore mentioned difficulties are not encountered with the alpha-amylase extraction. The enzyme, 2X crystalline protein, extracted from swine pancreas, is essentially free from any other carbohydrases. As a hydrolytic agent for starch, it is reproducible and more sensitive than perchloric acid, which did not detect starch levels below 1%. Due to the cost envolved, it is too expensive for routine analysis, but as a standard it is invaluable.

The relationship between starch extracted by alpha-amylase and the glucose extracted by other methods was investigated. This relationship was high with .2 $\underline{N}$ H_2SO_4 or taka-diastase (Table 3). The expression of glucose in either of these extracts as a starch value is questionable due to the previously shown difficulties with the acid on hemicellulose and taka-diastase on cellulose. Although errors

due to these drawbacks were not apparent in either experiment, their occurence should not be discounted.

If a given plant material stores only starch as reserve carbohydrate then it seems that a standard value of the reserve status would be established with an alpha-amylase extraction. Due to the pre-extraction heating with hot water, ethanol soluble sugars would be included. Using this criteria, the .2 $\underline{N}$ H_2SO_4 extraction glucose value combined with the ethanol soluble sugars would be the least expensive and most rapid on the plant materials tested. The experimental plant materials used were relatively low in starch; higher starch concentrations may cause even greater differences between methods than those reported here. On plant materials of unknown composition any given extraction should be compared against the alpha-amylase, if the plant material is thought to contain starch.

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VITA

The author was born in Cleveland, Ohio on April 18, 1942. His elementary and secondary education were obtained from the South Euclid-Lyndhurst public schools in Lyndhurst, Ohio. Upon graduation from high school, he enrolled at Iowa State University in September, 1960. In June, 1963 he was married to Judith K. Burkley. He received the Bachelor of Science in Horticulture degree in May, 1964, from the above university. In September of the same year, he enrolled at Virginia Polytechnic Institute where he has been pursuing graduate work leading to a degree of Master of Science in Agronomy.

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ABSTRACT

The Evaluation of Reserve Carbohydrates
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Midland bermudagrass was grown at 95 F and 60 F for 35 and 21 days respectively; with 300 and 30 pounds of nitrogen. The rhizomes were harvested at 7 day intervals and reserve carbohydrates determined by .2 N H_2SO_4, 2% H_2SO_4, Taka-diastase, Alpha-amylase and Perchloric acid extractions.

A preliminary study showed the two acids capable of degrading hemicellulose and the taka-diastase having a beta-glucosidase present. In this study it was not possible to attribute definite values to the structural materials extracted although either acid extracted a higher percentage of carbohydrate than taka-diastase or alpha-amylase.

The glucose extracted by either acid or taka-diastase was compared with starch extracted by alpha-amylase. It was found that at the 5% level the glucose extracted by .2 N H_2SO_4 and taka-diastase was the same as the starch; while the 2% H_2SO_4 was higher. This correlation may not be applicable to other studies or other plant materials where weak structural materials may contribute glucose to the acid extractions.