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FORAGE CARBOHYDRATES

Some Nonstructural Carbohydrates in Forage Legume Herbage

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The influence of stage of maturity on the content of some carbohydrate constituents in the herbage of alfalfa, red clover, Ladino clover, and birdsfoot trefoil was studied at Madison, Wis. Samples were taken at six stages of maturity and freeze-dried for subsequent analysis. Percentages of takadiastase enzyme extractable and 2% H_2SO_4 hydrolyzable carbohydrates generally were highest at early stages of maturity and tended to be higher in red clover and Ladino clover than in alfalfa and trefoil. The interrelationships of glucose, fructose, and sucrose were somewhat different in each of the four legumes.

IDENTIFICATION and quantitative determination of the major carbohydrate constituents of most forage legumes have been accomplished. General reviews include those of Hansen *et al.* (*3*) and Sullivan and Garber (*12*). Detailed studies have been made of alfalfa herbage by Hirst, Mackenzie, and Wylam (*5*) and Nalewaja and Smith (*8*), of red clover by Bailey (*1*), and of Ladino clover by Wilkins *et al.* (*14*). Little information is available regarding the carbohydrate composition of birdsfoot trefoil.

Results obtained by different investigators for the same species rarely are directly comparable because of seasonal and varietal differences, differences in geographic location, and variations in analytical methods used. To be most meaningful, comparisons of carbohydrate content in relation to species and stage of maturity should be made within a single study. Few studies have been found, however, where the carbohydrate contents at successive maturity stages of two or more forage legume species have been compared under the same experimental conditions. This study was conducted to provide such comparative information.

Materials and Methods

Alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), Ladino clover (*Trifolium repens* L.), and birdsfoot tre-

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foil (*Lotus corniculatus* L.) were sown in separate trials on May 25, 1959, and on May 14, 1960, on silt loam soil on the University Hill Farms, Madison, Wis. Both areas were limed and fertilized before seeding, and were topdressed with P and K as indicated by tests performed by the Wisconsin Soil Testing Lab. Fertilizers were applied as 0–0–60 and 0–15–45 formulations.

Vernal alfalfa, Dollard medium red clover, Ladino clover, and Empire birdsfoot trefoil were sown from certified seed at the rates of 12, 10, 2, and 10 pounds per acre, respectively, in a latin square design with 4 replications. Individual plots were 20 × 20 feet square. The plots were sprayed as needed to control insects.

Samples were harvested at six stages of the spring growth during 1960 and 1961, at the vegetative, prebud, early bud, $^1/_{10}$ bloom, full bloom, and green seed pod stages of maturity of each species, with the exception of Ladino clover. Ladino clover was sampled when alfalfa reached the stages previously mentioned. Plants representing a given maturity stage were arbitrarily chosen from the four replicates. No random sampling pattern was used. Sample size was limited to 50 to 75 grams of dry matter equivalent by the equipment used for freeze-drying.

Herbage yields were obtained from samples clipped from two 2- by 3-foot quadrats taken at random in the plots on each harvest date. The legume plants enclosed by the quadrat were clipped at about two inches above the soil surface with hand clippers, freed of weeds, placed in cloth bags, and dried at about 63° C. The dry weight of each sample was determined and the yield per acre calculated.

For carbohydrate analysis, material representative of the maturity stage being sampled was chosen from the four replicates and combined to form one sample. To simplify sampling procedure, all species were sampled on the same day if there was sufficient material of a given growth stage available from which to choose a representative sample. Whenever this was not possible, separate sampling dates were used. For convenience, average sampling dates for the two years are given in Table I. Duplicate subsamples were carried through a chemical analysis to the point of reducing power or spectrophotometric determination whereupon duplicate or triplicate determinations were made for each subsample.

The forage was cut about two inches above the soil surface with hand clippers, freed of weeds and extraneous material, cut into 2- to 3-inch pieces, frozen immediately with liquid air or liquid nitrogen, and surrounded with dry ice. The mixture of forage and dry ice was ground through a chilled meat grinder and placed in a freezer kept at −10° C. until the dry ice had evaporated. The samples were freeze-dried, ground in a Wiley mill through a 40-mesh screen, bottled, and stored over P_2O_5 in a vacuum desiccator in a freezer kept at −2° C. or colder.

Total nonstructural carbohydrates were extracted by two general methods; the takadiastase enzyme method of Weinmann (*13*) as modified by Lindahl, Davis, and Shepherd (*7*); and the hot 2% H_2SO_4 (0.8*N*) method as described by Smith (*10*). Aliquots were used to determine reducing power, and the results were expressed as per cent total nonstructural carbohydrates. The first of these extraction methods is commonly used for extracting carbohydrates metabolically available to the plant, including starch. The second has been used as a simplified means of extracting these carbohydrates from storage organ tissues and was included here in a comparative study of the two methods when used with forage legume herbage. Recent work (*11*) has shown that 0.2*N* H_2SO_4 more closely approximates the values obtained with the takadiastase enzyme, at least in tests made on alfalfa root and timothy stem base tissue.

Sugars were extracted with 80% ethanol and determined by anthrone and copper reduction methods as described previously (*9*).

All determinations of reducing power were made with the Shaffer-Somogyi copper reduction method as described by Heinze and Murneek (*4*). Glucose was used as the standard and results were calculated on a dry weight (70° C.) basis.

Results

Dry Matter Yields. Sampling dates, plant heights, and dry matter yields are shown in Table I. Yields generally increased with each advance in stage of maturity. The highest yields were produced by alfalfa and red clover followed, in order, by trefoil and Ladino clover. In alfalfa, and in red and Ladino clovers, only small increments in yields occurred between the full bloom and green seed pod maturity stages and substantial increases occurred between all other successive stages. In trefoil, by contrast, dry matter yields increased substantially between all maturity stages. Trefoil maintained a greater proportion of its foilage in a green and vegetative condition at mature stages than did alfalfa and red clover because of new growth produced by axillary buds on mature stems (*10*).

Total Nonstructural Carbohydrates. The levels of carbohydrates determined using enzyme and acid extractions are shown in Figure 1. Percentages of carbohydrates generally were highest at early maturity stages and lowest at advanced stages except in red clover in 1960 when this trend was slightly reversed. In general, the two clovers contained higher levels of carbohydrates than did alfalfa or trefoil, especially from early bud through the green seed pod stages of maturity. Comparisons

Table I. Cutting Dates, Plant Heights in Inches, and Yields in Pounds per Acre of Alfalfa, Red Clover, Ladino Clover, and Birdsfoot Trefoil during 1960 and 1961

Stage of Growth	Year	Average Date	Alfalfa		Red Clover		Ladino Clover		Birdsfoot Trefoil	
			Height	Yield	Height	Yield	Height	Yield	Height	Yield
Succulent	1960	4/27	6.6	833	4.2	817	3.9	844	...[a]	...[a]
	1961	5/10	6.5	1145	3.9	851	2.4	176	...[a]	...[a]
	Av.		6.6	989	4.1	834	3.2	510		
Prebud	1960	5/11	11.7	1581	6.8	1906	7.4	1672	...[a]	...[a]
	1961	5/17	12.3	1790	6.6	1517	4.0	506	4.0	579
	Av.		12.0	1686	6.7	1712	5.7	1089		
Early bud	1960	5/24	20.5	2841	16.5	3064	13.2	2713	6.7	521
	1961	6/5	28.3	4126	16.2	3414	5.9	935	10.6	1733
	Av.		24.4	3484	16.4	3239	9.6	1824	8.7	1127
$^1/_{10}$ Bloom	1960	6/13	32.1	3913	30.3	5423	14.7	2975	14.5	1695
	1961	6/12	34.2	5098	22.3	4253	7.2	1205	14.4	2684
	Av.		33.2	4506	26.3	4838	11.0	2090	14.5	2172
Full Bloom	1960	6/27	44.0	6289	40.5	6441	15.2	3654	23.2	3245
	1961	6/26	36.7	6293	25.1	5358	9.6	2363	19.0	4984
	Av.		40.4	6291	32.8	5900	12.4	3009	21.1	4115
Green Seed Pod	1960	7/11	42.8	6017	37.0	6820	14.3	3654	26.3	4546
	1961	7/10	38.1	6565	24.5	5570	7.9	2463	20.0	5750
		Av.	40.5	6291	30.8	6195	11.1	3059	23.2	5148

[a] Growth was not sufficient to sample.

among species were more difficult to make at the first two stages, probably because growing conditions varied more widely during these periods. Levels of total nonstructural carbohydrates in all species generally fluctuated more widely and seemed less related to stage of maturity in 1961 than in 1960.

Amounts of total nonstructural carbohydrates extracted by 2% H_2SO_4 generally were slightly higher than those extracted by the enzyme method, especially at the later stages of maturity. Smaller amounts of carbohydrates were extracted by 2% H_2SO_4 than by the enzyme method from Ladino clover at the succulent stage and from birdsfoot trefoil at the early bud stage in 1960, and from Ladino clover at the succulent and pre-bud stages in 1961.

Glucose, Fructose, and Sucrose. The results of anthrone determinations of chromatographically separated glucose, fructose, and sucrose are shown in Figure 2. Percentages of glucose and fructose were similar and generally were lower than those of sucrose in alfalfa at all stages of maturity during both years. Percentages of sucrose generally were highest at early maturity stages during both years.

Percentages of glucose and fructose were similar in red clover at early maturity stages but percentages of glucose consistently were higher at the three most advanced stages during both years. Percentages of sucrose varied little in 1960 but diverged widely in 1961. Results for the three sugars varied more in red clover in the two years than in any of the other species.

Percentages of glucose were lower than those of fructose in Ladino clover at succulent and prebud stages in 1960 and at succulent, pre-bud and early bud stages in 1961 but were higher at advanced maturity stages during both years. In contrast to alfalfa, percentages of glucose and of fructose generally were higher than those of sucrose.

Percentages of glucose were lower than those of fructose in trefoil at all stages of maturity during both years. Percentages of sucrose, glucose, and fructose generally were similar, and there was little variation in relation to advance in maturity during either of the two years.

Discussion

Dry matter yields generally increased with each successive advance in maturity, as was expected. However, alfalfa, red clover, and Ladino clover showed little or no increase between full bloom and green seed pod stages. These results, coupled with generally decreasing percentages of the various carbohydrates, diminished the readily available energy yield per acre at late maturities. Dry matter yields of trefoil not only increased substantially from full bloom to green seed pod stages but percentage of the various carbohydrate fractions decreased only slightly. The added yields of total nonstructural carbohydrates plus the tendency of trefoil to remain partially vegetative longer resulted in less deterioration in energy value of this species at the most advanced maturity stage.

Some of the percentage values for total nonstructural carbohydrates and sugar fractions found in this study are higher than many reported in the literature. The reduction of respiratory losses of carbohydrates and the improved extractability of freeze-dried herbage are considered to be largely responsible. Recently, Burns, Noller, and Rhykerd (*2*) reported large differences in the recoveries of "soluble carbohydrates" from freeze-dried *vs.* oven-dried plant tissues. Raguse and Smith (*9*) also compared the effects of various drying temperatures *vs.* freeze-drying on the carbohydrate constituents of alfalfa herbage. Their results indicated that freeze-dried herbage yielded larger amounts of total nonstructural carbohydrates, although not to the extent reported by Burns, Noller, and Rhykerd (*2*). The rapidity and efficiency of oven drying and the method subsequently used for extraction may markedly influence the final results.

Percentages of total nonstructural carbohydrates in trefoil were higher than in alfalfa at early maturity and

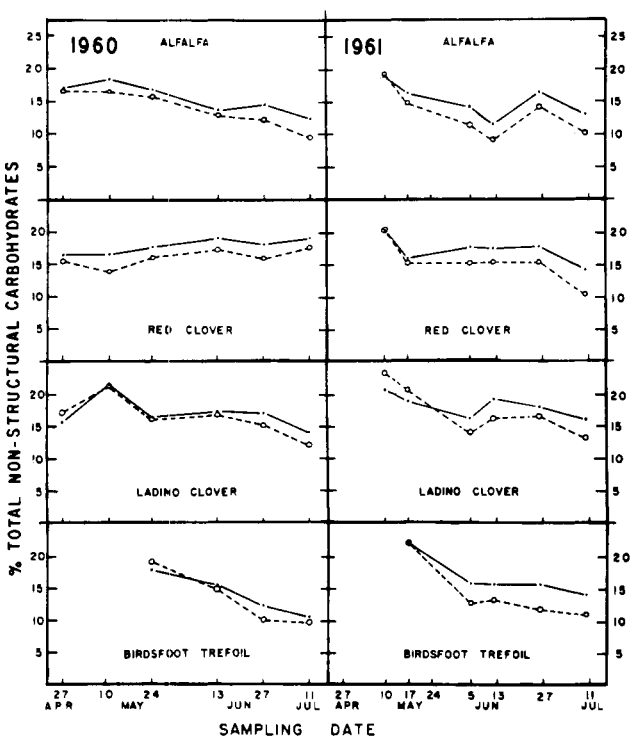

Figure 1. Trends of total nonstructural carbohydrates

Per cent of dry weight in the herbage of four forage legumes with advance in maturity. Extractions performed with takadiastase enzyme (O---O---O) and with hot 2% H_2SO_4 (·—·—·)

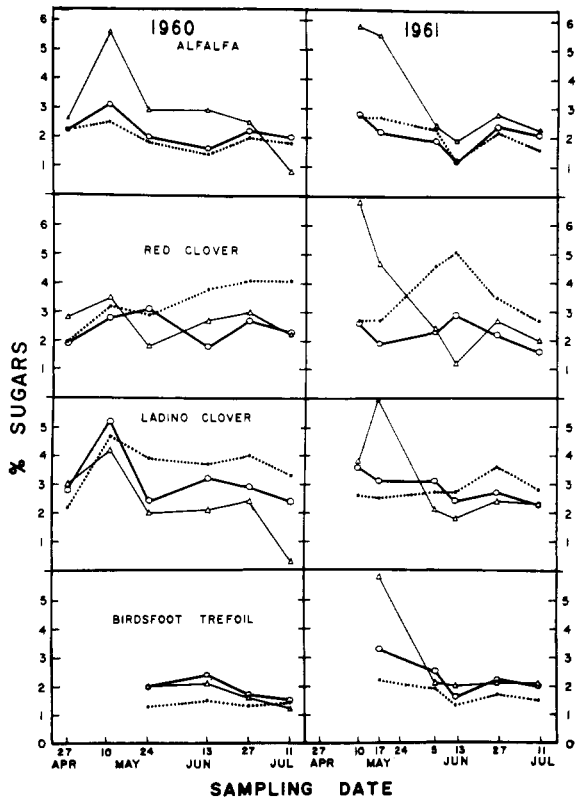

Figure 2. Trends of glucose (●····●····●), fructose (O—O—O), and sucrose (△—△—△)

Per cent of dry weight in the herbage of four forage legumes with advance in maturity

somewhat similar at late maturity. Trends of sugars in trefoil showed little change from early to later maturity and were much less responsive to environmental conditions. These results seem to fit well with the observations of Smith (*10*) that cyclic trends of carbohydrates readily available as energy to the plant in the roots of trefoil were much less pronounced than in the roots of alfalfa and red clover.

The closest agreement between values for total nonstructural carbohydrates from the two extraction methods occurred for samples harvested at early stages of maturity. In some instances at the early stages, smaller amounts of carbohydrates were extracted by 2% H_2SO_4 than by the enzyme method. The effect, limited to early maturity stages and most pronounced in Ladino clover, may have been due to the destruction of reducing substances in these extracts. In addition, 2% H_2SO_4 may have extracted variable amounts of hemicellulose, depending on the species and maturity stage of the sample. A superficial investigation of some 2% H_2SO_4 extracts by qualitative paper chromatography suggested that pentoses were being extracted. These would be included in carbohydrate determinations as reducing sugars. No attempt was made to determine amounts of these sugars, so the importance of this consideration remains uncertain. The fact that freeze-dried tissue was used must again be stressed since this may be of considerable importance in facilitating a more complete extraction of oligosaccharides, dextrins, or other carbohydrates that otherwise might be occluded within structural material or rendered less extractable in other ways by oven drying.

The interrelationships of percentages of glucose, fructose, and sucrose generally were not consistent with advance in maturity, particularly when the results of one year were compared with those of the other. Trefoil was a notable exception in that percentages of glucose consistently were lower than those of fructose during both years. In Ladino clover, levels of glucose during both years were lower than those of fructose during early stages of maturity with a reversal of that situation during later maturity stages. Levels of sucrose appeared to respond more readily than those of glucose or fructose to seasonal environmental differences and often varied more extremely over the range of maturity stages.

Determinations of total nonstructural carbohydrates and sugar fractions were made on whole-plant samples. The results, therefore, are most meaningful in relation to the use of legume forages as silage or in green feeding. The importance of fermentable carbohydrates in satisfactory silage production usually is emphasized. Stage of maturity may be important. Lanigan and Catchpoole (*6*) noted in laboratory studies with ryegrass and white clover that the proportion of legume could be increased with later maturity stages. The generally higher levels of nonstructural carbohydrates in red and Ladino clovers may be of importance to successful silage-making since they are readily fermentable.

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SEED MEAL HYDROLYSIS

Variation in Enzymatic Degradation Products from the Major Thioglucosides in *Crambe abyssinica* and *Brassica napus* Seed Meals

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A recent review by Kjaer (*9*), indicated that the thioglucosides of Cruciferae and related plant families generally yield isothiocyanates upon enzymatic hydrolysis. Oxazolidinethiones (goitrins) are formed if the thioglucoside contains a hydroxyl group properly located in the aglycon to facilitate ring closure. References are given concerning the occasional formation of nitrile plus sulfur or thiocyanate instead of the isothiocyanate. Glucose and acid sulfate ion are also released from thioglucosides (*11, 13*), but these products are not often mentioned because they do not vary with the nature of the thioglucoside.

Figure 1 shows the major thioglucosides from *Crambe abyssinica* and *Brassica napus* seeds, and the known products derived from the aglycon (excluding HSO_4^- which is always present). Daxenbichler, VanEtten, and Wolff (*3*) identified the principal thioglucoside of crambe as *epi*-progoitrin (I). (R)-goitrin (II), enzymatically derived from this thioglucoside (*3*), is the enantiomer of (S)-goitrin (II) derived from progoitrin (I) of *B. napus* (*10*). Later Daxenbichler, VanEtten and Wolff (*4*), reported the formation of (S)-1-cyano-2-hydroxy-3-butene (III) when isolated *epi*-progoitrin was hydrolyzed by white mustard myrosinase at pH 3; the (R) enantiomer was formed from progoitrin.

The variation in the kind and amount of aglycon products (other than HSO_4^-) derived from enzymatic hydrolysis of the thioglucosides when the seed meals of crambe and of *Brassica napus* (rapeseed) are autolyzed under various conditions is reported.