Changes in Carbohydrate and Nitrogen Concentrations During Storage of Heat- and Freeze-Dried Alfalfa Root Tissue

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Alfalfa root tissue that had been freeze-dried or oven-dried was stored at several temperatures and sampled periodically. Changes in carbohydrate fractions during storage were consistent for all treatments. Percentages of reducing and nonreducing (sucrose) sugars increased during storage, while starch decreased. However, the total of carbohydrate fractions remained nearly constant during storage. Total free amino acids increased during storage, although aspartic acid decreased. Glutamine and asparagine both increased markedly. Kjeldahl nitrogen decreased slightly during storage. In all cases, there was more variability due to method of drying and length of storage than to method of storage. This indicates the desirability for immediate analysis of dried tissue or compensation for the interconversion.

Plant tissue is usually dried and stored for a period of time before analysis for constituents due to time or economic reasons instead of immediately analyzing the fresh material. It is thus necessary to use a means of preservation that will permit minimal changes during drying and storage. Heat-drying has been widely used due to its simplicity, but evidence has indicated that analyses of freeze-dried tissue compare more favorably with those from fresh tissue (Laidlaw and Wylam, 1952). Raguse and Smith (1965) compared heat-drying methods and found that oven drying at 100° C for 1 hr and then at 70° C to dryness compared most favorably with composition of freeze-dried tissue.

Storage conditions for dried tissue may also be very important, especially since freeze-drying does not inactivate many of the enzymes present. Even with heat-drying, enzymes may not be inactivated. Blackman and Templeman (1940) found that rapid drying of white clover (*Trifolium repens* L.) and ryegrass leaves (*Lolium perenne* L.) at 95° C did not inactivate sucrose and starch-hydrolyzing enzymes. They hypothesized that the sample temperature did not reach 95° C because of the short period of rapid water loss and that the dehydrated enzymes were then more thermostable.

Several methods of preservation of ryegrass tissue were compared by Collins and Shorland (1945). They reported that tissue dried rapidly by heat (20 min at 120° C) was similar in carbohydrate composition to tissue treated with boiling alcohol. However, large increases in free sugars were observed in the heat-dried samples after 36 days of storage at room temperature.

Laidlaw and Wylam (1952) reported erratic interconversations within the water-soluble carbohydrate fraction when freeze-dried ryegrass herbage tissue was stored at 0° C or at room temperature. Perkins (1961) observed changes in chemical composition during storage of freeze-dried wheat (*Triticum aestivum* L.) leaves at room temperature and of fresh-frozen wheat leaves at −20° C. He found changes in both free sugars and free amino acids were similar under either type of storage. Sucrose in some varieties decreased to less than 5% of the original value during 12 months of storage. Free amino acid content nearly doubled during the first 3 months, but asparagine decreased. Also, changes in carbohydrate composition were not consistent among varieties.

The current study was devised to ascertain the effect of storage conditions on changes in the concentrations of some carbohydrate and nitrogen constituents following heat- and freeze-drying of alfalfa root tissue.

MATERIALS AND METHODS

Plants of alfalfa (*Medicago sativa* L. cultivar Vernal) were removed from the field on May 14, 1965, when they were in the prebud stage (20–25 cm tall) of growth. Roots were severed at the cotyledonary node and then cut to 15 cm in length. The root tissue was torn lengthwise and cut crosswise into small pieces to facilitate drying. The pieces were thoroughly mixed. About half of the tissue was dried on cheesecloth at 100° C for 1 hr and then at 70° C to dryness (35 hr) in forced-draft ovens. The remainder of the tissue was quick frozen in chipped dry ice and stored in a freezer at −16° C until the dry ice had sublimed. Drying was accomplished in a freeze-dryer at 0.1 mm vacuum and at a condenser temperature of −45° C. Drying was complete (<5% moisture) in 36 hr.

Both samples were ground to 40-mesh size, thoroughly mixed, and subsampled into glass bottles. Heat-dried subsamples were tightly capped and stored at room temperature (*ca.* 27° C), at −2° C, and at −16° C. The freeze-dried subsamples were stored uncapped over P_2O_5 and under vacuum in desiccators at the same temperatures. In addition, one freeze-dried subsample was stored at −16° C in a tightly capped bottle that was not placed in a desiccator.

Triplicate analyses for dry matter, total nitrogen, reducing sugars, total sugars, and starch were performed on the main samples immediately after grinding. Duplicate analyses of these constituents were made on each subsample following 3 and 6 months of storage. Duplicate determinations for free amino acids and amides were also made on the freeze-dried tissue following grinding. Single determinations were made on each of the freeze-dried subsamples after 3 and 6 months storage.

Vernal alfalfa roots were also harvested as described above

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Table I. Changes in Percentage of Dry Matter and of Carbohydrate Fractions due to Length of Storage of Freeze-Dried and Oven-Dried Alfalfa Root Tissue Harvested May 14, 1965

Constituents	Storage temperature and months storage								
	0 mo	25° C[a]		−2° C[a]		−16° C[a]		−16° C[b]	
		3 mo	6 mo	3 mo	6 mo	3 mo	6 mo	3 mo	6 mo
Freeze-dried tissue									
Dry matter	95.4	98.7	99.2	98.4	98.9	97.0	98.0	95.6	96.0
Reducing sugars	0.4	0.4	0.6	0.5	0.6	0.5	0.6	0.5	0.6
Sucrose	6.6	7.2	7.3	6.9	7.3	7.0	7.4	6.9	7.5
Starch	3.1	2.9	2.2	3.4	2.1	2.8	2.4	3.1	2.5
Total	10.1	10.5	10.1	10.8	10.0	10.3	10.4	10.5	10.6
Oven-dried tissue									
Dry matter	96.9	96.2	95.4	96.2	96.6	97.9	97.9		
Reducing sugars	0.5	0.5	0.6	0.5	0.6	0.5	0.6		
Sucrose	6.5	6.9	6.8	6.8	6.8	6.7	6.8		
Starch	2.4	2.0	1.5	2.2	1.5	1.8	1.6		
Total	9.4	9.4	8.9	9.5	8.9	9.0	9.0		

[a] Freeze-dried tissue stored in uncapped bottles in a vacuum desiccator over P_2O_5. Heat-dried tissue stored in capped bottles. [b] Stored in capped bottle without desiccator.

on April 6, 1965, just as spring growth was initiated. Before drying, the taproots were separated into the wood and bark fractions. Outer bark was peeled from the inner wood tissue at the cambium layer. The inner wood tissue constituted 55% of the taproot weight. The samples were then freeze-dried and ground as described above. Carbohydrate fractions were determined on duplicate samples after 1 and 9 months of storage at −16° C in a vacuum desiccator over P_2O_5.

Two root samples (upper 15 cm) of Vernal alfalfa were also harvested on September 20, 1965, when the plants were in the vegetative stage (15–18 cm tall). Part of the tissue was heat-dried and part was freeze-dried as described above. The dried samples were ground to 40-mesh size. Freeze-dried subsamples were stored in open glass bottles in vacuum desiccators over P_2O_5 held at room temperature and at −16° C. The heat-dried sample was stored at room temperature in a tightly-capped bottle. Duplicate analyses for dry matter and carbohydrate fractions were made following grinding and after 3.5 months of storage.

Dry matter was determined through weight loss by drying (*ca.* 500 mg) at 98° C with 25 mm of pressure for 4 hr. All subsequent analyses were corrected to 100% dry matter. The Kjeldahl method, with adaptations by Hiller *et al.* (1948), was used to determine total nitrogen on 500-mg samples.

Carbohydrate fractionations were made on 500-mg samples in a manner similar to that described by Raguse and Smith (1965). Reducing sugars and sucrose were extracted with hot 80% ethanol. Sucrose was determined by the increase in reducing power following acid hydrolysis. Starch was removed and hydrolyzed from the residue left after alcohol extraction with commercial takadiastase (Clarase 900, Miles Laboratory) as described by Smith (1969). Chromatographic analysis of the carbohydrate removed from the residue by the enzyme showed glucose to be the major (>95%) component. Reducing power of all solutions was determined by the copper reduction–iodine titration method according to Heinze and Murneek (1940). Carbohydrate fraction data were expressed as percentage of corrected dry weight.

Free amino acids were extracted with acetone–water (1:1) from the freeze-dried tissue according to the procedure of Wilding *et al.* (1960). The extract was deproteinized with picric acid, as outlined by Stein and Moore (1954). Amino acids were separated with the long column of a Beckman–Spinco analyzer. Amides were determined by the increase in aspartic and glutamic acid following autoclaving for 30 min at 1.1 kg/cm² pressure in 10% HCl. Free amino acids and amides were expressed as mg/g of corrected dry weight.

RESULTS

Changes in percentages of dry matter and individual carbohydrate fractions during storage of alfalfa root tissue harvested on May 14 are shown in Table I. The freeze-dried tissue in desiccators over P_2O_5 lost moisture at different rates, dependent upon the storage temperatures. Tissue stored at room temperature lost the most moisture while tissue stored at −16° C lost the least. The oven-dried tissue stored in capped bottles changed very little in moisture content.

Changes in individual carbohydrate fractions during storage were similar for all treatments. Percentages of reducing and nonreducing sugars increased during storage, while percentages of starch decreased. However, the total of all carbohydrate fractions remained relatively constant. Increase in sugar percentages during 6 months of storage was greater in the freeze-dried tissue (*ca.* 1.0%) than in the oven-dried tissue (*ca.* 0.4%). Starch decrease was similar for both types of drying on an absolute change basis (*ca.* 1.0%). However, on a percentage basis, the decrease was greater in the oven-dried tissue (35%) than in the freeze-dried tissue (26%).

Similar changes during storage were observed in the carbohydrate fractions of root tissue harvested on September 20 (Table II). During 3.5 months of storage, concentrations of sucrose plus reducing sugars in freeze-dried tissue increased 13%, while in oven-dried tissue they increased 7%. At the same time, starch decreased 11 and 12% in the oven-dried and freeze-dried tissue, respectively.

Composition of freeze-dried root tissue harvested on April 6, 1965, and analyzed following 1 and 9 months storage at −16° C is shown in Table III. Samples of the wood portion and the bark portion increased in sucrose plus reducing sugars 19 and 18%, respectively, and decreased in starch 21 and 23%, respectively. This indicates that both sites of carbohydrate accumulation (Ueno and Smith, 1970) change in composition during storage in a similar manner. Although these data were not corrected for moisture changes, the cor-

Table II. Influence of Drying Method and Storage Temperature on Changes in Percentage of Dry Matter and Percentage of Carbohydrate Fractions of Alfalfa Root Tissue Harvested September 20, 1965

	Storage temperature and months storage				
	Oven-dry[a]		Freeze-dry[b]		
	27° C		27° C		−16° C
Constituent	0 mo	3.5 mo	0 mo	3.5 mo	3.5 mo
Dry matter	96.2	95.6	95.9	99.7	98.7
Reducing sugars	1.5	1.6	0.4	0.5	0.5
Sucrose	5.7	6.1	5.2	5.9	5.8
Starch	4.6	4.1	8.2	7.1	7.3
Total	11.8	11.8	13.8	13.5	13.6

[a] Stored in capped bottles. [b] Stored in vacuum desiccator over P_2O_5.

Table III. Influence of Storage at −16° C on Percentage of Carbohydrate Fractions of Freeze-Dried Alfalfa Root Samples Harvested April 6, 1965. Samples were Stored in Open Bottles in Vacuum Desiccator over P_2O_5

	Inner wood		Outer bark	
Carbohydrate fraction	1 Month	9 Month	1 Month	9 Month
Reducing sugars	0.2	0.4	0.4	0.5
Sucrose	14.4	17.0	14.2	16.8
Starch	13.2	10.4	9.9	7.8
Total	27.8	27.8	24.5	25.1

rection would likely be less than 3% and would not compensate for observed changes. The correction would decrease slightly the percentage change of sugar fractions, but would increase slightly the percentage change of starch.

Free amino acids in the freeze-dried tissue after grinding and following 3 and 6 months storage are shown in Table IV. Regardless of storage temperature or condition, most of the amino acids increased in concentration (mg/g) or changed erratically, except aspartic acid. The decrease in aspartic acid concentration was greatest in the tissue stored at −16° C and least in the tissue stored at room temperature. Asparagine increased during the first 3 months and then appeared to remain steady or decrease. Glutamine also increased during the first 3 months and then decreased.

Table V. Effect of Storage Time at Different Temperatures on Kjeldahl Nitrogen. Data Presented as Percent N on Dry Weight Basis

	Time of storage (months)		
	0	3	6
Freeze-dried[a]			
Stored at 27° C	1.965	1.926	1.819
Stored at −2° C		1.952	1.901
Stored at −16° C		1.956	1.908
Stored at −16° C[b]		1.963	1.911
Oven-dried[c]			
Stored at 27° C	2.006	1.957	1.921
Stored at −2° C		2.036	1.968
Stored at −16° C		1.974	1.954

[a] Stored in uncapped bottles in vacuum desiccator over P_2O_5. [b] Stored in capped bottle without desiccator. [c] Stored in capped bottles.

Total nitrogen decreased slightly during storage under all conditions (Table V). This indicated that protein or nitrogen compounds were being broken down and probably lost due to volatilization.

DISCUSSION

Moisture loss was expected in the freeze-dried tissues since they were stored in vacuum desiccators over P_2O_5. The amount of moisture lost depended on the storage temperature. Tissues stored at room temperature dried faster than those stored at −16° C, while tissue stored at −2° C was intermediate. Moisture changes in the capped bottles were probably due to vapor pressure differences in the storage environment. Vapor pressure generally was higher at room temperature than in the freezers. Although the samples were tightly capped with plastic screw caps that had paper liners, slight amounts of water vapor exchange apparently occurred.

Table IV. Effect of Length of Storage at Different Temperatures on the Free Amino Acid Composition of Freeze-Dried Alfalfa Roots. Results are Expressed as mg/g of Dry Samples. Samples Harvested May 14, 1965

		25° C[a]		−2° C[a]		−16° C[a]		−16° C[b]	
	0	3 mo	6 mo	3 mo	6 mo	3 mo	6 mo	3 mo	6 mo
Aspartic acid	1.41	1.35	1.05	1.10	1.01	1.30	0.93	1.82	0.94
Threonine	0.43	0.45	0.49	0.36	0.52	0.44	0.54	0.42	0.55
Serine	0.59	0.56	0.64	0.51	0.64	0.57	0.62	0.60	0.63
Glutamic acid	0.78	0.74	0.90	0.70	0.91	0.77	0.91	0.81	0.90
Proline	0.69	0.87	0.78	0.85	0.57	0.79	0.71	0.81	0.80
Glycine	0.09	0.08	0.11	0.07	0.10	0.08	0.10	0.09	0.10
Alanine	0.36	0.34	0.46	0.33	0.44	0.36	0.46	0.37	0.43
Valine	0.14	0.14	0.21	0.10	0.21	0.15	0.21	0.17	0.19
Isoleucine	0.10	0.11	0.12	0.10	0.13	0.10	0.12	0.13	0.12
Leucine	0.08	0.09	0.11	0.09	0.11	0.10	0.11	0.09	0.09
Tyrosine	0.52	0.46	0.60	0.49	0.60	0.54	0.65	0.36	0.62
Phenylalanine	0.13	0.10	0.17	0.10	0.14	0.14	0.15	0.12	0.21
Subtotal	5.32	5.29	5.64	4.80	5.38	5.34	5.51	5.79	5.58
Asparagine	5.96	8.61	8.60	9.00	8.53	9.03	8.44	8.71	8.37
Glutamine	0.22	0.54	0.18	0.59	0.10	0.56	0.17	0.47	0.13
Subtotal	6.15	9.15	8.78	9.59	8.63	9.59	8.61	9.18	8.50
Total	11.47	14.44	14.42	14.39	14.01	14.93	14.12	14.97	14.08

[a] Stored in vacuum desiccator over P_2O_5. [b] Stored in capped bottle without desiccator.

The consistent increase in sugar fractions and decrease in starch during storage was not surprising. A conversion of starch to sugars should be expected as a normal physiological response to moisture and temperature stress (Jung and Smith, 1961) in the partially active tissue. Even though the tissue contained very little removable water, the proteins (enzymes) apparently were still hydrated sufficiently to maintain their activity. Since oven drying at a more extreme temperature than used in this study did not inactivate β-D-fructofuranosidase or starch-hydrolyzing enzymes (Blackman and Templeman, 1940; Collins and Shorland, 1945) it is not surprising that the oven-dried tissue in the current study showed a change similar to that of the freeze-dried sample but at a lower rate.

Free amino acids changed little or increased in concentration with storage time, except for aspartic acid, which decreased. The decrease in aspartic acid probably was associated with the increase in amide concentration. Perkins (1961) also reported an increase in amino acids during storage, but a decrease in asparagine. The reason for the lack of agreement concerning asparagine is unknown.

Several of the amino acid changes during storage were similar to those found by Wilding *et al.* (1960) during the cold-hardening of alfalfa root tissue. This also indicates that the plant tissue was responding to stress conditions. The large increase in amide concentration during the first 3 months of storage probably occurred because protein synthesis was stopped by the drying processes, but the incorporation of reduced nitrogen continued. This incorporation generally occurs through the amide fraction. In addition, protein breakdown was occurring which would add nitrogen to the metabolic pool to be used for amide synthesis.

It is therefore apparent that changes do occur in both freeze-dried and oven-dried samples over a wide range of storage temperatures. To alleviate this problem the assays probably should be performed as soon as possible after drying. Analysis of fresh material obviously is best and would also eliminate some of the problems associated with drying (Collins and Shorland, 1945; Laidlaw and Wylam, 1952; Raguse and Smith, 1965). A method of eliminating the problems that occur during drying and storage might be to immediately freeze the fresh tissue in the field with dry ice to rupture cells. Upon returning to the laboratory, the samples should be dropped into boiling ethanol (corrected to proper concentration) and ground in a blender. The hot ethanol would enter the ruptured cells readily stopping enzymatic activity (Collins and Shorland, 1945). The residue could be separated by filtration, and the residue and filtrate stored until analysis.

If fresh tissue is to be dried and stored, some of the tissue could be analyzed immediately and then repeated periodically to construct a "decay" curve. This would be acceptable for data correction, if rates of change were consistent for all tissue. However, Perkins (1961) reported different rates of change in carbohydrate fractions among varieties of wheat, and the current work indicates different rates of change among sampling times with tissue of the same variety. A difference among varieties would be suspected since they may respond differently to environmental conditions or may have been at different stages of physiological maturity. Physiological maturity would also be a factor within a variety since storage changes would be affected by enzyme, as well as substrate, concentrations which normally change during growth. These factors would limit the usefulness of the "decay" curve proposal.

Method of drying appears to have more effect on the subsequent changes during storage than does storage temperature or condition. This probably reflects the stability of the enzymes to the temperature effects before and during the drying process, since heat- and freeze-drying removed about the same amount of water.

This research substantiates the results of others (Blackman and Templeman, 1940; Laidlaw and Wylam, 1952; Perkins, 1961) that changes within tissue occur during storage; and the longer the storage time, the larger the changes are likely to be. Thus, analyses should be conducted as soon as possible after sampling the tissue. Under the same storage conditions, the greatest changes are likely to occur in freeze-dried tissue where the enzymes are functional, and are less likely to occur in heat-dried tissue. The least change will probably occur with storage in alcohol where the enzymes are denatured (Collins and Shorland, 1945).

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