[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL CHEMISTRY, UNIVERSITY OF WISCONSIN, AND THE OFFICE OF CEREAL INVESTIGATIONS, UNITED STATES DEPARTMENT OF AGRICULTURE]

THE EXTRACTION OF NITROGENOUS CONSTITUENTS FROM PLANT CELLS[1]

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Some investigators in plant metabolism seem to have overlooked the necessity for inhibition of autolytic changes in composition of the tissue, physiological interpretations having been based by them upon chemical analyses of material treated by various uncertain methods for preservation. In a recent publication[2] one of the present writers coöperated in demonstrating the possibility of desiccating certain plant tissues by heat under such conditions as to entail little or no disturbance of the original distribution of carbohydrates. These conditions could not, of course, be recommended for use in investigations of the nitrogenous constituents, without tests in relation thereto. However, incident to observations with spinach leaves, Osborne and Wakeman[3] concluded that rapid aeration of the fresh tissue at 60° probably accomplished desiccation without altering the distribution of protein constituents.

Pursuant to investigations of nitrogenous metabolism in leaves we found it desirable to accumulate preserved samples for subsequent chemical analysis. It also became important to determine the relative efficiency of various methods of extraction. Tests of these matters, here reported, have been based upon the two gross determinations of total nitrogenous compounds soluble in water and soluble nitrogenous compounds coagulable by boiling.

Preservative Treatment

A major difficulty encountered in the extraction of nitrogenous constituents of plant tissue relates to avoidance of coagulation of the soluble proteins. Because of their precipitating effect in this respect, the use of alcohol and freezing treatment as means of preservation are inapplicable.

Table I shows the effects of immediate packing in a freezing mixture and of slow desiccation by heat upon the recovery of water-soluble nitrogenous compounds from leaves of the sugar mangold. Extraction was accomplished by thoroughly macerating 100 g. of the blade tissue with fine spherical sand in a mortar, with the addition of a little ether, filtering on paper pulp and washing with water to a volume of 2 liters. In the case

[1] Published with the permission of the Director of the Wisconsin Agricultural Experimental Station, and the Chief of the Office of Cereal Investigations, U. S. Department of Agriculture.

[2] Link and Tottingham, THIS JOURNAL, **45**, 441 (1923).

[3] Osborne and Wakeman, *J. Biol. Chem.*, **42**, 1 (1920).

of this particular tissue, such treatment removed all but traces of the α-amino nitrogen. The results show that these methods do not preserve the original composition of the tissue. Freezing seems to have entailed chiefly the coagulation of soluble proteins, while desiccation at this low temperature has permitted extensive modification as to the amount and form of total soluble nitrogen.

TABLE I

RELATION OF PRESERVATIVE TREATMENT TO DISTRIBUTION OF SOLUBLE NITROGENOUS COMPOUNDS IN LEAVES OF THE SUGAR MANGOLD

Nitrogen in dry matter

Forms of nitrogen	Treatment of tissue: Extracted fresh %	Dried at 40° without aeration %	Frozen by ice-salt mixture %
Total soluble	4.0	2.2	3.3
Soluble protein	3.4	0.5	2.3

Results from tests of the desiccation effects at various temperatures and with different rates of aeration are presented in Table II. Rapid aeration was provided by the equipment already described,[4] while slow aeration was accomplished by similar exposure of the samples in drawers, with bottoms of wire netting, of a heated cabinet with an air current faintly perceptible to the moistened skin.

TABLE II

EFFECTS OF DESICCATION BY HEAT UPON THE DISTRIBUTION OF NITROGENOUS COMPOUNDS IN LEAVES

	Nitrogen in Dry Matter										
		Treatment of tissue									
		Extracted fresh		Dried at 55–60° slow aeration		Dried at 55–60° rapid aeration		Dried at 65–66° rapid aeration		Dried at 70–72° rapid aeration	
Species of plant	Total %	Tot. Sol. %	Sol. Prot. %	Tot. Sol. %	Sol. Prot. %	Tot. Sol. %	Sol. Prot. %	Tot. Sol. %	Sol. Prot. %	Tot. Sol. %	Sol. Prot. %
Barbery[a,b]	3.18	2.15	1.43	1.20	0.33	..	..	..	..	..	..
	2.60	0.43	0.15	..	..	0.30	0.06	..	..	..	..
Sugar beet	3.04	1.61	1.26	1.20	0.54	..	..	..	..	..	..
	2.82	1.81	1.39	1.28	0.67[c]	..	..	..	..	..	..
	2.30	1.50	1.09	..	..	1.30	0.65	..	..	..	..
	..	1.56	0.94	..	..	..	..	0.71	0.33	..	..
	..	4.07	3.46	..	..	..	..	1.16	0.52	..	..
	4.90	3.76	3.02	..	..	..	..	..	..	1.42	1.20

[a] This tissue presents abnormal conditions, due to high acidity.

[b] Sampled in August. Leaves dry and tough.

[c] Preliminary desiccation at 98°, to inhibit enzyme action.

These data indicate the difficulties, at least with these particular species of leaf, in preserving tissue for the *dissection* of the nitrogenous compounds. That the variations of both soluble and coagulable nitrogen in sugar-beet leaves dried with rapid aeration at about 65° are nearly equal, as compared with extracts from fresh material, indicates that the chief disturbance in this case is precipitation of protein. The same may be stated

[4] Ref. 2, p. 445.

of the barberry tissue dried at 55° to 60°. It seems hardly possible that a critical temperature exists between those tested here at which the soluble protein does not coagulate. The only alternative from direct extraction of fresh material, therefore, seems to be desiccation of amenable tissues at temperatures which allow only coagulation of the soluble protein, and recovery of the latter with that originally insoluble.

Methods of Extraction

In recovering the soluble constituents of plant cells it becomes important to compare the expressed sap with the extract obtained with water, giving special attention to possible disturbing effects of the latter treatment upon the distribution of solutes. The pressure applied here varied from mild to severe wringing by hand of the tissue, wrapped in cheese cloth, and subsequent use of a hand-operated screw press capable of developing a pressure of 150 kg. per sq. cm. Finally, the residue from the press was macerated with water in the usual manner. The sum of this series of extractions was therefore comparable with the usual direct watery extract of the tissues. Table III contains the results.

Table III

Distribution of Nitrogenous Constituents in Various Extracts of Sugar-Beet Leaves

			Method of extraction					
Moisture	Total nitrogen in dry matter		Hand wringing Portion I	Hand wringing Portion II	Mechanical pressure	Final maceration with water	Total preceding fractions	Direct maceration with water
%	%	Vol., cc.	31	19	..	..	..	..
84.2	3.10	Tot. N, %[a]	0.97	0.53	..	0.77	2.27	2.50
		Prot. N, %	.77	0.44	...	0.54	1.75	1.65
84.4	3.04	Vol., cc.	..	31[b]	24	..	..	..
		Tot. N, %	..	0.90	0.46	0.25	..	..
		Prot. N, %	..	.71	0.39	0.16	..	..
..	3.00	Vol., cc.	..	30[b]	20	..	..	..
		Tot N, %	..	0.50	0.25	1.01	1.76	2.17
82.1	3.20	Vol., cc.	..	..	55	..	..	..
		Tot. N, %	..	..	1.30	0.16	1.46	2.50
		Prot. N, %	..	..	1.02	0.07	1.09	2.10
..	4.91	Vol., cc.	..	..	40	..	..	..
		Tot. N, %	..	..	1.90	1.66	3.56	3.76
		Prot. N, %	..	..	1.60	1.34	2.94	3.02

[a] Based on dry matter of tissue.

[b] Hand extract complete.

Successive portions of the extracts obtained by pressure show decreasing concentration in all cases. It is therefore essential that complete removal of the sap be attained. This is far from possible, as indicated by comparison with the moisture values, under the conditions here applied. We had supposed it to be possible that direct application of water to the tissue

might, by dilution effects, alter the recovery of solutes, as compared with preliminary removal of the sap. As shown by the data, this anticipation was not realized. The recovery by direct extraction was somewhat greater than the sum of the fractional extractions. In view of this favorable showing of the method of watery extraction it appears to be suitable for use generally.

Comparison with Chibnall's Treatment

Chibnall[5] proposes to favor the isolation of cell proteins by previous removal of simpler solutes. This is accomplished by immersing the tissue in a cytolytic agent, followed by subjection to heavy pressure. We have compared the recovery of nitrogenous constituents by Chibnall's method, that is, in the watery extract from cells ground after subjection to pressure, with the method of direct extraction by grinding with water. The cytolyzed cells were treated in the mechanical press already mentioned. As a phase of the direct extraction the sap expressible from the crushed cells was analyzed separately from the subsequent recovery by water. Table IV presents the results.

TABLE IV

COMPARISON OF CHIBNALL'S TREATMENT WITH DIRECT EXTRACTION OF LEAF TISSUE

Plant species	Total nitrogen in dry matter %		Chibnall's extraction By pressure	By water	Total	Direct extraction By pressure	By water	Total
Barberry	3.18	Vol., cc.	64	..	..	52	..	..
		Tot. N, %	0.54	1.80	2.34	0.55	1.60	2.15
		Prot. N, %	.02	1.30	1.32	.13	1.30	1.43
	..	Tot. N, %	.07	0.16	0.23	.11	0.20	0.31
Sugar beet	3.00	Vol., cc.	70	..	..	50	..	..
		Tot. N, %	.57	1.60	2.17	0.75	1.01	1.76
	3.04	Vol., cc.	51	..	..	55	..	..
		Tot. N, %	.27	1.05	1.32	1.36	0.25	1.61
		Prot. N, %	.07	0.72	0.79	1.10	0.16	1.26
	3.20	Vol., cc.	42	..	..	55	..	..
		Tot. N, %	.26	0.67	0.93	1.30	0.16	1.46
		Prot. N, %	.07	.40	0.47	1.02	0.07	1.09

In the case of sugar beet leaves protein forms a considerable part of the nitrogenous extractives from unground cells in Chibnall's preliminary extract. This is probably due to rupturing of some of the cells under pressure. A rather large proportion of the nitrogen recovered by water in the second and chief extract is non-protein in character, equaling 40 to 50% of the total extracted. This is appreciably more than the non-protein proportion by direct extraction, where the latter formed 22 to 33% of the total. The reason for this difference cannot readily be ascribed

[5] Chibnall, *J. Biol. Chem.*, 55, 333 (1923).

to any other source than some modifying action of the cytolytic agent, which was ether. It will be noted that this effect does not appear in the case of the highly acid barberry tissue. We regard the data, as a whole, as favorable to the use of the direct method of extraction for these particular tissues.

Incident to the tests just described we have supplemented the watery extraction with Osborne's[3] application of alcoholic solution of 0.2% sodium hydroxide and with brief hydrolysis by 20.0% hydrochloric acid, as used by Hamilton and others.[6] In the case of leaf mesophyll tissue of the sugar beet, containing 3.04% of total nitrogen in the dry matter the recovery by extraction from the crushed cells was as follows: water-soluble, 1.32%; alkali-soluble, 0.18%; acid-soluble, 0.25%; total-soluble, 1.75%.

These data show that only a small portion of the exposed protein of the cell fails to dissolve in water, as indicated by the slight solvent effect of alcoholic alkali. Even the rather drastic acid treatment leaves about 40.0% of the cell nitrogen in the tissue. It appears probable that this residual portion is composed of nitrogenous constituents inclosed by cellulose material and relatively inert in metabolic processes.

Summary

This investigation deals with the efficiency of various methods of desiccation and extraction, as related to the proportions of total soluble nitrogenous compounds and soluble proteins in leaves of the barberry and sugar beet. Data are given which show the disturbing effect of freezing and of drying in still-air at 40° on the distribution of nitrogen in plant cells. Of three temperature ranges, 55–60°, 65–66° and 70–72°, with rapid aeration, the lower ones produced least alteration in the constituents determined. The effect at 65°, in the case of the sugar beet, is apparently limited to coagulation of some of the soluble protein. If this effect of desiccation is avoidable with these tissues a rather precise adjustment of temperature is required.

It is shown that successive fractions of expressed sap from sugar-beet leaves contain decreasing concentrations of nitrogenous extractives. The results indicate the desirability of extracting with water as general usage in partitioning the nitrogenous constituents of plant cells.

Comparison of Chibnall's method of extraction with direct watery extraction of crushed leaf cells shows that the former does not sharply separate non-protein from protein nitrogen. A larger proportion of the nitrogen recovered by direct extraction is coagulable than by Chibnall's method.

Supplementary use of weak alkaline extraction and rather severe acid hydrolysis demonstrate that water extracts the greater part of the proteins

[6] Hamilton, Nevens and Grindley, *J. Biol. Chem.*, **48**, 249 (1921).

exposed by crushing the cells of fresh tissue. From 40 to 50% of the nitrogenous constituents appears to be included within the cell structural material, and are therefore metabolically inert.

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THE SPECTROSCOPY OF THE SULFONATED INDIGOTINS

BY WALTER C. HOLMES

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The potassium salts of the four indigotin sulfonic acids have been investigated with the purpose of defining the correlation between their constitution and various aspects of their absorption and general behavior, and of obtaining data bearing upon their spectrophotometric identification and evaluation. The materials examined were pure dyes prepared by M. X. Sullivan of the Hygienic Laboratory, United States Public Health Service. Their absorptions were measured throughout the visible spectrum by means of a commercial wave-length spectrometer provided with a standard photometer with such provisions as would demonstrate the influence of a variety of factors.

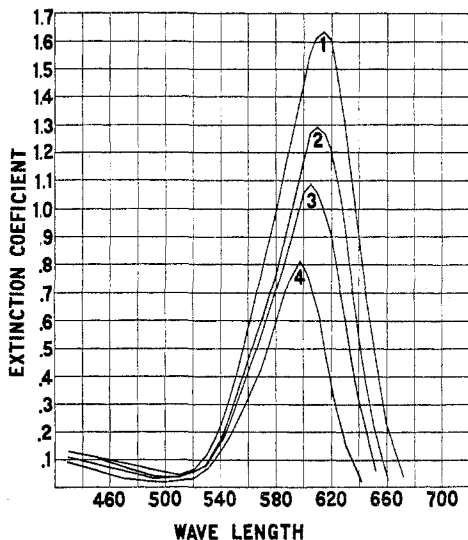

Fig. 1.—Potassium indigotin sulfonates. (Absorption of 40 parts of dye per million parts of 80% alcohol in a 1cm. cell.) (1) = Indigotin SO_3K; (2) = Indigotin $(SO_3K)_2$; (3) = Indigotin $(SO_3K)_3$; (4) = Indigotin $(SO_3K)_4$

The absorptions of the dyes in 80% alcohol are plotted in Fig. 1.

The dependence of the absorption upon constitution is clearly evident. With progressive sulfonation there occurs a displacement in the spectral location of the absorption maxima toward the violet together with a decrease in the intensities of absorption which is roughly proportional to the increase in molecular weights.

The absorptions of the dyes in distilled water are plotted in Fig. 2.

A relation between constitution and absorption similar to that noted in alcoholic solution is evident in the aqueous solutions of the di-, tri- and tetrasulfonic derivatives. The absorption of the monosulfonic derivative