(9) Parker, F. W., *Science*, **111**, 215 (1950).
(10) Posternak, T., *J. Biol. Chem.*, **188**, 317 (1951).
(11) Schoch, T. J., *J. Am. Chem. Soc.*, **64**, 2957 (1952).
(12) Schwimmer, S., J. Agr. Food Chem., **1**, 1063 (1953).
(13) Schwimmer, S., Bevenue, A., Weston, W. J., and Potter, A. L., *Ibid.*, **2**, 1300 (1954).
(14) Schwimmer, S., and Olcott, H. S., *J. Am. Chem. Soc.*, **75**, 4855 (1953).
(15) Umbreit, W. W., Burris, R. H., and Stauffer, J. F., "Manometric Techniques and Tissue Metabolism," Burgess Publishing Co., Minneapolis, 1949.
(16) Willigen, A. H. A. de, *Die Stärke*, **3**, 147, *Food Science Abs.*, **25**, 186 (1953).
(17) Yang, F., and Dju, M. Y., *Chinese J. Physiol.*, **14**, 473 (1939).

Received for review October 26, 1954. Accepted December 20, 1954.

PLANT TISSUE ANALYSES

Test for Pectin Based on Reaction of Hydroxamic Acids with Ferric Ion

R. M. McCREADY and R. M. REEVE
Western Utilization Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Albany 6, Calif.

The reaction of the ester groups in pectin with aqueous alkaline hydroxylamine at room temperature for 2 minutes produces hydroxamic acids. Water-insoluble, red-colored complexes are formed upon subsequent addition of ferric ion. These reactions serve satisfactorily as a histochemical test for pectin in plant tissues and as a qualitative test for pectic substances. The test seems to be specific for pectin under the conditions recommended.

Carboxylic acid derivatives, such as esters, lactones, anhydrides, amides, nitriles, and other compounds, react with hydroxylamine to form hydroxamic acids which give colored complexes with ferric ion (*8–11*). Acyl phosphates (*15*) and acetylcholine (*9*) have been determined quantitatively by this reaction. Anhydrides, lactones, and esters react with aqueous alkaline hydroxylamine at room temperature and can be detected by paper chromatography (*1*, *23*). Other compounds, such as amides, nitriles, and fatty acid esters, must be heated with alkaline hydroxylamine in high-boiling organic solvents before the reaction occurs (*10*, *11*, *19*).

Pectin esters react with hydroxylamine in aqueous alkali at room temperature, at a more rapid rate than de-esterification, and on addition of ferric ion an insoluble colored ferric–hydroxamic acid complex is formed.

Reagents and Procedure

The reagents are those used by Hestrin (*9*) for quantitative determinations of acetylcholine.

Hydroxylamine, 13.9 grams dissolved in 100 ml. of water.

Sodium hydroxide, 14.0 grams dissolved in 100 ml. of water.

Hydrochloric acid solution, 1 volume of concentrated hydrochloric acid reagent (specific gravity 1.18) diluted with 2 volumes of water.

Ferric chloride reagent, 10 grams of ferric chloride hexahydrate dissolved in 100 ml. of 0.1*N* hydrochloric acid.

To about 0.005 gram of the test substances suspended or dissolved in 1 ml. of water is added accurately 1 ml. of the hydroxylamine reagent, then 1 ml. of the sodium hydroxide solution. The reactants are allowed to stand for 2 minutes; then 1 ml. of the diluted hydrochloric acid is added, followed by 1 ml. of the ferric chloride reagent. A red precipitate is produced in the positive tests. A control is obtained by adding the test substances to the reactants after the addition of the hydrochloric acid, because the reaction with esters to form hydroxamic acids does not proceed in strongly acid solution.

Experimental

Application to Sections of Plant Tissues. Fresh fruit and vegetable tissues were sectioned serially on a sliding microtome at thicknesses (80 to 520 microns) dependent on their structure and cell size. All freshly cut sections were placed immediately in water containing about 300 p.p.m. of dissolved sulfur dioxide. All sections were tested in dishes of 10-ml. capacity, and the amount of each reagent was doubled in order to ensure complete coverage and suspension of the section. The containers were swirled after addition of each reagent to provide thorough mixing.

Positive color production was immediate following the addition of the ferric ion and color intensity was fully developed within 1 minute. The sections were then transferred to water and excess ferric chloride and salts were washed out before further examination.

Examinations at oil immersion magnifications (ca. 1200x) were most readily made after tested sections had been vacuum infiltrated with water to remove the profuse gas bubbles evolved during the test reaction. Transverse cuts of adjacent cell walls (at the section surface) showed that localization of the color complex was confined to the so called "compound middle lamella" (*7*).

Selectivity for Pectin and Color Reflection Measurements. The selectivity of the color test for pectin was determined as follows: Fresh sections of young Meyer lemon, ripe apple, and potato were tested immediately and some were de-esterified at room temperature for 16 hours in purified orange pectinesterase (*16*) solution at pH 5.5. Other sections were soaked for 30 minutes in 10% aqueous sodium hydroxide at room temperature. Some of the enzymatically de-esterified sections were dehydrated in absolute methanol and then re-esterified for 2 hours in 0.5*N* hydrochloric acid in absolute methanol at room temperature. These conditions were suggested by results of esterification of galacturonides by Jansen and Jang

(*12*). Alkali-treated sections were re-esterified by the same method, after thorough washing in water and dehydration with methanol. All treated sections were then tested, as previously described, and controls were run for each kind of treatment.

Color intensities were quantitatively evaluated directly on the washed sections. Reflection density was measured with a Photovolt reflectance and densitometer unit (No. 5013). With this instrument a beam of light about 3 mm. in diameter is directed at an angle on the specimen and the scattered light is reflected from the specimen through a filter to the photocell. Values so obtained are conveniently expressed as reflection density units (defined as log R_o/R_t, where R_o is the reflectance of a white surface, such as blank section, and R_t is the reflectance of a colored section). A 530-mμ filter was chosen because the absorption maxima of these colored complexes formed with esters in aqueous media were in the range of 520 to 540 mμ (*9*).

Reflection densities were measured on sections mounted either between a microslide and cover glass or between two cover glasses. Before the top cover glass was adjusted in position, one drop of water was added to the section to ensure a uniform section surface free from large, external air bubbles. All sections of each kind of tissue and of given thickness were kept essentially in the serial order in which they had been cut, to ensure uniform sampling.

Quantitative comparisons of the reflection densities in both fresh and variously treated sections are listed in Table I, where each value is an average obtained from four to six readings taken at different section areas of a single section. The readings varied about ±4% from the average. Care was taken to avoid areas which showed localized reactions of unusually great intensity, as in the skin and vascular tissues.

Comparisons of the values for the sections of apple tissue indicate that thicknesses of section from 300 to 500 microns gave about the same reflection densities. Parenchyma cells in these apples average about 200 microns in diameter and the intercellular spaces are very large (*18*). The young Meyer lemon rind showed darker colorations with increasing thickness of section. In this tissue the cells were much smaller (20 to 40 microns) and intercellular spaces were small because the cells had not yet developed the characteristic arms or branches of mature citrus rind.

The applicability of the test for quantitative determination of either the degree of esterification of pectin or the amount of pectin in situ is under investigation. Because the same color intensity can be given by varied amounts of pectin with different degrees of esterification, it would be necessary to know the anhydrouronic acid content or to esterify all of the carboxyl groups.

Alkali completely de-esterified the pectic substances in the apple tissue, while the enzymatic treatment did not. Pectinesterase is specific for the methyl ester groups of pectin (*16*), although some of these groups cannot be so hydrolyzed. Re-esterification of the enzymatically de-esterified sections restored a large portion (60 to 70%) of the original reactivity. Restoration of color reactivity in the alkali-treated sections was less than 50% because some of the pectin was solubilized and lost from the sections as a result of these treatments.

Young raspberry sections reacted strongly positive. A soluble red color produced in the reaction was washed out with water and the cell wall regions of the tissue remained darkly stained. De-esterified sections of tissue tested in the presence of glucose pentaacetate were negative after the soluble red color had been washed out. There is no evidence that cell wall material adsorbs soluble red colored materials to give a false positive test.

Verification of Specificity. A number of natural polymers, sugars and sugar derivatives, and other compounds were tested in solution or in suspension to verify the specity of the test for esterified pectin. Five milligrams of the test substance were mixed or dissolved in 1 ml. of water and tested as described (Table II).

The color reactivities obtained with these natural polymers and other substances show the specificity of the hydroxamic acid–ferric ion reaction for pectin esters with the exceptions noted. Of the natural polymers tested, only esterified pectins gave positive reactions in which the colored complexes formed were insoluble. A methyl glycoside of polygalacturonide methyl ester (*2*) with a molecular weight of about 6000 (*13*) gave a soluble dark red color. Colored complexes formed by other reacting substances, with the exceptions of some phenolics, were soluble. Most of the substances gave no color reaction with the test.

Slight color reactions (pink to red) were obtained when the pectic acid samples were not dissolved before testing. These pectic acids were known to contain about 0.5% of residual ester groups; when they were tested in solution, no color reaction was obtained. Iridophycin, a sulfuric acid ester of galactan from *Iridophycus flaccidum* (*22*), did not give a positive test under these reaction conditions.

The sensitivity of the test is in the range of 500 γ of pectin per ml. of solution tested. Below 500 γ no insoluble colored ferric–hydroxamic acid complex is produced.

Discussion

Qualitative chemical tests for the identification of pectins in solution or in mixture, such as the naphthoresorcinol (*21*) and carbazole (*5*) tests, have not been found suitable for histochemical localization of pectin in plant tissues. Dye-staining reactions, on the other hand, lack specificity and the natures of their reactions are often unknown. As a result, approaches to a study of the pectic substances within plant tissue have often been complicated (*3*, *14*, *20*).

Thorium nitrate (*4*) and neutral lead

Table I. Reflection Densities of Treated and Untreated Sections of Apple, Lemon, and Potato Tissues Tested for Pectin in situ

Tissue	Section Thickness, Microns	Reflection Density[a]: Untreated Sections vs. Blank at 0		Treated Sections vs. Blank at 0: Alkali-Treated		Enzymatically De-esterified		Re-esterified	
		Control	Test	Control	Test	Control	Test	Control	Test
Newtown Pippin apple parenchyma	320	0.05	0.37	..	..	0.085	0.165	0.025	0.265
	320	0.075	0.39	..	..	0.085	0.16	0.005	0.025
	520	0.05	0.35	..	..	..	..	..	..
	400	0.08	0.41	0.07	0.07	..	..	0.07	0.19
Immature Meyer lemon ca. 5/8-inch diameter, rind only	80	0	0.37	..	..	0.005	0.08	0	0.39
	120	0.005	0.47	..	..	0.005	0.23	0.01	0.44
Burbank Russet potato	240	0	0.08	..	..	0.01	0.03	0.002	0.11
	240	0.005	0.075	..	..	0.005	0.04	0.005	0.09

[a] Average obtained from 4 to 6 readings per section.

Table II. Color Reactions with Various Substances

Substances	Test	Color Reaction
Natural polymers		
Apple pectin	+	Very intense dark red, insoluble
Citrus pectin	+	Very intense dark red, insoluble
Raspberry pectin	+	Very intense dark red, insoluble
Strawberry pectin	+	Very intense dark red, insoluble
Pectic acid		
Enzymatically de-esterified	−	No color when tested in solution, red to pink when solid substances tested
Alkali de-esterified	−	
Acid de-esterified	−	
Gum arabic	−	Light brown, faded rapidly
Gum tragacanth	−	Brownish red, faded
Dextran (peach)	−	None
Gum sandarac	−	None
Agar	−	None
Guar mannogalactan	−	None
Hemicellulose (lima bean pod)	−	None
Iridophycin (from alga)	−	None
Starch	−	None
Xylan (lima bean pod)	−	None
Sugars and derivatives		
Glucose pentaacetate	+	Soluble, intense dark red
Gulonic lactone	+	Soluble, dark red to brown
Fructose 1,6-diphosphate	+	Soluble, faintly red
Methyl glycoside of polygalacturonic methyl ester (Link's Cpd., *2*)	+	Soluble, iodine brown
Glucose 1-phosphate	−	None
Galacturonic acid	−	None
Methyl glucoside	−	None
Fructose	−	None
Glucose	−	None
Sucrose	−	None
Miscellaneous compounds		
Acetonitrile	−	None
Casein	−	None
Saponin (alfalfa)	−	None
Urea	−	None
Coumarin	+	Soluble, light greenish brown, faded
Hesperidin	+	Soluble, brownish to black
Chlorogenic acid	+	Soluble, dark red-brown
Quercetin	+	Soluble, then ppt. green-yellow to brown
Quercitrin	+	Soluble, then ppt. dark green-yellow
Rutin	+	Soluble, then ppt. green-brown
3-3′,4-4′-Tetrahydroxybiphenyl	+	Insoluble, muddy purplish brown

acetate have been used to test for the presence of pectin in solution. Techniques developed with these reagents reportedly use gel properties as a basis for differentiating among pectin substances, hemicelluloses, and gums.

The basic lead acetate test for pectic substances (*6*) provides good differentiation between pectin (yellow precipitate) and other gums. A modification of this method was proposed (*17*), in which the test substances are treated in solution with potassium oxalate and commercial pectinase for 20 hours, and the lead acetate test for galacturonic acid is then applied. The use of heat and the formation of obscuring precipitates when solid materials are so tested, however, vitiate the use of such tests for microscopic purposes.

Methods for detecting pectic substances in solid mixtures include chemical separation into water-soluble and insoluble fractions and treatment with specific pectic enzymes. Much of the earlier work on pectins was accomplished by chemical separation methods augmented with microscopic techniques in which ruthenium red and other dyes were used to stain plant tissues before and after chemical treatments (*3*, *7*, *14 20*). However, these dyes are not specific for pectins. The staining of pectic materials with ruthenium red appears to depend upon carboxyl groups, and pectic acids, pectates, and nonuronide carboxyl-containing compounds are indiscriminately stained (*20*). Thus, additional chemical treatments are required to differentiate the pectic substances from the nonuronide components of the cell wall materials.

These disadvantages are readily overcome with the proposed hydroxamic acid–ferric ion test for esters. The test requires only a few minutes, is specific, and may be applied equally well for determining esterified pectins in solid mixtures or as a histochemical test.

Acknowledgment

The authors thank W. Z. Hassid, Department of Plant Biochemistry, University of California, for the sample of iridophycin, and T. E. Rawlins, Department of Plant Pathology, University of California, for his interest and helpful suggestions.

Literature Cited

(1) Abdel-Akher, M., and Smith, F., *J. Am. Chem. Soc.*, **73,** 5859 (1951).
(2) Baur, S., and Link, K. P., *J. Biol. Chem.*, **109,** 293 (1953).
(3) Branfoot, M. H., "Critical and Historical Study of the Pectic Substance of Plants," Gt. Brit. Dept. Sci. Ind. Research, *Spec. Rpt.* 33 (1929).
(4) Bryant, E., *Ind. Eng. Chem., Anal. Ed.*, **13,** 103 (1941).
(5) Dische, Z., *J. Biol. Chem.*, **167,** 189 (1947).
(6) Ehrlich, F., *Ber.*, **65B,** 352 (1932).
(7) Esau, Katherine, "Plant Anatomy," Wiley, New York, 1953.
(8) Feigl, F., "Qualitative Analysis by Spot Tests," 3rd ed., p. 359, Nordemann Publishing Co., New York, 1946.
(9) Hestrin, Shlomo, *J. Biol. Chem.*, **180,** 249 (1949).
(10) Hill, U., *Anal. Chem.*, **19,** 932 (1947).
(11) Hill, U., *Ind. Eng. Chem., Anal. Ed.*, **18,** 317 (1946).
(12) Jansen, E. F., and Jang, Rosie, *J. Am. Chem. Soc.*, **268,** 1475 (1946).
(13) Jansen, E. F., MacDonnell, L. R., and Ward, W. H., *Arch. Biochem.*, **21,** 149 (1949).
(14) Kertesz, Z. I., "The Pectic Substances," Chap. 8 and 10, Interscience, New York, 1951.
(15) Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **161,** 415 (1945).
(16) MacDonnell, L. R., Jang, Rosie, Jansen, E. F., and Lineweaver, H., *Arch. Biochem.*, **28,** 260 (1950).
(17) Owens, H. S., McCready, R. M., Shepherd, A. D., Schultz, T. H., Pippen, E. L., Swenson, H. A., Miers, J. C., Erlandsen, R. F., and Maclay, W. D., Western Regional Research Lab., U. S. Dept. Agr., Albany, Calif., **AIC-340** (1952).
(18) Reeve, R. M., *Food Research*, **18,** 604 (1953).
(19) Soloway, Saul, and Lipschitz, A., *Anal. Chem.*, **24,** 898 (1952).
(20) Tetley, Ursula, *J. Pomol. Hort. Sci.*, **8,** 153 (1930).
(21) Tollens, B., *Ber.*, **41,** 1788 (1908).
(22) Tseng, C. K., *Science*, **101,** 597 (1945).
(23) Whittaker, V. P., and Wijesundera, S., *Biochem. Soc. Proc.*, xlv., *Biochem. J.*, **49** (1951).

Received for review October 25, 1954. Accepted January 3, 1955. Mention of trade names does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.