LIBRARY
OF THE
UNIVERSITY
OF ILLINOIS

630.7
Il6b
no.621-640

AGRICULTURE

NON CIRCULATING

CHECK FOR UNBOUND
CIRCULATING COPY

30.1
I l6b
UNIVERSITY OF ILLINOIS
AGRICULTURE LIBRARY
Ag Lib
B

A Review of the CARBOHYDRATE CONSTITUENTS OF ROUGHAGES

By R. G. Hansen, R. M. Forbes, and Don M. Carlson

LIBRARY OF THE
DEC 12 1958
UNIVERSITY OF ILLINOIS

Bulletin 634
UNIVERSITY OF ILLINOIS AGRICULTURAL EXPERIMENT STATION

NORTH CENTRAL REGIONAL PUBLICATION 88

Agricultural Experiment Stations of Illinois, Indiana, Iowa, Kansas, Michigan, Minnesota, Missouri, Nebraska, North Dakota, Ohio, South Dakota, and Wisconsin

and

U. S. Department of Agriculture, Cooperating

November, 1958

This publication is a contribution of the NC-25 committee on "Factors Affecting the Utilization of Feed by Ruminants."

COMMITTEE MEMBERS

Illinois . . . . . R. G. Hansen, R. M. Forbes
Indiana . . . . . G. D. Goetsch, D. L. Hill
Iowa . . . . . W. E. Burroughs
Kansas . . . . . L. R. Fina
Michigan . . . . . C. W. Duncan
Minnesota . . . . . M. O. Schultze
Missouri . . . . . W. H. Pfander, W. C. Ellis
Nebraska . . . . . T. W. Dowe, J. H. Pazur
North Dakota . . . . . W. E. Dinusson
Ohio . . . . . O. G. Bentley
South Dakota . . . . . L. B. Embry, L. D. Kamstra
Wisconsin . . . . . E. M. Foster
U. S. Department of Agriculture,
State Experiment Stations Division . . . . . R. E. Ely

ADMINISTRATIVE ADVISER

Tom S. Hamilton, Associate Director, University of Illinois,
Agricultural Experiment Station

The authors wish to express their appreciation for suggestions made by members of the NC-25 committee and in particular for the critical review of the manuscript by Professors Max O. Schultze and Fred Smith of the University of Minnesota.

Urbana, Illinois November, 1958

CONTENTS

GENERAL CONSIDERATIONS 6

CLASSICAL ANALYSIS OF PLANT MATERIAL 7

HOLOCELLULOSE: CELLULOSE AND THE HEMICELLULOSES 9

- Constitution of Cellulose 9
- Constitution of Hemicelluloses 10

ISOLATION AND QUANTITATIVE ESTIMATION OF THE POLYSACCHARIDE FRACTIONS 12

- Preparation of Forage Samples 12
- The Holocellulose Fraction 12
- Cellulose 18
- The Hemicelluloses 23

FRACTIONATION OF HEMICELLULOSE 28

- Identification of Various Sugars 28
- Estimation of Pentoses and Pentosans 31
- Estimation of Uronic Acids 31

SOLUBLE SUGARS 33

- Monosaccharides and Oligosaccharides 33
- Fructans 33

THE PECTIC SUBSTANCES 39

LIGNIN 40

- Constitution 40
- Estimation of Lignin 42

CONCLUDING REMARKS 44

BIBLIOGRAPHY 45

A Review of the Carbohydrate Constituents of Roughages

By R. G. Hansen, R. M. Forbes, and Don M. Carlson[1]

The unique digestive system of ruminants enables them to utilize roughage as a major source of energy, an ability not shared by monogastric animals. In order to take full advantage of this unique ability, it is necessary to understand completely the chemical nature of this class of feedstuffs. The major portion of roughages, whether fresh or dried grass, hay, silage, straw, or fibrous byproducts, consists of a mixture of complex carbohydrates whose intimate composition is as yet poorly defined. Techniques are being developed (80),[2] however, that will facilitate studies in this area.

Since about 1940, procedures have been developed that yield more precise quantitative information concerning the classification of roughage carbohydrates than that given by the method of proximate analysis designed by Henneberg and Stohmann in 1864 (29, 65). The evaluation of feeding stuffs undoubtedly has been aided by this classical determination of crude fiber and nitrogen-free extract, but it is surprising that methods which measure highly variable and complex fractions have been used for such a long period of time without major modification.

Although there are excellent broad reviews of the carbohydrate constituents of plants,[3] a satisfactory review of the material pertaining specifically to the carbohydrates of roughages is not, to the authors' knowledge, available, other than the brief account by Percival (97). The primary purpose of this publication is therefore to review the methods and results of the qualitative and quantitative analyses for the carbohydrate constituents of some of the common roughages.

---

[1] R. G. Hansen, formerly Professor of Biological Chemistry in Dairy Science now in the Department of Agricultural Chemistry, Michigan State University; R. M. Forbes, Professor of Animal Nutrition; and Don M. Carlson, formerly Assistant in Dairy Science, now in the Department of Agricultural Chemistry, Michigan State University. A major portion of the material in this publication was taken from a master's thesis by Mr. Carlson.

[2] Numbers in parentheses refer to bibliography, page 45.

[3] In the preparation of this review, extensive use was made of the following references: 25, 26, 68, 129.

GENERAL CONSIDERATIONS

The energy which supports the activities of most living creatures is derived directly or indirectly from the energy of sunlight through the process of photosynthesis. This process, consisting of the transformation of the carbon dioxide of the air into the carbon-containing organic materials of plants, makes these carbon compounds available as sources of energy both to the plant itself and to organisms incapable of using the energy of sunlight to synthesize their substance from carbon dioxide. Sugars, which are the principal products of photosynthesis, may either be stored as such in the plant or may be converted to other substances. Many of the materials which constitute the major portion of the plant — the polysaccharide components of the cell wall — are closely related chemically to the sugars produced in photosynthesis.

Through the metabolic activities of the plant, the primary sugars are transformed (1) to the polysaccharides, such as starch and fructans, which serve as energy reserves for plant growth; (2) to the substances which make up the structure of the cell wall, such as cellulose, hemicelluloses, lignin, and pectins; (3) to the nitrogen-containing proteins of the plant protoplasm; and (4) to the fats and waxes which respectively provide reserve food materials and the coatings for leaves and other surfaces. In addition to these major components, plant tissues also contain a variety of other substances which are often present in small concentrations.

The cell walls of the higher plant are typically made up of a mixture of a number of kinds of polysaccharides and polysaccharide derivatives. In the cell walls of mature tissues, nonpolysaccharide material, especially lignin, may also be incorporated into the structure of the wall. The characteristic component of the cell wall is cellulose, which occurs in nearly pure form in fibers. Cellulose is also a major constituent of essentially all the higher-plant cell walls that have been investigated.

In the dividing cell, polysaccharides are deposited in the initial membrane, recognized as the cell plate. Possibly the first polysaccharide formed in large amounts is pectin or calcium pectate, although xylan and other hemicelluloses are probably present with perhaps a submicroscopic frame of cellulose. During the early stages, the cell plate must also contain protoplasmic material and perhaps other noncarbohydrate substances. In any event, this primitive expanding plate reaching toward the walls of the mother cell is the middle lamella which will serve in part, when cell division is complete, to bind the two daughter

cells. In the mature tissue, this tenuous intercellular layer contains a large amount of lignin. Also present are pentosan, probably xylan and uronic acid-containing material, and a small amount of pectin.

Lignin functions as an intercellular cement, but in high concentrations it may cause the plant tissue to become unduly brittle. Thus corn stalks of high lignin content are found to break (lodge) more readily than those of varieties with a lower lignin content. Since small amounts of lignin are found in young tissue, it is not solely a product of aging.

Cell walls contain a variety of polysaccharides, historically called hemicelluloses, in addition to cellulose. The name "hemicellulose" was given to the polysaccharide fraction extracted from plant materials by dilute alkali by Schulze in 1892 (113). So scanty was the knowledge of carbohydrate chemistry at that time that these substances were regarded as degraded or imperfect celluloses, hence the name hemicellulose. It is now known that these alkali-soluble polysaccharides are complex mixtures which vary with the source and method of extraction.

CLASSICAL ANALYSIS OF PLANT MATERIAL

Proximate analysis is still the most widely used system for evaluating feedstuffs. This scheme of analysis was devised by Henneberg and Stohmann (65) at the Weende Experiment Station in Germany as a result of their investigations from 1858 to 1863. Crude fiber was the residue after digestion of the ether-extracted sample with acid and base. The sum of the percentages of moisture, crude protein (nitrogen × 6.25), ash, ether extract, and crude fiber subtracted from 100 gave the percentage of nitrogen-free extract. Henneberg felt it necessary to designate the residue from the acid and base digestion as crude fiber since the residues obtained from different substances by exactly the same procedures were different. He also realized the complexity of the nitrogen-free extract. It became one of the main purposes of the School of Agricultural Chemistry in Göttingen to investigate the nature of this extract.

A summary of the work published in 1897 by Tollens (119), director of the Göttingen station, listed the following substances in the nitrogen-free extract: sugars, polysaccharides, hexosans, pentosans, methyl pentosans, sugar alcohols, pectins, lignin, organic acids, tannin, vegetable colors, resins, and other aromatic substances. It was obvious that the determination of each of these substances for feed evaluation would involve impractical amounts of time. According to Tollens (119), Henneberg realized that crude fiber and even pure cellulose were susceptible to attack in herbivorous animals. A comparison of appropriate

data from a number of feeds showed that the sum of the digestible cellulose and digestible nitrogen-free extract agreed approximately with the analytical results obtained for nitrogen-free extract, a coincidence that was characterized by the term "compensation." The practical worth of the values found for crude fiber and nitrogen-free extract was not seriously questioned, but the diverse nature of those fractions isolated from different plants and by slightly altered methods was recognized. Since the digestible part of the crude fiber has about the same nutritive value as the digestible part of the nitrogen-free extract, and since the sum of these was approximated by the determination of the nitrogen-free extract, the practical value of this method has seemed sufficiently satisfactory to warrant the continuance of the Henneberg method of analysis. Tollens (119) gives an insight to Henneberg's opinions in the following quotation from Henneberg's lecture notes: "In order to ascertain the value of a feedingstuff for nutrition, it is necessary to determine the content of all the separate constituents, or at least of all the groups of similar value, and so far as the cellulose is concerned, the various modifications of the same. These requirements the customary analysis of vegetable feedingstuffs by no means fulfills. . . . The present method of fodder analysis needs greatly to be perfected, but in many respects accomplishes more than would be expected from its defectiveness."

Although he expected the system to be modified by the discovery of improved methods, the present method of feedingstuff analysis is practically the same as that announced provisionally by Henneberg in 1863.

The principal indigestible portion of forages for herbivora is lignin. Analysis of the proximate constituents of roughages shows that lignin is distributed to a varying degree in both the crude fiber and the nitrogen-free extract. It has been well substantiated that at times, in pasture herbage for example (33, 59), the crude fiber is even more digestible than the nitrogen-free extract. Abundant confirmation has also been reported for variations in the composition of plants and the digestibility of the proximate principals due to species, stage of maturity, and moisture supply, and an apparent difference due to methods of estimation. Obviously then, neither the crude fiber nor the nitrogen-free extract fractions represent discrete chemical entities, but are highly variable and complex. The suggestion has frequently been made that, as far as possible, the carbohydrate fractions should be partitioned into chemical or biological units. The problem is largely a consideration of the principal constituents of the cell wall: cellulose, hemicellulose, and lignin.

Crampton and Maynard (34) suggested that the partitioning of the carbohydrate portion into lignin, cellulose, and other carbohydrates may be of greater usefulness in predicting feeding values than the Henneberg division. They therefore proposed chemical isolation of lignin and of cellulose and an estimation of the other carbohydrates by difference. The chief objection to this system is the need for an entirely satisfactory method for lignin determination.

A modification of this scheme was subsequently proposed by Crampton and Whiting (35) wherein cellulose and other carbohydrates were determined, thus obviating the need for a direct lignin estimation. In spite of the authors' claims that these fractions more nearly approximated biologically related units and that the procedure is adaptable to routine analysis, the system has not been widely used since its announcement in 1942.

Without pursuing further the merits and limitations of a system of proximate analysis and of other proposed analytical procedures, the importance of defining as precisely as possible the chemical nature of the carbohydrate fraction should be obvious to the scientific investigator. A study of the metabolic behavior of plant carbohydrates is completely limited without such characterization. The concept of "holocellulose" has been developed in attempts to comprehend the complex nature of the polysaccharide fraction.

HOLOCELLULOSE: CELLULOSE AND THE HEMICELLULOSES

Constitution of Cellulose

The term "holocellulose" was devised to include the cellulose and hemicellulose fractions of plant cell polysaccharides. Cellulose occurs in plant cell walls with varying amounts of associated polysaccharides and other related materials and is the main substance accounting for physical properties of the cell wall. A precise account of cellulose structure is given by Whistler and Smart (129). Acid hydrolysis of cellulose gives a high yield of β-D-glucose. Controlled hydrolysis gives cellobiose (4-O-β-D-glucopyranosyl-D-glucose). This evidence together with the results of methylation studies and the presence of cellobiose indicates that cellulose consists of long chains of glucopyranose units linked uniformly by 1-4-β-D-glycosidic bonds. The chains appear to be very long and unbranched. Molecular weight studies indicate chains of 1,400 to 10,000 glucose units.

We may thus picture cellulose in plant cell walls as made up of

long chains of glucose residues. The chains are packed into well-oriented crystalline aggregates interspersed with amorphous regions. These aggregates, or micelles, are short in comparison with chain length, so each chain participates in the formation of many micelles (25).

In all cell walls the chains (of glucose units) run in the plane of the wall. Cell walls of mature cells consist of numerous superimposed layers, which comprise primary and secondary walls, the secondary wall being inside the primary and being formed only in the mature cells. This secondary wall may be thick and may contain several layers distinguishable microscopically. Between the primary walls of adjoining cells is the middle lamella, which consists primarily of pectins, whereas the primary and secondary walls are mainly cellulose, but may contain pectins, lignins, polysaccharides, polyuronides, silica, etc. Cellulose once laid down in the cell wall is not considered available as an energy source for the plant; cellulases of higher plant origin have been reported only in germinating seeds (128).

Constitution of Hemicelluloses

Typical hemicellulose preparations may include (68):

(a) Short-chain glucans, either removed from the cellulose fibrils by the extraction process or existing outside the fibrils in the encrusting polysaccharides.

(b) Polymers of xylose, arabinose, mannose, galactose, and possibly other monose units, such as rhamnose and fucose. Whether these exist as chains built up of like units or of mixed units is still uncertain in most cases. If they are built up of mixed units, it is probable that one unit predominates.

(c) Mixed polymers of sugar units and uronic acid units, usually methylated and sometimes acetylated.

(d) Residual pectin polysaccharides where not completely removed earlier. Separation of "pectin" araban and galactan from "hemicellulose" araban and galactan is almost always arbitrary.

(e) The special polysaccharides which form the bulk of the cell walls of certain plant materials may be classified as hemicelluloses or not, purely by expanding or contracting the scope of definitions. The mannan of the ivory nut is one example. Seaweed polysaccharides, although not usually considered as hemicelluloses, are nevertheless extracted and analyzed by much the same methods.

The structure and size of a few polysaccharides are discussed by Whistler and Smart (129).

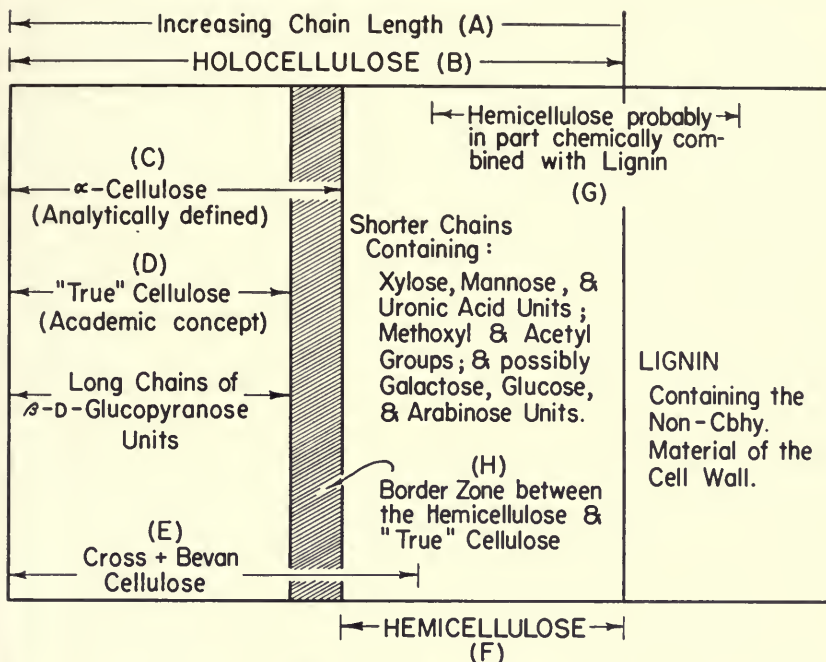


Fig. 1. — Relationship between cellulose and hemicellulose (refs. 86, 89, 131).

(A) The solubility of the different fractions may be used as an indication of the comparative length of the chain; for example, α-cellulose, which is closely related to true cellulose, is insoluble in 17.5% NaOH solution and has molecules of an estimated degree of polymerization greater than 200, whereas the hemicelluloses are insoluble in dilute alkali and are thought to have usually around 50 units in each chain.

(B) Holocellulose = cellulose + the hemicelluloses.

(C) As mentioned above, α-cellulose is the material that is insoluble in 17.5% NaOH. This is merely an arbitrary method; however, it will contain more than just glucose units which are contained in the "true cellulose."

(D) The "true cellulose" is long chains of β-D-glucopyranose units.

(E) "Cross and Bevan cellulose" is the term used to designate the cellulose fraction obtained by the method developed by these investigators (36). This fraction contains little lignin, but does contain other cell-wall constituents not attacked by boiling NaOH (1%) followed by chlorine and sodium sulfate treatment. Polyuronide hemicelluloses are removed with the lignin.

(F) Hemicellulose.

(G) The fact that all of the hemicelluloses cannot be obtained free from lignin without degradation of the hemicelluloses is sometimes explained by the theory that there may be a bond between lignin and hemicelluloses in some way.

(H) This shaded portion could be determined either with the cellulose (α-cellulose) or with the hemicelluloses, depending upon whether cellulose is determined as only D-glucose units (putting it in the hemicellulose category) or as a chemically standardized procedure (α-cellulose) which would put it in the cellulose fraction.

It should be evident from this listing of substances that may be included in a hemicellulose preparation and from the great variation in solubilities of the fractions that the separation of a pure molecular type of polysaccharide is difficult. It is possible that the greater variation may not be in the type of polysaccharide present, but in the molecular weight. The efforts in this field have not been entirely unrewarded as some individual polysaccharides have been separated from the hemicellulose fraction. The most important of these is xylan. Although xylan has been isolated as a pure molecular type (32), it also occurs polymerized with glucuronic acid, galactose, glucose, and arabinose (129). The relationship between cellulose and hemicellulose is shown in Fig. 1, which is derived from Norman (86), Norman and Jenkins (89), and Wise (131).

ISOLATION AND QUANTITATIVE ESTIMATION OF THE POLYSACCHARIDE FRACTIONS

Preparation of Forage Samples

The proper preparation of the forage sample prior to analysis is necessary in order to obtain accurate analytical results. For storage purposes or for subsequent extraction procedures, it is usually necessary to dry the sample. Enzymatic reactions which occur between the end of active vegetative growth and the time at which the plant is processed in the laboratory affect the chemical composition and extractability of the carbohydate constituents. The most common method of enzymatic inactivation is immersion of the plant material in boiling 80% ethanol. Other methods can be used with a similar effect, such as drying at the proper temperatures or rapid freezing in dry ice followed by lyophilization (75).

Improper drying can seriously affect solubility and chemical reactivity of some constituents. Optimum drying appears to require the rapid withdrawal of water at a relatively low temperature (about 0° to 40° C). The gradual removal of water, such as by air drying, is thought to allow adjacent polysaccharide molecules or chain segments to come into contact gradually and establish strong secondary bonds which later restrain the separation of molecules and hinder the penetration of water or other solvents.

The Holocellulose Fraction

The cellulose and hemicellulose fractions represent a substantial part of most forage materials and contribute the major portion of the

energy value of forages for ruminants. The fact that cellulose and hemicellulose are comparable in digestibility by ruminants seems to justify combining these fractions from a physiological viewpoint (54). The separation from forages of a single fraction containing both these components has been found to be desirable. Therefore one of the most important advances in the determination of cell-wall polysaccharides was the quantitative isolation of combined cellulose and hemicelluloses of extractive-free wood by Schmidt *et al.* (112).

For the extraction of polysaccharides, plant materials are often ground to pass a 40-60 (0.417-0.246 mm. opening) mesh screen, but this practice has not been standardized. In some reports fineness of grinding has not been stated, but mention is merely made of ground material.

The next step usually employed in preparation of the sample is the removal of lipids and other so called "extractives." For the lipid extraction of plant material in preparation for holocellulose isolation, an azeotropic mixture of benzene and ethanol (2:1) is usually preferred. A hydrophilic reagent is included to assist in the extraction from the intercellular spaces. Following the lipid extraction, a water extraction is frequently used, employing either hot water or water at room temperature. It is desirable, however, to maintain the pH near neutrality because an acidic solution may cause hydrolytic cleavage of labile glycosidic linkages. The material thus extracted up to this point is termed "extractive-free." Prior to separation of polysaccharides from plants of high pectin content, an ammonium oxalate extraction is generally used.

Schmidt's method involved the use of chlorine dioxide in pyridine and water and took about a month for the quantitative removal of lignin. Realizing that a method such as this is impractical for routine investigations, the Forest Products Laboratory initiated work to find a more rapid extraction method. Ritter and Kurth (108) developed a method at the Laboratory that isolated the cellulose and hemicelluloses from maplewood in about 10 hours. This was achieved by repeated alternate treatments of the extractive-free wood with chlorine and a solution of pyridine in ethanol, followed finally by treatment with a cold calcium hypochlorite solution, until the residue was white and free from lignin. This material was called "holocellulose," which means the whole or entire cellulosic material, including the hemicelluloses.

There have been many modifications and changes in techniques for the isolation of holocellulose. Jayme (67) used methanol-benzene for the extractions and sodium chlorite and acetic acid for delignification

and found that the lignin content of wood holocellulose was 2.8 to 3.5 percent. However, if this residual lignin was removed by more drastic action, polysaccharide material was lost also.

Chlorite holocellulose holds an important position in the determination of the carbohydrate components of plant material and is an excellent source of raw material for research on the individual components of the main carbohydrate fractions. One of the more recent methods of preparation of holocellulose, and in most instances the method applied to roughages, is the method of Wise, Murphy, and D'Addieco (132). This is a modification of Jayme's method which includes digestion at higher temperatures with repeated additions of chlorite to shorten the extraction time. The chlorite method has been studied for nonwoody plant material and has been found to be satisfactory for the preparation of holocellulose (50). It reduces the time required and simplifies the techniques used for the preparation of this carbohydrate fraction. The holocellulose still contains about 2 to 4 percent of lignin, and Wise *et al.,* like Jayme, found that if the holocellulose was extracted further, some of the carbohydrate material was removed.

While studies on wood holocellulose were made using the Wise *et al.* method, few if any were made on roughages until Bennett (18) in 1947 isolated the holocellulose fraction from Kentucky bluegrass, corn cobs, oat straw, timothy hay, and corn stalks. A product that had satisfactory color and gave low yields of furfural was isolated by this procedure in about 2 to 3 hours, whereas older procedures required about 2 to 3 days. The results showed that the holocellulose retained considerable ash and about one-third of the nitrogen content of the original plant material.

Later Adams and Castagne (2) prepared holocellulose from the straws of wheat, oats, barley, rye, and seed flax. When three chlorite treatments were used, the lignin content of the holocellulose was decreased to approximately 2.5 percent; the flax, however, retained 4 to 5 percent of the lignin with three treatments and 3.5 percent after five treatments. The effect of successive chlorite treatments (one to nine) is shown in Fig. 2. The data indicate a slight rise in the ash content of the holocellulose, a lowering of lignin content with a subsequent rise after about six treatments, and a reduction of carbohydrate material (not shown) after about four treatments. With more than seven treatments, a portion of the cellulose was removed. A gradual increase in pH from 4.19 for the starting material to 6.60 after the ninth treatment was also noticed. The rise in pH has a deleterious effect on chlorine dioxide as an oxidizing agent, but certainly alleviates much of the possible

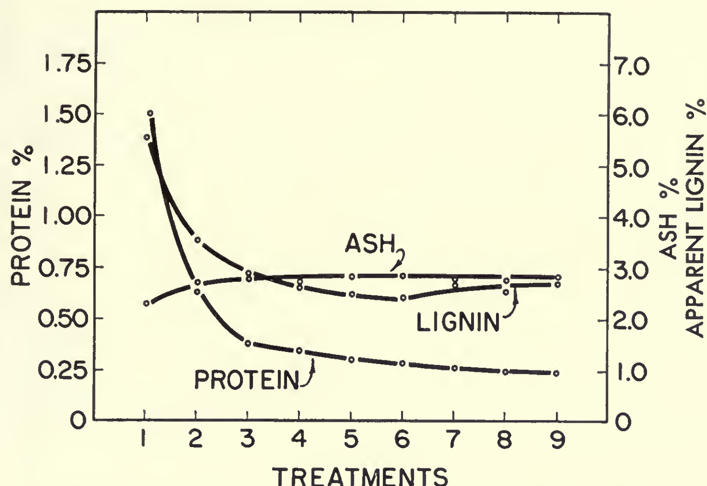


Fig. 2. — Effect of successive chlorite treatments (ref. 2).

destruction of the holocellulose normally found when polysaccharides are exposed to a low pH. Various other treatments were tried to remove the residual lignin from the holocellulose, but all were without success. It was considered preferable to leave the lignin in the holocellulose and make corrections for it.

Adams and Castagne obtained the following yields of holocellulose for five straws given various chlorite treatments. The theoretical yield was obtained by subtracting from 100 the sum of the ash-free lignin, protein, and ash contents of the extracted straws.

Straw	*Number of chlorite treatments*	*Actual yield*	*Theoretical yield*
		perct.	*perct.*
Wheat	3	76.1	76.0
Oats	3	76.6	76.5
Flax	5	71.8	73.7
Barley	3	78.1	77.8
Rye	4	77.9	77.0

The holocellulose, when compared with the extractive-free straw, showed a slightly higher ash content, a lower pentosan content, but similar uronic acid anhydride and acetyl contents. A lower pentosan content has been found by other workers (109) but not explained. Approximately 10 to 15 percent of the protein present in the straw was found in the holocellulose fraction. Reproducibility of results using larger quantities (70 gm.) of material was possible. The lignin,

ash, pentosan, and holocellulose contents of various straws reported by Adams and Castagne in a later work (5) show good agreement with their earlier work (2):

	Wheat	*Oat*	*Rye*	*Flax*
		(percent of dry weight)		
Ash	4.7	8.9	4.7	2.7
Lignin (apparent)	23.0	22.3	23.1	21.5
Lignin (ash free)	21.3	19.7	22.2	21.5
Pentosan	27.5	22.7	24.2	21.4
Holocellulose	71.0	69.3	66.0	71.0

It was evident that more work was needed to determine the validity of the chlorite procedure in preparing holocellulose from forages, as most of the studies had been performed on a somewhat limited type and number of crops. Ely and Moore (50) prepared holocellulose from seven hays, two silages, and one straw, which obviously varied in composition over a wide range, and concluded that the chlorite treatment could be used successfully with different forages. The holocellulose fraction represented 66 to 81 percent of the dry matter of the extractive-free samples. Two acid chlorite treatments gave essentially complete extraction of holocellulose from all forages, and again larger numbers of extractions caused a loss of carbohydrate.

The chlorite procedure, however, does not appear to be as successful in the isolation of holocellulose from feces. Ely and Moore (51) isolated the holocellulose from the feces of cows fed the ten roughages mentioned above (50). They concluded that a uniform number of acid chlorite treatments will not prepare holocellulose in theoretical yields with all samples of feces. A difference was also noted in the composition of holocellulose from forages and feces (53). Holocellulose prepared from feces contained more lignin than holocellulose prepared from forages. There was an increase of protein and apparent lignin content in holocellulose preparations from high-protein materials over those prepared from low-protein materials.

Corn cobs, probably because of their porous nature, are more easily extracted. This was shown by Whistler *et al.* (127) who used four treatments with sodium chlorite and acetic acid at 15-minute intervals (instead of the hourly intervals employed by Wise *et al.* (132) and obtained a yield of 81.5 percent holocellulose with a lignin content of only 0.4 percent.

As the duration and number of acid-chlorite treatments appeared to be of some significance, Harwood (62) compared the method of Wise *et al.* (132), as modified by Adams and Castagne (2), with the method used by Whistler *et al.* (127), in the preparation of holocellulose from wheat straw. Fig. 3 shows the composition and yields of holocellulose

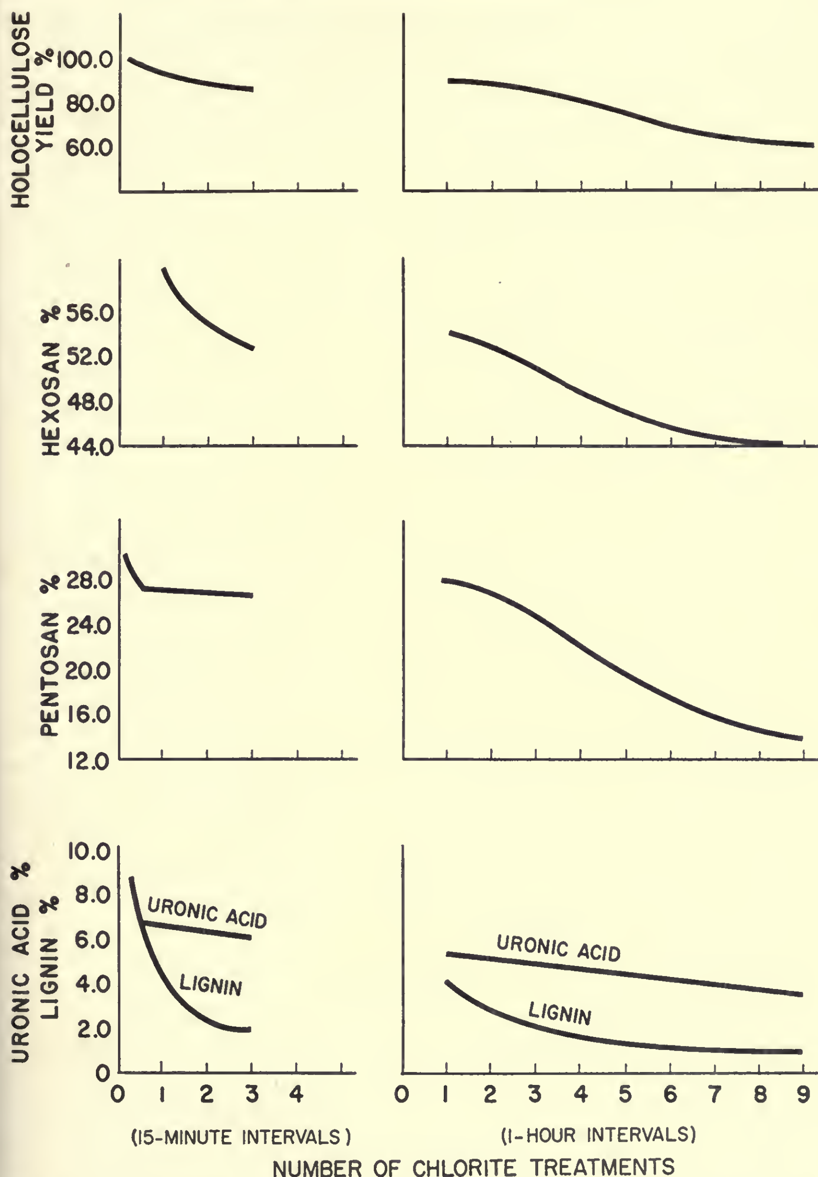


Fig. 3. — Yield and composition of holocellulose prepared with increasing number of chlorite treatments by Whistler method (left) and by Wise method (right) (ref. 62).

obtained by the two procedures when plotted against various numbers of chlorite treatments.

The lignin impurity which is constantly found in the holocellulose fraction may or may not be cause for concern. Jermyn and Isherwood (69) reported that 80 percent of the residual lignin from pear holocellulose was precipitated as a contaminant of hemicellulose-A. Wise *et al.* (132), however, in their classical report on chlorite holocellulose, reported that only small amounts of alkali-soluble lignin are precipitated with the hemicellulose fraction which is precipitated by neutralization with acetic acid and that this lignin is completely soluble in either alcohol or acetone. In view of the contradictory reports on the presence of lignin, it is apparent that a lignin analysis must be performed on the hemicellulose fractions unless experience has proved differently. There is usually a small amount of the hemicellulose fractions that is not accounted for.

Instances have been reported of a high nitrogen content in the holocellulose portion which, of course, indicates the presence of protein. Opinions differ as to the importance of this impurity. These analytical differences should be considered especially with regard to any further experimental use of the preparation. Ely, Melin, and Moore (49) incubated various forages with pepsin prior to the preparation of holocellulose in an effort to decrease the protein content. When compared with untreated samples, a reduction of protein content was found, but concomitant losses of carbohydrate also occurred. It was concluded therefore that pepsin pretreatment of forages to lower the protein content of the holocelluloses is unsatisfactory if a quantitative yield of carbohydrate is required.

The percentage of holocellulose obtained from some of the more common feeds is given in Table 1.

Cellulose

As it is now possible to prepare large samples of holocellulose from various materials, the fractionation of this mixture of polysaccharides can be undertaken. The most common method of fractionation is by separation into subfractions based upon the solubility of some of the components. The insoluble residue that remains after digestion with dilute alkali consists largely of cellulose or polymers of β-D-glucose.

Cellulose is frequently defined as a cell-wall fraction obtained by specific treatment and may also contain other constituents, particularly xylans. The classical treatment is that of Cross and Bevan (36) in which the plant material is treated with boiling 1% NaOH and then with chlorine and sodium sulfite. This treatment removes most of the

Table 1. — Holocellulose Content of Some Common Roughages[a]
(Expressed on dry basis)

Roughage	Holocellulose[a]	Reference
	perct.	
1. Alfalfa hay	75.4	50, 57
2. Alfalfa silage	71.4	50
3. Barley straw	78.4	2, 57
4. Buckwheat straw	60.7	57
5. Clover hay	69.6	50
6. Corn cobs	80.2	18, 129
7. Corn silage	79.4	50
8. Corn stalks	78.6	18
9. Flax straw	71.4	2, 3
10. Kentucky bluegrass	73.7	18
11. Lespedeza hay	63.2	50
12. Oat straw	74	2, 3, 18, 57
13. Orchard grass	70.6	50
14. Red clover hay	77.5	57
15. Rye straw	71.5	2, 3
16. Soybean hay	68.3	50
17. Timothy hay	75.3	18, 50
18. Wheat straw	78	1, 2, 3, 56, 57, 109

[a] Values given as averages when more than one estimation was available.

lignin and the polyuronide hemicelluloses, leaving as a residue cellulose and cellulosans. The latter fraction may be removed with 17.5% NaOH and constitutes about 10 to 25 percent of the total Cross and Bevan cellulose. The material undissolved by 17.5% NaOH is termed "true cellulose," or α-cellulose (Fig. 1). Of the 17.5% NaOH-soluble fraction, a portion is precipitable by acid and is termed β-cellulose and a portion is not precipitable by acid and is termed γ-cellulose. The widely used Norman-Jenkins procedure (87) is similar to the Cross and Bevan method and gives a similar product but employs hypochlorite rather than chlorine. The Crampton-Maynard modification (34) of the Kürschner and Hanak procedure relies on acetic and nitric acids to dissolve the tissue components other than cellulose. This procedure results in a residue containing much less of the cellulosans than the chlorite-sulfite method and has been favored by some investigators because it is claimed to yield a "purer" cellulose. Druce and Willcox (44) have employed a dichromate oxidation procedure for cellulose analysis which is reported to give an essentially "pure" hexosan product. This method yielded unreasonably low results when tried in our laboratory (Forbes).

The Norman-Jenkins method has been modified by Matrone, Ellis, and Maynard (78) to shorten the time and increase the efficiency of operation, and this method is in wide use today. Bennett (21) has also modified the Norman-Jenkins procedure using sodium chlorite rather than alternate treatments with hypochlorite and sulfite. The Bennett

procedure gives more consistent results, and although the percentage of crude cellulose is somewhat higher than that yielded by the Norman-Jenkins method, the lignin and pentose contents are lower. The Norman-Jenkins and the Matrone-Ellis-Maynard procedures yield similar results. Since ruminants seem to utilize cellulose and pentosans with equal gross efficiency, a method which groups these items as a "biological unit" has merit for purposes of describing feedstuffs in terms of probable usefulness to animals. Studies such as those of Ely, Kane, Jacobson, and Moore (48) in which an attempt was made to identify quantitatively the constituents of a "biological unit" such as Matrone cellulose should be continued, and further specific evidence of the comparative efficiency and mechanism of utilization of the specific chemical entities comprising the carbohydrate fractions of forages should be accumulated.

In Table 2 is presented a compilation of data showing the cellulose content of roughages commonly used as animal feeds in this country.

Table 2. — Percentage of Lignin and Cellulose in the Dry Matter of Forage Crops

Description of sample	Lignin	Cellulose	Reference
Alfalfa hay	9.9	28.3	52, 102
second cutting	9.2	25.0	116
green, young	3.0	....	58
green, mature	10.5	....	58
8 samples, simulated grazing	3.8–6.8	....	59
preflowering 6–7″	3.6	17.7	9
preflowering 10–12″	4.1	18.0	9
preflowering 12–14″	6.7	24.7	9
preflowering 16–18″	8.0	29.9	9
late flowering	7.2	25.9	9
Alfalfa meal	9.6	23.4	60
Alfalfa silage	11.7	....	52
Barley plants, 7 days	1.5	....	100
21 days	1.7	....	100
35 days	2.9	21.9	100
49 days	5.1	28.6	100
63 days	6.8	28.7	100
77 days	7.0	30.3	100
86 days	7.7	31.6	100
Barley straw	15.0	48.0	11, 85
Bluegrass, Kentucky			
av. 5 stages of maturity	5.7	27.4	104
young	2.4	....	28, 58
mature	11.0	....	14, 26, 28, 58
10 samples, simulated grazing	5.6	....	59
heads just emerged	4.7	33.0	116
Bromegrass, 6 samples			
simulated grazing	5.2	....	59
5 stages of maturity	6.0	28.9	104
heads just emerged	6.3	41.5	116
hay	7.6	31.0	102

Table 2. — Continued

Description of sample	Lignin	Cellulose	Reference
Bromegrass, 2 varieties, young	4.4	20.0	96
mature	14.3	33.4	96
Clover hay, alsike	11.4	31.6[a]	102
Ladino	5.7	22.0[a]	116
Ladino, 7 samples, simulated grazing	5.5	....	59
Ladino, young	2.3	....	58
Ladino, mature	4.6	....	58
hay, red	11.0	28.8[a]	102
Kenland Red, simulated grazing	5.0	....	59
Kentucky 215, simulated grazing	5.1	....	59
Corn cobs, fine chaff	11.1	49.4	92
cobs, coarse chaff	11.6	59.8	92
cobs, woody ring	8.9	58.1	92
cobs, pith	9.8	56.7	92
silage	6.6	....	52
stalks	15.7	45.4	21, 107
U.S. 13 blades	11.6	45.1	92
sheathes	12.0	48.4	92
shell	16.4	55.2	92
nodes	13.0	32.7	92
husk	7.4	54.4	92
ear shanks	10.9	46.6	92
rudimentary ears	8.4	41.5	92
tassels	14.0	49.5	92
pith	9.0	60.2	92
vascular bundles	14.4	51.9	92
dent, S. Dakota	18.8	44.5	92
flint, S. Dakota	9.2	42.4	92
leaves, dent, S. Dakota	12.5	37.2	92
leaves, flint, S. Dakota	10.3	39.0	92
Fescue, tall, av. 5 stages of maturity	6.3	28.8[a]	104
av. 17 samples	6.0	....	59
simulated grazing			
young	2.2	....	58
mature	7.6	....	58
preflowering 8–10″	4.5	33.8	9
late preflowering	3.8	38.8	9
full flower	6.4	39.9	9
seeding	7.5	35.8	9
Lespedeza hay	12.8	27.2[a]	52, 102
Oat grass, tall, av. 5 stages of maturity	6.3	30.0[a]	104
young	4.1	20.6[a]	96
mature	11.7	29.8[a]	96
Oat hay	8.5	30.5[a]	102
hulls	10.5	48.7	60
straw	15.9	52.9	85
straw	10.5	....	8
plant, 7 days	1.6	....	101
21 days	1.6	21.0	101
35 days	1.9	23.3	101
49 days	3.2	28.8	101
63 days	6.7	38.4	101
77 days	8.0	37.5	101
91 days	9.0	42.0	101
105 days	10.0	47.0	101

[a] Data reported in terms of "true" cellulose.

Table is concluded on next page

Table 2. — Concluded

Description of sample	Lignin	Cellulose	Reference
Orchard grass, av. 5 stages of maturity	6.6	30.5[a]	104
young	4.9	....	52
heads, just emerged	6.1	37.5	116
av. 6 successive cuttings	6.3	27.0[a]	116
15 samples, simulated grazing	6.1	....	59
young	3.0	....	58
mature	10.5	....	58
preflowering 5–7″	5.5	35.2	9
preflowering 8–10″	4.9	36.7	9
early flowering	6.1	40.6	9
late flowering	8.3	41.2	9
seeding	9.7	43.4	9
hay, 6–7″	7.8	34.0	47
hay, 8–10″	6.7	34.7	47
hay, 10–12″	8.0	37.7	47
hay, 12–14″	9.7	40.3	47
Redtop, av. 5 stages of maturity	6.3	28.8[a]	104
2 samples, simulated grazing	4.8	....	58
Reed canary grass, av. 5 stages of maturity	5.0	27.6[a]	104
Ryegrass, perennial, preflowering, 4–5″	4.7	34.1	9
preflowering, 6–8″	3.7	35.5	9
early flowering	4.5	35.0	9
late flowering	6.9	42.6	9
seeding	12.7	46.2	9
young	4.1	28.5	84, 90
mature	13.0	42.6	84, 90
Rye straw	13.3	49.5	107
Soybean hay	12.6	35.2[a]	52, 102
stalks	13.7	50.5	52
Sudan grass	8.2	27.9[a]	102
Timothy, 1st cut, 8–9″	3.9	18.2[a]	103
2d cut, 12″	4.6	24.3[a]	103
1st cut, 9–12″	3.4	19.0[a]	103
2d cut, 10″	4.0	22.6[a]	103
3d cut, 8″	5.9	26.2[a]	103
mature, headed	8.1	31.4[a]	103
mature, bloom	8.3	32.1[a]	22, 99, 103
hay, 10 samples	9.8	31.3[a]	102
av. 5 stages of maturity	7.3	28.9[a]	104
heads just emerged	7.5	41.0	116
headed	7.4	44.0	116
early bloom	9.8	46.0	116
past bloom	10.5	49.0	116
Wheat straw	11.6	46.6	52, 60
Wheat straw	14.9	51.9	85, 115
Wheat straw	20.7	52.9	107
Ill., combined, good	20.1	49.1	11
Ill., threshed, good	20.8	51.7	11
S. Dakota, threshed, good	18.1	45.5	11
slightly weathered	17.8	49.2	11
medium weathered	21.5	43.1	11
badly weathered	34.0	24.4	11
Wheatgrass, 3 varieties, young	5.4	21.8[a]	96
mature	14.2	32.5[a]	96

[a] Data reported in terms of "true" cellulose.

The Hemicelluloses

The fractionation of the alkali-soluble portion remaining after precipitation of cellulose is attained by neutralization and additions of ethanol. Xylan and mannan have been obtained in a fairly pure state by repeated solution and fractional precipitation. Most of the remainder of this complex mixture called hemicelluloses has yet to be unequivocally separated. Williams and Benevue (130) have ably discussed the problems of isolating the hemicellulosic materials.

The lack of information regarding the constitution and properties of the hemicelluloses can be ascribed to (1) the lack of sufficiently good methods for unequivocal separation, (2) loss of identity of the isolated hemicelluloses, (3) differences in composition of the hemicelluloses extracted from different plants, (4) the fact that the polysaccharides isolated may constitute only fragments of the original polysaccharide, and (5) the fact that the hemicellulose fractions investigated by different workers have not always been comparable. Even though most of the hemicellulose preparations so far obtained constitute mixtures, they have a number of properties in common such as alkali solubility and ease of hydrolysis in acid. The presence of different kinds of molecules, combined with the possibility of different degrees of polymerization of each type, causes increased difficulty in separation. The evidence of Leech (76) for the chemical union between glucose and mannose in gymnosperm cellulose makes it appear that mixed chains may occur in certain cases at least. It is apparent that some difficulty is experienced in differentiating a pure polysaccharide from the hemicellulose fraction. Jermyn (68) states that, "The minimum requirements that must at present be satisfied for a polysaccharide to be considered 'pure' are those of a reasonably narrow range of molecular weights and a constant ratio between sugar-residues, if more than one is present, on subfractionation. If the polysaccharide chains are branched, the branches must all be of the same type. The 'purity' of a polysaccharide molecule must still be defined by specifying criteria satisfactory for a certain purpose; when a polymer molecule, such as cellulose, cannot be dissolved except after degradation, it is difficult to assign any meaning at all to 'chemically pure.' Within these limitations the only polysaccharides that may be considered as having been isolated from the holocellulose fraction of the cell wall in a pure condition are cellulose, xylan, and mannan. The literature reveals many preparations of 'hemicellulose' that have been realized by the authors to be complex mixtures."

Hemicelluloses A and B. Although there are, of course, no standard procedures for the complete separation of the various components by solubility, the fractionation of the hemicelluloses into high-molecular-weight glycans and low-molecular-weight glycans and those containing uronic acid units is obtained by the method of O'Dwyer (93). In the discussion of this method, O'Dwyer relates the hemicelluloses more closely to the pectins than to cellulose because of the presence of uronic acid units. The method is described as the neutralization of the alkali solution upon which a flocculent precipitate occurs. This initial precipitate is called hemicellulose-A and contains the high-molecular-weight glycans. The second fraction is precipitated by the addition of ethanol to the neutralized filtrate and contains the low-molecular-weight glycans. This second fraction is designated hemicellulose-B. The uronic-acid-containing constituents may be distributed to a varying degree in both fractions but are usually found to the greatest extent in the ethanol precipitate. In some cases, the notation is extended as additional purification steps are introduced; but the extent of designation is not uniform and frequently similarly designated fractions isolated by different workers will not possess similar compositions. From most plants the A fraction is considerably the largest because within this fraction occur the abundant and widely distributed xylans. Experimental evidence indicates that xylan is often the only constituent of the A fraction.

Polyuronides. Precise knowledge of the constitution of the somewhat ill-defined polyuronide hemicelluloses is almost nonexistent, owing to the difficulty of isolating homogeneous products. In many cases, the uronic acid constituent has not been identified with certainty.

Much interest is attached to the mode of union of the other sugar units present in the polyuronides and the way in which the uronic acid is joined, especially when the proportion of uronic acid is small. An investigation of the methylated glucoses obtained by the hydrolysis of a methylated hemicellulose fraction of Iceland moss (61) revealed the existence of glucose residues linked through the 1:3, 1:4, 1:6, and the hitherto unreported 1:2 positions. Mannose, galactose, and glucuronic acid were also detected in the products of hydrolysis of the hemicelluloses. An example of the more precise methods of attacking the problem of the mutual union of the sugar units in hemicelluloses is that of Aspinall and Ferrier (12). They isolated hemicellulose from barley husks by solution in N NaOH after extracting them with ethanol-benzene, hot and cold water, and 0.01N NaOH. The hemicellulose was precipitated with acetone after neutralization of the alkaline extract.

The total yield was 7.2 percent of the husk, and the material was found to contain 67 percent xylose, 11 percent arabinose, 2 percent glucose, 1 percent galactose, 3.7 percent uronic anhydride, 7.8 percent lignin, 1.2 percent ash and 1.2 percent methoxyl. From methylation and other studies, it was concluded that the polysaccharide is composed of chains of 1-4-β-D-xylopyranose residues to which are attached side chains of L-arabofuranose and 2-O-D-xylopyranosyl-L-arabofuranose residues through position 3, and glucopyranuronic acid residues through position 2. There appears to be a small degree of branching in the backbone of D-xylose residues.

Occurrence of hemicelluloses in common forages. The first work on the hemicelluloses of timothy hay, using holocellulose as starting material, was done by Flanders (56). In the first of two papers, Flanders described the extraction of the hemicellulose fractions and their pentose and uronic acid contents and gave the following summative analysis of timothy hay on a moisture-free basis:

Constituent	*Percent*
Ash	6.4
Benzene-alcohol extractives	9.9
Water extractives	18.2
Pectic substances as calcium pectate	0.4
Hemicellulose I[a] (5% K_2CO_3)	7.9
Hemicellulose II[a] (2.5% KOH)	6.0
Hemicellulose III[a] (10% KOH)	6.3
Hemicellulose IV[a] (20% KOH)	1.2
α-Cellulose	29.8
Lignin	11.6
Protein	5.1
Total	102.8

[a] Corrected for ash.

Since none of the plant constituents listed above was determined by difference, Flanders concluded that the sum of these constituents is

Table 3.—Percent Yield of Hemicellulose Fractions From Holocellulose[a]
(All values corrected for ash)

Solvent	Alfalfa hay	Barley straw	Buckwheat straw	Oat straw	Red clover hay	Wheat straw
2.5% K_2CO_3	10.9	16.8	8.3	9.5	14.5	11.4
5% KOH	8.7	14.8	11.7	6.8	7.0	13.9
10% KOH	9.3	14.5	18.8	9.7	12.4	16.6
20% KOH	5.2	4.6	8.8	4.5	8.6	5.1
Total	34.1	50.7	47.6	30.5	42.5	47.0

[a] Reference 57.

Table 4. — Percent of Uronic Acid Anhydride and Pentose Obtained From Hemicellulose Fractions[a]

Source of hemicellulose fraction	Solvent			
	2.5% K_2CO_3	5% KOH	10% KOH	20% KOH
Alfalfa hay				
U.A.A.[b]	33.8	17.8	13.3	10.4
Pentose	34.1	64.3	60.8	68.9
Barley straw				
U.A.A.	13.2	8.4	6.5	5.6
Pentose	66.8	71.0	78.0	69.2
Buckwheat straw				
U.A.A.	22.5	13.1	10.8	8.7
Pentose	64.1	62.1	75.9	63.7
Oat straw				
U.A.A.	13.0	8.7	5.5	5.1
Pentose	74.7	76.1	76.2	73.8
Red clover hay				
U.A.A.	30.4	26.5	14.6	13.0
Pentose	22.5	51.9	60.9	61.4
Wheat straw				
U.A.A.	9.7	7.0	5.4	5.0
Pentose	71.1	76.8	75.5	72.9

[a] Reference 57.
[b] Uronic acid anhydride.

Table 5. — Hemicellulose Content of Some Common Feeds[a]
(Expressed on dry basis)

Feed	Hemicellulose	Reference
	perct.	
Alfalfa hay	28.9	57
Barley straw	39.9	57
Beet pulp	5.9	19a
Buckwheat straw	28.9	57
Citrus pulp	9.2	19a
Corn stalks	23.8	19a, 124
Cranberry pulp	13.1	19a
Mixed hay	23.1	19a
Oat hay	27.5	19a
Oat hulls	35.0	71
Oat straw	21.3	57
Orchard grass	12.8	22
Red clover hay	32.9	57
Sheep's fescue	18.1	17
Sweet vernal grass	12.1	17
Timothy hay	22.9	56
Wheat straw	25.2	1, 5, 57

[a] Values given as averages where more than one estimate was available.

as close to 100 percent as could be expected. The molar ratios of pentose:uronic acid range from 10.4 for the more easily extracted hemicelluloses (5% K_2CO_3) to 19.6 for the more difficultly extracted portion (20% KOH).

In his second paper, Flanders (57) dealt with the successive extraction of the hemicellulose fractions obtained from solvents of increasing alkalinity from the chlorite holocelluloses of alfalfa and red clover hays and of barley, buckwheat, oat, and wheat straws. The percentages of holocellulose obtained from the extracted plant materials were: alfalfa hay, 85.0 percent; red clover hay, 77.5 percent; barley straw, 78.7 percent; buckwheat straw, 60.7 percent; oat straw, 69.6 percent; and wheat straw, 79.4 percent. The percentages of hemicellulose obtained from each of these holocelluloses by various alkaline extractions are given in Table 3. The author suggested that before comparisons are made between the plant sources, it should be noted that three different plant families are represented. Alfalfa and red clover are legumes; barley, oats, and wheat are grasses; and buckwheat is a member of the buckwheat family. In all cases, the percent of uronic acid anhydride decreased as the alkalinity of the solvent was increased (Table 4). The pentose content of the hemicellulose fractions did not show this regularity of change (Table 4).

The pentose:uronic acid anhydride ratios increased from the 5% K_2CO_3 fraction through the 20% KOH fraction, but the differences

Table 6. — Pentosan and Uronic Acid Anhydride (Polyuronide) Content of Hemicellulose[a]

Feed	Pentosan	Uronic acid anhydride	Reference
	perct.	*perct.*	
Alfalfa hay	67.1	15.5	57, 98
Barley straw	71.2	8.4	57
Buckwheat straw	66.4	13.7	57
Corn cobs	83.2	4.6	19, 31
Corn stalks	84.5	7.1	20, 124
Lima bean pods	73.3	6.3	31
Oat hulls	85.9	3.8	71
Oat straw	72.7	8.1	4, 57
Red clover hay	49.2	21.1	57
Rye straw	85.8	3.7	19
Sheep's fescue	50.9	6.2	17
Sugar cane fiber	95.6	5.0	38
Timothy hay	80.4	6.5	56
Wheat straw	77.4	7.2	4, 5, 20, 57

[a] Usually determined as a percentage of the hemicellulose fraction; however, many different methods of determination and extraction were used and should be taken into consideration when viewing the results. Values given are averages when more than one investigator is cited.

were usually small. With few exceptions the 5% K_2CO_3 solution yielded the greatest amount of hemicellulosic material, the 10% KOH next, the 2.5% KOH third, and the 20% KOH the least.

The percentages of hemicelluloses and pentosans and uronic anhydrides found in some of the more common feeds are given in Tables 5 and 6 respectively.

FRACTIONATION OF HEMICELLULOSE

While no standard procedure has been outlined for the fractionation of holocellulose, there are some general considerations that may be helpful. It is Jermyn's opinion (68) that the holocellulose should be extracted with alkali of increasing strength (for example, 2, 5, 10, 15, and 24% KOH) and the composition of each of the resulting fractions examined. If they form a continuous series with no indication of abrupt changes of composition, it is probably sufficient for analytical purposes to extract with 24% KOH to divide holocellulose into cellulose and hemicellulose. The α-cellulose preparation, or the alkali-insoluble portion, is usually analyzed for ash and volatile material; lignin, nitrogen, methoxyl, and uronic acid determinations are ordinarily negative.

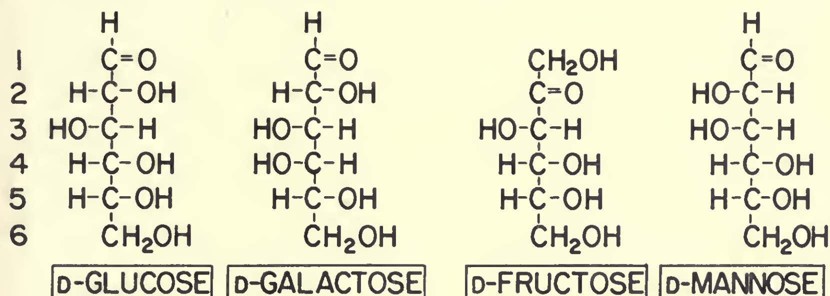
After isolation of the hemicellulose fractions (by the neutralization of the alkali-soluble portion and the addition of ethanol), the hydrolysis of these fractions must be considered. The chemical relation of the hydrolysis products of hemicelluloses is given in Fig. 4.

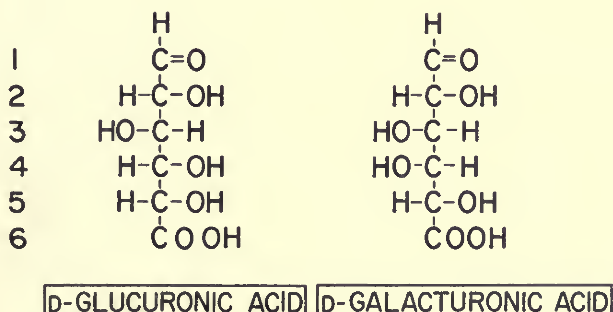
Various methods have been tried for the hydrolysis of the hemicelluloses. Jermyn (68) uses 3% nitric acid and gradually raises the digestion temperature from room temperature to boiling over about a half-hour period. The hydrolysis is carried out for 3.5 hours, and at the end of this time any undissolved material is tested for the presence of carbohydrate. If a negative test is found, the hydrolysis is regarded as being complete.

Identification of Various Sugars

Bishop and Adams (23) hydrolyzed the hemicellulose fraction of wheat straw by heating with 1% sulfuric acid in a boiling water bath. The progress of hydrolysis was followed by changes in reducing power and was stopped when this value reached a maximum, which usually required a period of 12 hours. A noncarbohydrate residue amounting to as much as 7.0 percent of the hemicellulose fractions was noted.

For examination of the constituent sugars, the acid hydrolyzate of each hemicellulose fraction was neutralized with barium carbonate, an aliquot chromatographed, then eluted from the paper, and the reducing value of the eluent determined. On the basis of analyses for individual





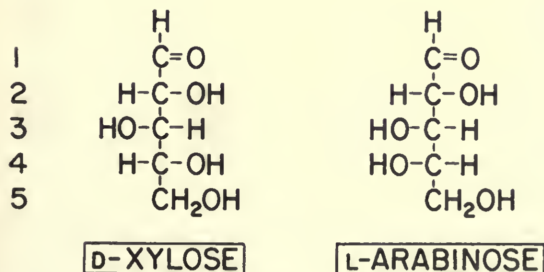


Fig. 4.— Chemical relation of hydrolysis products of hemicellulose.

sugars, all of the hemicellulose fractions contained D-xylose, L-arabinose, D-glucose, D-galactose, and hexuronic acid in molar ratios of 40:7:2:1:4.

The sugars from the hemicelluloses of corn stalks were obtained by Bennett (20) by the hydrolysis of the hemicellulose fractions A, B, C, and D (in order of resistance to extraction) for 10 hours with 4% sulfuric acid in a weight:volume ratio of 1 to 50 in an all-glass assembly in a boiling water bath. Nitrogen gas was bubbled through the mixture to reduce oxidation to a minimum. Fig. 5 shows the combined paper chromatogram of the sugars obtained from hemicellulose fractions A, B, C, and D as compared with a control. The molar ratios of L-arabinose, D-xylose, and D-glucose plus D-galactose in the hydrolyzates of the hemicellulose fraction given by Bennett were:

Fraction	*L-arabinose*	*D-xylose*	*D-glucose + D-galactose*
A	1.0	7.4	1.7
B	2.3	6.3	1.0
C	2.3	9.6	1.0
D	1.0	13.0	1.0

The same sugars were present in all fractions, but in different proportions.

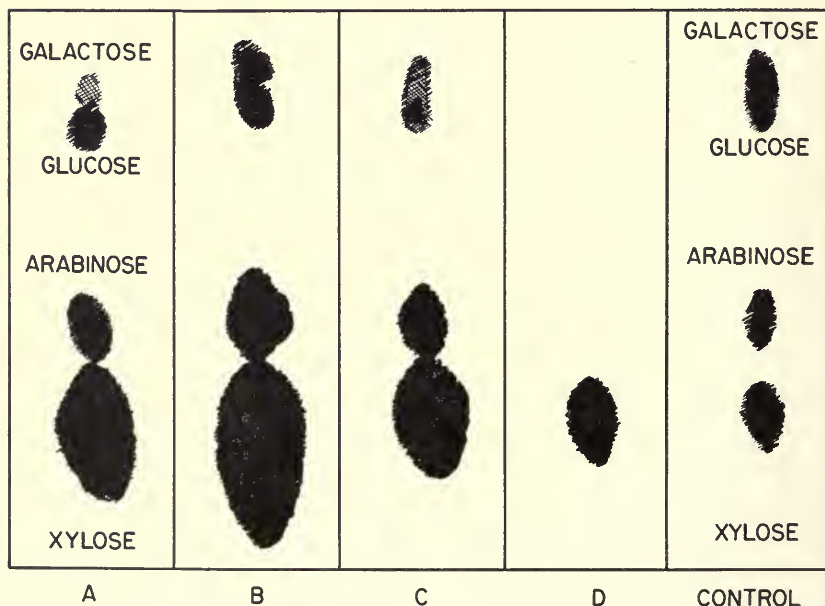


Fig. 5. — Paper chromatograms of sugars from hemicellulose fractions.

Estimation of Pentoses and Pentosans

The chromatographic technique of identification and isolation of the sugars of the hemicelluloses is time-consuming. A good colorimetric method for use directly on the hydrolyzate would be a great advantage. However, a quantitative estimate of pentoses and pentosans, which make up the larger portion of the hemicelluloses, is very difficult to obtain with the present methods of analysis. The classical method of determination by destructive distillation with 12% hydrochloric acid and measurement of furfural formed can only be used to advantage when a relatively "pure" pentose or pentosan material is analyzed. When used in the analysis of the cell-wall polysaccharides, however, the yield of furfural is not quantitative as it includes arabinose, xylose, and uronic acids, all with different correction factors. Any hexoses or hexosans present will cause further error in the test.

Various methods for the estimation of the pentosan fraction of hemicelluloses are used, but none is without a possible error or assumption. The most common method used is that outlined in the A.O.A.C. (14) which includes the addition of 12% hydrochloric acid and the determination of furfural by the addition of phloroglucinol. Correction factors are given.

Tracey (121) described a colorimetric method for the estimation of pentoses in the presence of large amounts of hexoses and uronic acids. This is accomplished by allowing the reaction to take place at room temperature and taking advantage of the fact that pentoses, especially xylose, react faster. Factors influencing color development, nature of the reaction, sensitivity, and specificity are given. Difficulty can still be encountered, however, when using this method for the estimation of cell-wall polysaccharides as it is not completely specific.

McRary and Slattery (79) outlined a colorimetric determination of pentoses and pentosans which has the same limitations as other tests in reference to accuracy of estimation of the material present in hemicelluloses.

Estimation of Uronic Acids

A number of methods have been described for the determination of uronic acids. Most of them depend on the fact that the acid may be more or less quantitatively decarboxylated when heated with sufficiently concentrated mineral acids and the carbon dioxide liberated can be determined as a measure of the uronic acid, free or combined, originally present. Unless the substances being investigated are relatively pure, however, it is highly unlikely that the yield of carbon dioxide will give a true measure of the uronic acid content. It may

therefore be quite feasible to use the above method for a substance such as pectin, which is a relatively pure polygalacturonic acid, but when the amount of uronic acid is low, the error encountered will be decidedly greater. This is particularly true in the analysis of the hemicelluloses where only traces of uronic acids may be present.

The method most commonly used for uronic acid determinations of hemicelluloses is that of Tracey (120). The uronic acid is decarboxylated by heating with strong acid. The carbon dioxide produced is measured manometrically using a Van Slyke-Neil apparatus. This method, however, requires considerable time and intricate manipulations and would be difficult to use for routine investigations.

For routine determinations, the micro titrimetric procedure described by Ogston and Stanier (94) is probably more applicable. This method involves the absorption of carbon dioxide in barium hydroxide in a closed system and titration with acid using a microburette. The mixtures should be stirred with a stream of carbon dioxide-free nitrogen.

In a method described by Browning (30) the carbon dioxide evolved is trapped in an absorbing tube and the difference weighed. This method is seemingly accurate for large amounts of carbon dioxide, but ineffective for the usual small quantities that are dealt with in routine laboratory investigations.

Many of the errors arising from the evolution of carbon dioxide from substances other than uronic acid may be eliminated by the methods of Nevell (83). Here the course of carbon dioxide evolution is plotted against time, and the resultant curve is found to fall into two parts, an initial steeply rising portion caused by carbon dioxide evolution from uronic acids, followed by a second, more linear portion caused by the slower evolution of carbon dioxide from other carbohydrates. The latter portion of the curve is extrapolated to zero time, and the intercept gives the amount of carbon dioxide evolved from uronic acids alone. When sufficient time and material are available, this may be the most accurate method of estimation.

The presence of naturally occurring carbonates may also render uronic acid analyses invalid. When hemicelluloses have been prepared by alkaline extraction, stringent precautions are usually necessary to prevent the presence of alkali carbonate in the hemicellulose preparations.

Another method used for the quantitative determination of the hemicellulose constituents is that of selective fermentation (15). This method, like chromatography, takes considerable time, labor, and equipment, but the possibilities of accuracy may warrant its use in some cases.

SOLUBLE SUGARS

Monosaccharides and Oligosaccharides

A relatively small amount of free glucose is present at any one time in most plants (about 1 percent). Fructose is usually found in somewhat larger amounts in the free form in plants than is glucose and is more subject to seasonal variations (42, 74). Fructose, like glucose, is present mainly in the polymeric form. Clover and alfalfa appear to contain the same free sugars as those found in grasses (74). In most analyses, glucose and fructose are combined under the heading of hexoses, and galactose, if present, may also be included. The presence of D-mannitol in perennial ryegrass has been demonstrated (63).

Different types of oligosaccharides have been reported to be present in plants, as may be expected because of the varying degrees of polymerization of some of the polysaccharides. Sucrose is by far the most important, both in quantity and in its importance to plant metabolism. There is evidence that sucrose is utilized in the formation of fructans by enzymatic transfructosylation. The presence of melibiose, raffinose, and stachyose has also been demonstrated.

Lagowski *et al.* (72) isolated from alfalfa, and identified by paper chromatography, a nonreducing substance which was tentatively identified as sucrose. However, attempts to crystallize the substance proved unsuccessful. The lack of a sufficient amount of material prohibited identification by optical rotation or the preparation of a derivative. This is probably the first indication of the presence of a nonreducing sugar in alfalfa.

Fructans

During the period 1870-1900, a number of fructose polymers were discovered in the monocotyledons. These were distinguished from inulin by their great solubility in cold water. The discovery of fructans in the cereal grains was largely due to attempts by agricultural chemists to isolate dextrins as intermediate products between sugar (glucose) and starch. The chief difference noted in this new compound was that it was levorotatory. The significance of the levorotatory fructans was overlooked for about 20 years mainly because the preparations were not pure (the main impurity was sucrose) and because of the controversy as to the presence or absence of dextrins. Since about 1928 the significance of the fructans has been appreciated. The reserve function and occurrence in storage organs were stressed at first although the existence of fructans in the leaves was recognized quite

early. A comprehensive review of the studies on fructans up to 1940 was written by Archbold (7).

The fructans are the main reserve substances in perennial grasses but are not found in alfalfa or clover, where they seem to be replaced by araban and galactan with small amounts of water-soluble polyglucosans (74). From the nutritional and reproductional standpoint of the perennial grasses, the fructans are of prime importance. The ease of hydrolysis, coupled with the ready fermentation of the hydrolytic products, suggests their importance in contributing to the silage-making qualities of many grasses. Fructans are described by Whelan (126) as levorotatory, amorphous or microcrystalline, of varying solubility in cold water, very soluble in hot water, insoluble in absolute alcohol, and not precipitated by barium hydroxide, either directly or on addition of alcohol. They are nonreducing, unfermented by yeast (this may not be true of low-molecular-weight fructans), resist amylase action, and are very susceptible to hydrolysis by acid. They do not color iodine, but hydrochloric acid vapor imparts a purple color which distinguishes them from polysaccharides not containing fructose.

Plants contain enzymes which readily hydrolyze fructans (133). A very rapid degradation of fructan is also noted during experimental ensiling procedures. Fructans are usually categorized into two main divisions (111): those having a 1, 2′ linkage or the inulin type, and those with a 2, 6′ linkage or the phlein type. Intermediate stages having both types of linkages are also found. The phlein type is the one occurring for the most part in grasses and is distinguished from the inulin type in that the molecules are thought to lie like the pages of a book while in the phlein type the molecules are end to end or linear (95).

Fructans occur in many of the grasses, especially perennial grasses, and have been shown to occur in all parts of the plant — roots, stems, and leaves. The amount found in any one part of the plant has been definitely shown to be related to season, effects of defoliation, influence of fertilizers, and grazing methods (114, 117). These are discussed at some length by Weinmann (125).

Although many specimens of fructans have been isolated and named (7, 129), detailed analysis has been done by only a few, with H. H. Schlubach being foremost in this field.[1] Hirst, McGilvray, and Percival (66) and Cuendet (37) were the first to postulate that sucrose was present as the end-group of inulin. It is the general opinion that sucrose is involved in the synthesis of fructans through enzymatic transfructosylation (6, 16, 39, 40, 41, 45, 64, 110).

[1] H. H. Schlubach and co-workers have published 48 papers on the fructans, which appeared in *Annalen.*

One major difference among the fructans is their molecular weight. Bacon and Edelman (16) chromatographed components from the Jerusalem artichoke. Altogether there were seven nonreducing components with R_f values ranging from that of sucrose to zero. The component not moving was assumed to be inulin. This is indicative of the presence of lower-molecular-weight polysaccharides, and when the implications are known there may be a great deal of light thrown on the synthesis and breakdown of fructans in plants. Porter and Edelman (106) also have demonstrated through the use of chromatography the presence of compounds ranging from sucrose to possibly phlein.

A method of calculating the molecular weight of fructans is given by Palmer (95) in which the material is hydrolyzed and the glucose content is determined. On the assumption that there is one glucose unit per molecule of fructan, the molecular weight is calculated from the amount of glucose present.

Fructans have been isolated from various grasses (129) — couch or quack grass, bent grass or red top, oat grass, wild rye, barley, ryegrass, wheat, rough stalk bluegrass, and meadow grass. They are particularly abundant in artichoke, chicory, dahlia tubers, and sea onion. Waite and Boyd (122, 123) have found that the stems of growing plants commonly contain much higher levels of fructose polymers than does the leaf portion of the same plant.

Norman, Wilsie, and Gaessler (91) surveyed some of the more important grasses adapted to Iowa with regard to percentage of fructan at different seasons of the year and different stages of maturity. In general the average content was of the order of 2 to 4 percent fructan. Sudan grass, smooth bromegrass, Kentucky bluegrass, orchard grass, reed canary grass, and domestic ryegrass were analyzed. Analyses of individual plant materials varied more than the fructan content of the various species, indicating that the stage of growth was more closely related to the fructan content of the grass than was the species. The limits of the values determined were from 1.69 to 7.6 percent of the dry matter. The averages were: sudan 4.14, smooth bromegrass 2.16, Kentucky bluegrass 3.29, orchard grass 2.54, reed canary 2.76, and domestic ryegrass 3.26. These values merely serve to indicate the variability of the fructan content. These values, and those reported by Johns (70) in Australia are to be contrasted with those recorded by Waite and Boyd (122, 123) and results of analyses done in the USSR by Morosov (82).

Data showing the variation of fructan content to be expected between species, season, or plant part are presented in Table 7.

Table 7. — Variation of Fructan Content of Some Common Plants Due to Species, Season, and Part of Plant
(Expressed on dry basis)

Plant	Part	Average	Range	Period	Date of high	Date of low	Reference	Number of determinations
		perct.	*perct.*					
Couch grass	Rhizomes	14.8	....	Apr. 48	....	....	10	1
Domestic ryegrass	Aerial	3.26	2.93–3.60	9 June–20 June 39	9 June	20 June	91	2
Fescue	Aerial	4.0	.6–11.6	17 May–2 Oct. 51	17 May	14 Aug.	123	8
Fescue	Aerial	1.9	.2–5.2	28 Apr.–29 Sept. 52	19 June	22 July	123	7
Fescue	Leaf	1.3	.2–4.8	28 Apr.–29 Sept. 52	19 June	22 July	123	7
Fescue	Stem	5.4	1.0–13.9	28 Apr.–29 Sept. 52	19 June	22 July	123	7
Kentucky bluegrass	Aerial	3.29	2.12–3.82	2 May–11 July 39	9 June	20 June	91	5
Orchard grass	Aerial	2.54	....	29 May 39	....	....	91	1
Orchard grass	Upper ⅔ of leaf blade	7.6	....	Greenhouse	....	....	114	1
Orchard grass	Lower ⅓ of leaf blade	22.0	....	Greenhouse	....	....	114	1
Orchard grass	Upper ½ of stubble	23.7	....	Greenhouse	....	....	114	1
Orchard grass	Lower ½ of stubble	36.2	....	Greenhouse	....	....	114	1
Orchard grass	Roots	8.2	....	Greenhouse	....	....	114	1
Orchard grass	Aerial	2.3	.2–10.7	15 June–17 Sept. 51	15 June	17 Sept.	123	6
Orchard grass	Aerial	.8	0–2.4	7 May–29 Sept. 52	7 May	2 July	123	7
Orchard grass	Leaf	.3	0–0.5	7 May–29 Sept. 52	7 May	2 July	123	7
Orchard grass	Stem	.3	0–4.4	7 May–29 Sept. 52	7 May+ 24 Sept.	2+22 July	123	7
Orchard grass	Aerial	3.0	....	26 May 49	....	....	13	1
Orchard grass	Aerial	3.6	2.0–4.9	3 May–6 June 49	6 June	3 May	74	3

Table 7. — Concluded

Plant	Part	Average	Range	Period	Date of high	Date of low	Reference	Number of determinations
		perct.	*perct.*					
Reed canary grass	Aerial	2.76	2.38–3.11	2 May–9 June 39	9 June	29 May	91	4
Ryegrass	Stubble	15.04	14.33–16.17	17 March 41	....	....	117	3
Ryegrass	Roots	1.81	1.64–1.92	17 March 41	....	....	117	3
Ryegrass	Aerial	4.44[a]	....	3 May 49	....	....	73	1
Ryegrass	Aerial	16.6	15.8–17.4	6–7 June 51	Noon	6:00 p.m.	122	8
Ryegrass	Aerial	4.1	.5–16.1	15 June–18 Sept. 51	15 June	15 Aug.	123	6
Ryegrass	Aerial	5.4	.9–12.7	20 May–21 Oct 52	25 June	11 July	123	6
Ryegrass	Leaf	4.1	.4–12.1	20 May–21 Oct. 52	25 June	11 July	123	6
Ryegrass	Stem	14.1	5.4–20.9	20 May–21 Oct. 52	21 Oct.	11 July	123	6
Ryegrass	Aerial	14.3	5.0–22.5	23 April–28 June 48	7 June	23 April	36	4
Ryegrass	Aerial	9.6	....	3 May 52	....	....	133	1
Ryegrass	Aerial	9.7	6.8–12.1	3 May–6 June 49	6 June	3 May	74	3
Smooth bromegrass	Aerial	2.16	1.69–2.89	2 May–29 May 39	2 May	29 May	91	3
Sudan grass	Aerial	4.14	2.33–7.60	29 May–5 Aug. 39	3 July	20 June	91	17
Timothy	Aerial	4.3	.6–9.3	18 June–17 Sept. 51	18 June	17 Sept.	123	4
Timothy	Aerial	1.8	.3–5.7	12 May–30 Sept. 52	12 May	29 July	123	7
Timothy	Leaf	.8	.0–2.3	12 May–30 Sept. 52	12 May	12 July	123	7
Timothy	Stem	4.9	1.9–10.0	12 May–30 Sept. 52	30 Sept.	21 Aug.	123	7

[a] Wet weight.

Some interesting analytical studies on the carbohydrates of grasses and clovers have been done in Britain. Laidlaw and Reid (74) discussed the development of methods for the estimation of the free sugar and fructan contents. They employed an 80% ethanol extraction followed by hydrolysis of the fructan in 1% oxalic acid and separation and identification of the components qualitatively and quantitatively using paper chromatography. They obtained the following variations in concentrations, due to date of cutting and maturity of the plant:

	Percent sucrose	*Percent glucose*	*Percent fructose*	*Percent fructan*
Perennial ryegrass	3.6–7.4	1.2–1.4	1.3–2.6	6.8–12.1
Cocksfoot (orchard grass)	2.6–3.7	0.9–1.2	1.3–2.1	2.0–4.9*

* Difference principally in the fructan composition.

Proper preparation of grass samples for analysis is of utmost importance. Laidlaw and Wylam (75) found that satisfactory results for the free sugar and fructan contents of grasses were obtainable in freeze-dried specimens if the analyses were performed immediately after freeze-drying and milling. They found the following variations in results when samples were not analyzed immediately:

	Percent sucrose	*Percent glucose*	*Percent fructose*	*Percent fructan*
Initial analysis of freeze-dried grass	5.3	0.6	0.8	4.0
Kept 1 week at room temperature	5.4	0.4	1.4	3.6
Kept 1 week at 0° C.	4.0	0.6	1.5	3.9
Kept 5 weeks at room temperature	5.1	0.5	1.6	3.5
Kept 5 weeks at 0° C.	4.9	0.8	0.9	3.5
Dried further at 35° C. and 0.01 mm. Hg over P_2O_5, and kept 4 weeks *in vacuo* at room temperature	4.4	1.2	1.7	3.8

Probably of more immediate practical concern is a study by Wylam (133) on the carbohydrate breakdown during wilting and ensilage. The implications of the enzymatic breakdown of sucrose and fructan that occurs during wilting and laboratory ensiling of grasses are discussed with respect to the collection of grass samples for analysis and the preparation of the silage. The effect of wilting is readily seen in his figures:

	Dry matter perct.	*Percent of dry weight*			
		Sucrose	*Glucose*	*Fructose*	*Fructan*
Fresh grass	18.4	6.2	1.3	1.7	9.6
Wilted 2 hours	20.6	5.1	1.3	1.7	9.2
Wilted 24 hours	44.6	5.4	1.9	2.9	5.2
Wilted 8 days	78.1	5.2	1.6	1.4	3.5
Bottled 4 hours	18.4	4.8	1.7	2.1	7.1
Bottled 96 hours	18.4	3.4	3.2	2.4	2.8

Wylam also shows the changes of the various sugars present with respect to time and pH:

	Percent of dry weight of fresh grass		
	Fresh grass	*Ensiled 8 days pH 3.8*	*Ensiled 8 months pH 3.5*
Glucose	6.7	8.3	3.0
Fructose	4.5	6.2	10.8
Sucrose	5.2	0.3	nil
Fructan	8.4	1.8	nil
Oligosaccharides	present	present	absent
Galactose	nil	1.0	0.7
Arabinose	nil	nil	1.2
Xylose	nil	nil	1.3
Total soluble CHO (excluding oligosaccharides)	24.8	17.6	17.0

Sprague and Sullivan (114) found that, in general, the fructan is largely concentrated in the stem and lower stem portion of the plant in rather high amounts with as much as 36 percent of the lower portion of the stem being fructans. Their results with orchard grass grown in the greenhouse were:

	Percent of weight of entire plant	*Percent of dry weight*		
		Reducing sugars	*Sucrose*	*Fructan*
Upper ⅔ of leaf blades	14.0	1.4	8.4	7.6
Lower ⅔ of leaf blades	12.1	1.2	5.8	22.0
Upper ½ of stubble*	9.4	1.9	3.6	23.7
Lower ½ of stubble*	23.6	0.7	2.6	36.2
Roots	40.9	1.2	8.9	8.2

* All parts other than leaf blades and roots.

Sprague and Sullivan also report changes in composition of orchard grass with respect to sucrose, reducing sugar, and nitrogen (soluble and insoluble) at various intervals after cutting, especially as affected by low-nitrogen and high-nitrogen fertilization. One result of particular interest is that high-nitrogen fertilization tended to reduce the percentage of fructan and low-nitrogen fertilization tended to increase it.

THE PECTIC SUBSTANCES

Another group of cell-wall materials is that of the pectic substances, derivatives of pectic acid, which in turn is made up of long chains of D-galacturonic acid residues. The galacturonic acid residues all possess the 6-membered pyranose ring structure. In pectic acid the carboxyl groups are free and so are able to combine with available cations such

as calcium, in which case the insoluble calcium pectate is precipitated. In pectin and the protopectin, both of which are derivatives of pectic acid, the carboxyl groups are masked and are in fact esterified with methyl alcohol. Thus the chain structure of pectin and protopectin is similar to that of pectic acid, except that the carboxyl groups are methylated.

In the living plant, protopectin is almost exclusively confined to the cell wall. Pectin, however, is found dissolved in the plant juices and is presumably present in the protoplasm of the cell. Pectic acid, in the form of its calcium and magnesium salts, appears to make up much of the middle lamella of the cell wall and promotes the adhesion of adjacent cells. When the pectate of the middle lamella is removed, for example through precipitation of its calcium with oxalate or by hydrolysis with the appropriate enzyme, pectinase, the cells of the tissue concerned tend to separate from one another. The process of maturation of fruit involves the conversion of protopectin to pectin.

Although the amount of pectic substances in forages is usually small, between 1.0 and 1.5 percent (55), a total pectin content of alfalfa of about 17 percent has been reported (98).

LIGNIN

Constitution

Although included in the carbohydrate fraction by reason of association, lignin is not carbohydrate in nature. It is a high-molecular-weight condensation product of one or more types of aromatic compounds intermixed in the cell wall with cellulose and other constituents. Woody plants may contain up to 50 percent lignin, but roughages will contain from 2 or 3 percent up to about 20 percent, depending on the stage of maturity (Table 2, page 20). Pigden (105) has shown that distribution of lignin within range grasses may vary, and this, as well as amount of lignin, influences curing properties.

We cannot yet assign a correct chemical structure to lignin, but many studies of degradation products have provided much information about its structural units. As might be expected, the products vary both with the source of lignin and with the type of degradative reaction employed. Corn cob lignin has been found to yield on dry distillation a mixture of catechol, guiacol, p-cresol, o-cresol, n-propylguiacol, and other compounds. Most of these same products may be obtained by distillation with zinc dust in an atmosphere of hydrogen. Varied theories about the intimate structure of lignin have been developed

from studies of degradation products and of chemical and physical properties of the parent material. Attempts to synthesize lignin on the basis of these findings have been only partly successful. A more complete description of the problems of lignin structure is given in the excellent book by F. E. Brauns (27).

Brauns summarizes his discussion of the structure of lignin in the following vein: Results of the hydrogenation of lignin, whereby up to 50 percent cyclohexyl-propane derivatives are obtained, show that a large part is made up of phenyl-propane derivatives, because there can be no doubt that the cyclohexyl ring results from the hydrogenation of the benzene rings. That benzene rings occur in lignin is shown not only by the analysis (which indicates a strong unsaturation) but also by the fact that on mild alkaline oxidation up to 25 percent vanillin has been obtained. The formation of vanillin from lignin further proves that, in at least a part of the lignin, the phenyl ring contains a methoxyl group in the m-position and a hydroxyl group or phenyl ether linkage in the p-position to the side chain.

There is little doubt that lignin is a high polymer which is formed to the extent of at least 75 percent from phenylpropyl derivatives. The manner of their linkage is not clear, but in at least a part of the lignin molecule, the carbon atom at the 5-position of the benzene ring is connected with the side chain of another unit by a C-C linkage. On the other hand, the formation of 5-iodovanillin by the iodination of lignin is evidence that some of the 5-carbon is free (this is undoubtedly so in end groups and may also be true in central groups linked by phenol ether linkage).

The presence of about 2 percent phenolic hydroxyl indicates that the lignin molecule is built up from 4 to 5 phenylpropyl units. Other evidence points to molecular weight of the building units of about 840.

We are still far from the solution of the problem of lignin structure, as may be seen from consideration that in 1820 Braconnot discovered that glucose is the primary unit of cellulose. It was more than 100 years later that the correct mode of combination of the glucose anhydride units in cellulose was elucidated. In the chemistry of lignin, we do not even know the exact structure of the primary units.

Elementary analysis of lignin reveals 62 to 63 percent carbon and 5 to 6 percent hydrogen, again emphasizing its aromatic nature. Methoxyl groups occur in amounts varying from 4 to 20 percent, depending on species of plant, maturity, and method of isolation of lignin (27). Free hydroxyl and phenolic groups are present in varying amounts, as are ether linkages. The lignin isolated from feedstuffs always contains nitrogen, but the hypothetical formulas for lignin structure have ignored

this. It is thought by some investigators, that the nitrogen is not an integral portion of the lignin molecule but represents mainly protein in the feedstuffs that cannot be removed, and many investigators determine "true lignin" by correcting the "crude lignin" content by the amount of N × 6.25 it contains, assuming the N to be of protein origin. Others, such as Bondi and Meyer (24), believe the N to be an integral portion of the molecule; these latter authors interpret their data to indicate presence of ring-bound, tertiary nitrogen. It seems likely that both kinds of N are present in the lignin isolated by quantitative procedures. The evidence that amino acids may be obtained by hydrolysis of crude lignin (Thomas and Armstrong (118), De Man and de Heus (43), Forbes and Hamilton (60)) is convincing. It is also true that the lignin studied by Bondi and Meyer represents only that portion of the lignin which is solubilized from plant tissue by ethanolic alkali and does not represent all of the lignin present in the sample extracted by this method.

Estimation of Lignin

An excellent brief account of the more common methods used in quantitative determination of lignin is given by Thomas and Armstrong (118). The procedures generally employed have the common feature of removal of nonlignin matter, leaving the lignin as a residue to be measured gravimetrically. For this purpose the major reagent is either fuming (42-43%) HCl or 72% (by weight) H_2SO_4 for hydrolysis of cellulose. Under appropriate conditions neither of these reagents will appreciably degrade lignin, but care must be taken to prevent the conversion of nonlignin matter into insoluble products or into products that will react with lignin and thus increase the apparent lignin content. Recent modifications of these two major methods have been directed toward methods effecting a more complete removal of these interfering materials. The 72% H_2SO_4 method is most commonly used today, and further comments on methodology will relate to this procedure.

Norman and Jenkins (88, 89) performed much of the fundamental work in demonstrating the effects of carbohydrate and protein on the lignin determination. Following the lead of Ritter, they showed that pentosans may be hydrolyzed and converted to furfuraldehyde slowly in the presence of 72% H_2SO_4 and that furfuraldehyde may combine with lignin, resulting in high apparent lignin yields. Under appropriate conditions, 2 hours at 20° C., this effect was minimal, and this is the basis of the present recommendation for 72% H_2SO_4 treatment. Norman and Jenkins also observed that lignin isolated from plant materials of high protein content or isolated in the presence of added protein, contained more nitrogen than that from low-nitrogen plant

materials and more "apparent lignin" than when no protein was added to the sample. They adopted a pretreatment with 5% H_2SO_4 to hydrolyze the protein and to remove the major portion of the pentoses. They also recommended following the 72% H_2SO_4 acid treatment with a 3% H_2SO_4 treatment to complete hydrolysis and solution of nonlignin material. The widely used method of Ellis, Matrone, and Maynard (46) is essentially the method of Norman and Jenkins, with the addition of an acid pepsin digestion prior to the 5% H_2SO_4 treatment. Neither of these groups of investigators advocates the use of a correction factor for the nitrogen remaining in the lignin. Armitage, Ashworth, and Ferguson (8) advocate the use of an alkaline trypsin digestion following the dilute acid treatment in place of the acid-pepsin treatment prior to dilute acid treatment. They also correct the crude lignin for protein ($N \times 6.25$). In a series of careful studies, Thomas and Armstrong (118) have shown that correction for N, considering it as of protein origin, eliminates most of the differences found in lignin content of grasses determined by different modifications of the 72% H_2SO_4 method. They have also investigated the conditions necessary for removal of fats and waxes which may be resistant to 72% H_2SO_4 and have found that the 4-hour alcohol-benzene extraction procedure of Ellis *et al.* (46) gives much less extractive material and yields somewhat more lignin than does the 30-hour extraction with alcohol-benzene recommended by MacDougall and DeLong (77). A final important consideration in lignin analysis is the manner in which the sample is treated prior to chemical analysis. MacDougall and DeLong showed clearly that drying of grass samples at 105° C. yielded significantly higher results for lignin than did drying at air temperature or at 60° C. They concluded that the higher temperature permitted reactions between lignin and both nitrogenous and carbohydrate components of the plant and also caused the formation of insoluble artifacts, probably from carbohydrates. Ellis *et al.* (46) have confirmed this effect of temperature on lignin analysis of plants, and Thomas and Armstrong have done so with feces.

Moon and Abou-Raya (81) have studied in great detail the various sources of error in the 72% H_2SO_4 method and have concluded that an "acid-insoluble" lignin may be determined by altering the customary procedure to eliminate reprecipitation by dilute acid of lignin-like matter dissolved by 72% H_2SO_4. Their procedure does not yield a "purer" lignin than, for example, the Ellis (46) or Armitage (8) methods, and only yields a portion of the total lignin. The major advantage of the Moon method is a relative simplicity of procedure by facilitating filtration and omission of hydrolysis with 5% and with 3% H_2SO_4.

CONCLUDING REMARKS

It has been the purpose of this publication to review critically the nature and distribution of the carbohydrates of roughages as revealed by the more recent methods of analysis. Emphasis has also been placed on the effects of alterations in analytical methods on the qualitative and quantitative results.

One of the more important advances in this area has been the development of improved methods for isolating holocellulose from plant materials. Holocellulose, consisting of cellulose, hemicelluloses, and some lignin, serves as a good starting material for studies of the structural chemistry of these complex carbohydrates. It remains important, however, to specify the method of analysis used, since holocellulose shares with crude fiber the characteristic of varying in quality and quantity, depending on the method of analysis as well as on the plant source.

Progress has been made in unraveling the complex constitution of the hemicelluloses. This interesting and baffling problem will be solved only after means of isolating "pure" polysaccharide polymers have been improved.

The presence of fructans in plants has been recognized for many years, but their structure and distribution in the plant have been extensively studied only in recent years, and it is suggested that the fructans are probably related to ensiling qualities of perennial grasses.

The unique position of lignin among the plant carbohydrates is due to its unreactivity, as a result of which its presence may strongly affect the availability of plant nutrients to animals and also may affect the curing properties of forages. Current lignin studies have mainly been devoted to modifying the classical methods of analysis, having the aim of procuring quantitative yields of true lignin. These studies are difficult, since no good standard exists with which to judge lignin purity. There is interesting evidence that the anatomic distribution of lignin in forages as well as the absolute amount influences their curing qualities. Continued interest and investigation into the roughage carbohydrates will eventually permit a more adequate description of this major fraction of plant organic matter, and as a consequence a better understanding of plant physiology and of the significance of plant carbohydrates in animal nutrition will be attained.

BIBLIOGRAPHY

1. ADAMS, G. A. Can. J. Chem. **32**:186. 1954.
2. ADAMS, G. A., and CASTAGNE, A. E. Can. J. Res. **26B**:325. 1948.
3. ADAMS, G. A., and CASTAGNE, A. E. Can. J. Res. **27B**:907. 1949.
4. ADAMS, G. A., and CASTAGNE, A. E. Can. J. Res. **27B**:924. 1949.
5. ADAMS, G. A., and CASTAGNE, A. E. Can. J. Chem. **29**:109. 1951.
6. ALLEN, P. J., and BACON, J. S. D. Biochem. J. **63**:200. 1956.
7. ARCHBOLD, H. K. New Phytol. **39**:185. 1940.
8. ARMITAGE, E. R., ASHWORTH, R. B., and FERGUSON, W. S. J. Soc. Chem. Ind. **67**:241. 1948.
9. ARMSTRONG, D. G., COOK, H., and THOMAS, B. J. Ag. Sci. **40**:93. 1950.
10. ARNI, P. C., and PERCIVAL, E. G. V. J. Chem. Soc. p1822. 1951.
11. ARONOVSKY, S. I., YOUNGER, J. O., and NELSON, G. H. Paper Trade J. **120** (8):124. 1945.
12. ASPINALL, G. O., and FERRIER, R. J. J. Chem. Soc. p4188. 1957.
13. ASPINALL, G. O., HIRST, E. L., PERCIVAL, E. G. V., and TELFER, R. G. J. J. Chem. Soc. p337. 1953.
14. ASSOC. OFF. AG. CHEM. Official methods of analysis. 8th edition. 1955.
15. AUERNHEIMER, A. H., WICKERHAM, L. J., and SCHNIEPP, L. E. Anal. Chem. **20**:876. 1948.
16. BACON, J. S. D., and EDELMAN, J. Biochem. J. **48**:114. 1951.
17. BENNETT, E. J. Biol. Chem. **146**:407. 1942.
18. BENNETT, E. Anal. Chem. **19**:215. 1947.
19. BENNETT, E. J. Ag. Res. **75**:43. 1947.

19a. BENNETT, E. Anal. Chem. **20**:642. 1948.

20. BENNETT, E. Arch. Biochem. **27**:99. 1950.
21. BENNETT, E. Anal. Chem. **24**:1510. 1952.
22. BINGER, H. P., SULLIVAN, J. T., and JENSEN, C. O. J. Ag. Food Chem. **2**:696. 1954.
23. BISHOP, C. T., and ADAMS, G. A. Can. J. Res. **28B**:753. 1950.
24. BONDI, A., and MEYER, H. Biochem. J. **43**:248. 1948.
25. BONNER, J. Plant biochemistry. Acad. Press, Inc., New York. 1950.
26. BONNER, J., and GALSTON, A. W. Principles of plant physiology. W. H. Freeman and Company, San Francisco. 1952.
27. BRAUNS, F. E. The chemistry of lignin. Acad. Press, Inc., New York. 1952.
28. BROWN, E. M. Missouri Ag. Exp. Sta. Res. Bul. 360. 1943.
29. BROWNE, C. A. J. Assoc. Off. Ag. Chem. **23**:102. 1940.
30. BROWNING, B. L. Tappi **32** (3):119. 1949.
31. CARSON, J. F., and MACLAY, W. D. J. Am. Chem. Soc. **70**:293. 1948.
32. CHANDA, S. K., HIRST, E. L., JONES, J. K. N., and PERCIVAL, E. G. V. J. Chem. Soc. p1289. 1950.
33. CRAMPTON, E. W. Sci. Ag. **19**:345. 1939.
34. CRAMPTON, E. W., and MAYNARD, L. A. J. Nutr. **15**:383. 1938.
35. CRAMPTON, E. W., and WHITING, F. J. An. Sci. **2**:278. 1943.
36. CROSS, C., and BEVAN, E. Cellulose. Longmans Green and Co., 2d edition. New York. 1911.
37. CUENDET, L. S. Thesis, Univ. of Minnesota, 1950, cited by Boggs, L. A., and Smith, F., in J. Am. Chem. Soc. **78**:1880. 1956.
38. DAVIS, B. L., and PHILLIPS, M. J. Ag. Res. **63**:241. 1941.
39. DEDONDER, R. Compt. Rend. **230**:549. 1950.
40. DEDONDER, R. Compt. Rend. **232**:1134. 1951.
41. DEDONDER, R., and BUVRY, C. Compt. Rend. **231**:790. 1951.
42. DE MAN, T. J., and DE HEUS, J. G. Rec. Trav. Chim. Pays-Bas. **68**:43. 1949.
43. DE MAN, T. J., and DE HEUS, J. G. Rec. Trav. Chim. Pays-Bas. **69**:271. 1950.

44. DRUCE, E., and WILLCOX, J. S. J. Ag. Sci. **39**:145. 1949.
45. EDELMAN, J., and BACON, J. S. D. Biochem. J. **49**:446. 1951.
46. ELLIS, G. H., MATRONE, G., and MAYNARD, L. A. J. An. Sci. **5**:285. 1946.
47. ELY, R. E., KANE, E. A., JACOBSON, W. C., and MOORE, L. A. J. Dairy Sci. **36**:334. 1953.
48. ELY, R. E., KANE, E. A., JACOBSON, W. C., and MOORE, L. A. J. Dairy Sci. **36**:346. 1953.
49. ELY, R. E., MELIN, C. G., and MOORE, L. A. J. Dairy Sci. **39**:1742. 1956.
50. ELY, R. E., and MOORE, L. A. J. Ag. Food Chem. **2**:826. 1954.
51. ELY, R. E., and MOORE, L. A. J. Dairy Sci. **38**:1017. 1955.
52. ELY, R. E., and MOORE, L. A. J. An. Sci. **14**:718. 1955.
53. ELY, R. E., and MOORE, L. A. J. Dairy Sci. **39**:1706. 1956.
54. FERGUSON, W. S. Biochem. J. **36**:786. 1942.
55. FERGUSON, W. S. Ag. Prog. **23**:129. 1948.
56. FLANDERS, C. A. Arch. Biochem. Biophys. **36**:421. 1952.
57. FLANDERS, C. A. Arch. Biochem. Biophys. **36**:425. 1952.
58. FORBES, R. M., and GARRIGUS, W. P. Univ. of Kentucky (unpublished data). 1949.
59. FORBES, R. M., and GARRIGUS, W. P. J. An. Sci. **9**:354. 1950.
60. FORBES, R. M., and HAMILTON, T. S. J. An. Sci. **11**:480. 1952.
61. GRANICHSTÄDTEN, H., and PERCIVAL, E. G. V. J. Chem. Soc. p54. 1943.
62. HARWOOD, V. D. Tappi **35** (12):549. 1952.
63. HARWOOD, V. D. J. Sci. Food Ag. **5**:453. 1954.
64. HARWOOD, V. D., LAIDLAW, R. A., and TELFER, R. G. J. J. Chem. Soc. p2364. 1954.
65. HENNEBERG, W., and STOHMANN, F. Beiträge zur Begründung einer rationellen Fütterung der Wiederkäuer. Zweites Heft. Ueber die Ausnutzung der Futterstoffe durch das volljährige Rind und über Fleischbildung im Körper desselben. C. A. Schwetschke und Sohn, Braunschweig. 1864.
66. HIRST, E. L., MCGILVRAY, D. I., and PERCIVAL, E. G. V. J. Chem. Soc. p1297. 1950.
67. JAYME, G. Cellulosechem. **20**:43. 1942.
68. JERMYN, M. A. Modern methods of plant analysis. Paech, K., and Tracey, M. V., editors. Vol. 2, Springer-Verlag, Berlin. 1955.
69. JERMYN, M. A., and ISHERWOOD, F. A. Biochem. J. **64**:123. 1956.
70. JOHNS, A. T. New Zealand J. Sci. Tech. **37A**:301. 1955.
71. KRZNARICH, P. W. Cereal Chem. **17**:457. 1940.
72. LAGOWSKI, J. M., SELL, H. M., HUFFMAN, C. F., and DUNCAN, C. W. Arch. Biochem. Biophys. **76**:306. 1958.
73. LAIDLAW, R. A., and REID, S. G. J. Chem. Soc. p1830. 1951.
74. LAIDLAW, R. A., and REID, S. G. J. Sci. Food Ag. **3**:19. 1952.
75. LAIDLAW, R. A., and WYLAM, C. B. J. Sci. Food Ag. **3**:494. 1952.
76. LEECH, J. G. Tappi **35** (6):249. 1952.
77. MACDOUGALL, D., and DELONG, W. A. Can. J. Res. **20B**:40. 1942.
78. MATRONE, G., ELLIS, G. H., and MAYNARD, L. A. J. An. Sci. **5**:306. 1946.
79. MCRARY, W. L., and SLATTERY, M. C. Arch. Biochem. **6**:151. 1945.
80. MONTGOMERY, R., and SMITH, F. J. Ag. Food Chem. **4**:716. 1956.
81. MOON, F. E., and ABOU-RAYA, A. K. J. Sci. Food Ag. **3**:399, 407, 595. 1952.
82. MOROZOV, A. S. Compt. Rend. Acad. Sci., U.S.S.R. **24**:407. 1939.
83. NEVELL, T. P. J. Text. Inst. **39**:T118. 1948.
84. NORMAN, A. G. Biochem. J. **30**:1354. 1936.
85. NORMAN, A. G. Biochem. J. **31**:1597. 1937.
86. NORMAN, A. G. The biochemistry of cellulose, the polyuronides, lignin, &c. Oxford University Press, London. 1937.
87. NORMAN, A. G., and JENKINS, S. H. Biochem. J. **27**:818. 1933.
88. NORMAN, A. G., and JENKINS, S. H. Biochem. J. **28**:2147. 1934.

89. NORMAN, A. G., and JENKINS, S. H. Biochem. J. **28**:2160. 1934.
90. NORMAN, A. G., and RICHARDSON, H. L. Biochem. J. **31**:1556. 1937.
91. NORMAN, A. G., WILSIE, C. P., and GAESSLER, W. G. Iowa State Col. J. Sci. **15**:301. 1941.
92. NORTHERN REGIONAL RESEARCH LABORATORY. Peoria, Illinois. Mimeo. March, 1945.
93. O'DWYER, M. H. Biochem. J. **20**:656. 1926.
94. OGSTON, A. G., and STANIER, J. E. Biochem. J. **49**:591. 1951.
95. PALMER, A. Biochem. J. **48**:389. 1951.
96. PATTON, A. R. J. An. Sci. **2**:59. 1943.
97. PERCIVAL, E. G. V. British J. Nutr. **6**:104. 1952.
98. PHILLIPS, M., and DAVIS, B. L. J. Ag. Res. **60**:775. 1940.
99. PHILLIPS, M., DAVIS, B. L., and WEIHE, H. D. J. Ag. Res. **64**:533. 1942.
100. PHILLIPS, M., and GOSS, M. J. J. Ag. Res. **51**:301. 1935.
101. PHILLIPS, M., GOSS, M. J., DAVIS, B. L., and STEVENS, H. J. Ag. Res. **59**:319. 1939.
102. PHILLIPS, T. G., and LOUGHLIN, M. E. J. Ag. Res. **78**:389. 1949.
103. PHILLIPS, T. G., and SMITH, T. O. New Hampshire Ag. Exp. Sta. Tech. Bul. 81 (Part 1). 1943.
104. PHILLIPS, T. G., SULLIVAN, J. T., LOUGHLIN, M. E., and SPRAGUE, V. G. Agron. J. **46**:361. 1954.
105. PIGDEN, W. J. Can. J. Ag. Sci. **33**:364. 1953.
106. PORTER, H. K., and EDELMAN, J. Biochem. J. **50**:xxxiii. 1952.
107. REID, J. D., NELSON, G. H., and ARONOVSKY, S. I. Ind. Eng. Chem., Anal. Ed. **12**:255. 1940.
108. RITTER, G. J., and KURTH, E. F. Ind. Eng. Chem. **25**:1250. 1933.
109. SCHENK, W. A., and KURTH, E. F. Ind. Eng. Chem. **33**:1398. 1941.
110. SCHLUBACH, H. H., and LÜBBERS, H. Angew. Chem. **66**:744. 1954.
111. SCHLUBACH, H. H., and SINH, O. K. Annalen **544**:111. 1940.
112. SCHMIDT, E., TANG, Y.-C., and JANDEBEUR, W. Cellulosechem. **12**:201. 1931.
113. SCHULZE, E. Z. Physiol. Chem. **16**:387. 1892.
114. SPRAGUE, V. G., and SULLIVAN, J. T. Plant Physiol. **25**:92. 1950.
115. STRAKA, R. P., NELSON, G. H., and LEVINE, M. J. Ag. Res. **64**:129. 1942.
116. SULLIVAN, J. T. J. An. Sci. **14**:710. 1955.
117. SULLIVAN, J. T., and SPRAGUE, V. G. Plant Physiol. **18**:656. 1943.
118. THOMAS, B., and ARMSTRONG, D. G. J. Ag. Sci. **39**:335. 1949.
119. TOLLENS, B. Exp. Sta. Rec. **8**:641. 1897
120. TRACEY, M. V. Biochem. J. **43**:185. 1948.
121. TRACEY, M. V. Biochem. J. **47**:433. 1950.
122. WAITE, R., and BOYD, J. J. Sci. Food Ag. **4**:197. 1953.
123. WAITE, R., and BOYD, J. J. Sci. Food Ag. **4**:257. 1953.
124. WEIHE, H. D., and PHILLIPS, M. J. Ag. Res. **64**:401. 1942.
125. WEINMANN, H. Proc. 6th Intern. Grassland Congr., Vol. 1, p655. 1952.
126. WHELAN, W. J. Modern methods of plant analysis. Paech, K., and Tracey, M. V., editors. Vol. 2, Springer-Verlag, Berlin. 1955.
127. WHISTLER, R. L., BACHRACH, J., and BOWMAN, D. R. Arch. Biochem. **19**:25. 1948.
128. WHISTLER, R. L., and CORBETT, W. M. The carbohydrates. W. Pigman, editor. Academic Press, Inc., New York. 1957.
129. WHISTLER, R. L., and SMART, C. L. Polysaccharide chemistry. Academic Press, Inc., New York. 1953.
130. WILLIAMS, K. T., and BENEVUE, A. J. Assoc. Off. Ag. Chem. **39**:901. 1956.
131. WISE, L. E. Pulp and Paper Mag. Can. **50**:179. 1949.
132. WISE, L. E., MURPHY, M., and D'ADDIECO, A. A. Paper Trade J. **122** (2):35. 1946.
133. WYLAM, C. B. J. Sci. Food Ag. **4**:527. 1953.

5M—3-59—67331

UNIVERSITY OF ILLINOIS-URBANA
Q.630.7IL6B C001
BULLETIN. URBANA
621-640 1957-59

3 0112 019528790