# THE COMPOSITION OF THE MUCILAGE FROM THE SEED OF CHIA GRANDE *HYPTIS SUAVEOLENS* (L.) POIT.

by

John K. Stille

---

A Thesis

submitted to the faculty of the

Department of Chemistry

in partial fulfillment of

the requirements for the degree of

Master of Science

in the Graduate College

University of Arizona

1953

---

Approved: M. B. Seeley, May 8, 1953

Major Professor Date

This thesis has been submitted in partial fulfillment of requirements for an advanced degree at the University of Arizona and is deposited in the Library to be made available to borrowers under rules of the Library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the dean of the Graduate College when in their judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED:

John K. Stille

ACKNOWLEDGEMENT

This research was suggested and supervised by Dr. M. G. Seeley.

TABLE OF CONTENTS

ABSTRACT OF THE THESIS

THE COMPOSITION OF THE MUCILAGE
FROM THE SEED OF CHIA GRANDE,
HYPTIS SUAVEOLENS (L.) POIT.

by

John K. Stille

The unpurified mucilage from the seed of the chia grande is a calcium or magnesium salt of a complex polyuronide and is composed of a mixture of two mucilage fractions with different solubilities but with the same ash-free equivalent weight. This complex polyuronide is composed of one uronic acid united with about three pentosan units, one methylpentosan unit, and one hexosan unit with the loss of ten molecules of water.

Preparation of the free acid from the crude mucilage indicates that the hexosan unit is the first sugar molecule to be removed by hydrolysis.

Hydrolysis of the mucilage offered further proof that the hexosan unit is the first sugar molecule to be removed and indicated that the methylpentosan sugar molecule is near the end of the uronic acid molecule.

Paper chromatography of the hydrolysis products showed the sugars in the mucilage to be d-galactose, l-arabinose, l-xylose, and l-rhamnose. The uronic acid was identified as galacturonic acid by oxidation to mucic acid.

## INTRODUCTION

The chia grande plant*, Hyptis suaveolens, grows in tropical Sonora, Mexico, near Guasaremos, Rio Mayo, and Chihuahua. It favors moist canyon slopes between 1,000 and 4,000 feet, grows to the height of a meter, and produces a lavender flower. The plant is browsed to a limited extent by livestock. The disc-like seeds, which are about 4 mm. in diameter, swell in water to five times their original volume, contain a very mucilaginous coat, and, like certain species of Salvia, are used in Mexico as food under the name "Chia".(1) The Warihios Indians cultivate the plant and use the sweetened water-soluble extract of the seeds as a drink and as a treatment for fevers and intestinal disorders. The seeds, mixed with a little saliva, are used to remove foreign objects from the eye.

The purpose of this investigation was to determine the kinds and relative amounts of the monosaccharides in the mucilage extracted from the seeds of the chia grande.

Mucilages belong to a class of carbohydrates known as polyuronides. Anderson and Sands(2) have defined polyuronides as polysaccharides that contain one or more uronic

---

*The seed used in this investigation was collected by Dr. S. B. Detwiler of the U. S. Department of Agriculture, Soil Conservation Service.

acid units in their molecular structure. Polyuronides are extracted by water and dilute alkali from water-soluble plant exudates, mucilages, and most plant materials. Mucilages are secreted by the hairs of many plants and occur in the plant as membrane or cell mucilages.[3,4] In many cases they are obtained in large quantities from certain seeds,[5] either as endosperm mucilages or as a seed coat mucilage. The seed coat mucilage reduces moisture loss and induces water absorption from the air, thus providing the embryo with water during germination.

Many of the seed coat mucilages have been studied in the past twenty years and have all been found to be polyuronides. Extraction of the mucilage from the seeds with water and precipitation with ethanol was the most often used method in the isolation of the mucilages.

One of the earliest seed mucilages investigated was that of the white mustard seed, Brassica alba. Bailey and Norris,[7,8] found it contained a mixture of cellulose and polyuronides. The crude mucilage was precipitated by ethanol from an aqueous extract. The addition of barium hydroxide to an aqueous solution of the mucilage precipitated a heavy gel, which was found to contain l-arabinose, d-galacturonic acid and a glycuronic acid. On hydrolysis, the soluble fraction yielded l-arabinose, d-galactose, l-rhamnose and d-galacturonic acid. The mucilage from the cress seed, Lepidium sativum, also investigated

by Bailey,[7] swells in water but cannot be easily separated from the seeds. Analysis of the mucilage obtained by precipitation with ethanol, showed 25 per cent uronic acid, 24 per cent furfuraldehyde, and 18 per cent cellulose. On hydrolysis with two per cent sulfuric acid at 80° for 20 hours, the cellulose fraction separated. From the hydrolysate, the barium salt of a complex uronic acid was precipitated, and l-arabinose and d-galactose were identified. Further hydrolysis showed the complex acid to be l-rhamnose and d-galactose linked to a hexuronic acid.

The mucilage extracted from the flax seed, Linum usitatissimum, was one of the first mucilages to find industrial importance in cases where water-soluble emulsifying agents, thickeners, or binders are needed.[5] It has been used to a small extent in pharmaceutical industries as a demulcent. Anderson and Crowder[9] found d-xylose and an aldotrionic acid to be the first products of hydrolysis. Further hydrolysis splits the aldotrionic acid into l-galactose and an aldobionic acid consisting of d-galacturonic acid and l-rhamnose. Anderson and Lowe[10] later showed that d-galacturonic acid, l-rhamnose, l-galactose, and d-xylose were present in equimolar amounts and that hydrolysis of the mucilage yielded d-xylose first and l-galactose second. The aldotrionic acid was shown to be composed of equimolar amounts of d-galacturonic acid, l-rhamnose and l-galactose. On the basis of the relative ease of hydrolysis

of the d-xylose and l-galactose, and the resistance to hydrolysis of the aldotrionic acid, Anderson postulated a branch chain structure for the mucilage.

The quince seed mucilage from the Persian quince, Cydonia vulgaris, is also of commercial interest since it shows the properties similar to those of the flax seed mucilage. Renfrew and Cretchner[11] reported 33 per cent cellulose and a mixture of aldobionic acids. The mucilage was extracted from the seeds with cold water and precipitated by the addition of ethanol and acetone. The mucilage acid was found to be insoluble in water. Mild hydrolysis yielded a mixture of aldobionic acids along with a small amount of l-arabinose. Analysis of the crude salt precipitated from the hydrolysate showed an aldobionic acid with one molecule of a hexuronic acid linked to d-xylose. The hexuronic acid was found to be 72 per cent monomethylated hexuronic acid, and 28 per cent unmethylated hexuronic acid.

Plantago seed mucilages have been found to be comparitively complex in structure. In general the yields of mucilages from these seeds are the highest obtained.[5] The psyllium seed, Plantago ovata Forsk, was shown by Anderson and Fireman[12] to contain a mixture of polyuronides. The mucilage is a white, fibrous material obtained in a 20 per cent yield by precipitation from aqueous extract with alcohol. The molecule is composed of galacturonic acid

combined with l-arabinose. The remaining portion of the molecule, linked to the l-arabinose, contains chains of d-xylose varying in length from 8 to 35 xylose units. The seed mucilage from Indian wheat, Plantago fastigiata, T., investigated by Anderson, Seeley, and Gillette,(13) was found to contain a mixture of polyuronides varying in composition from 8 to 17 pentosan molecules combined with one molecule of d-galacturonic acid. The mixture consists of various salts of d-galacturonic acid linked by a glycosidic union from its aldehyde group to a chain of several molecules of l-arabinose. The l-arabinose is joined to a longer chain of d-xylose molecules.

The composition of seed mucilages are not unlike that of the plant gums. The molecule in the cases reviewed seems to center around an aldobionic acid. The methods used in the determination of the composition of seed mucilages are, in general, the same. Extraction with water and precipitation with alcohol has been used in most cases. Purification to a high degree is difficult since crystallization cannot be used.(2) Purification is usually effected by successive precipitation, treatment with dilute acids, or dialysis. Fractional precipitation will sometimes separate mixtures of mucilages. Analytical determinations made on the polyuronide are those for uronic acid, pentosan, methylpentosan, methoxyl, and ash. The percentage hexosan is estimated by difference. Mineral acids are most often used for the

hydrolysis of the mucilages which proceeds very slowly. Sulfuric acid is especially suited, since it may be easily removed by carbonates or hydroxides of the alkaline earths. Fractional hydrolysis of the mucilage is advantageous since the arrangement of the sugar molecules in the polyuronide can be determined in many cases. It is believed that hydrolysis first removes the polysaccharide side chains and breaks one of the disaccharide linkages between the uronic acid and the sugar in the main chain. By varying the concentration of the acid, the temperature, and the length of hydrolysis from the mildest to the most severe conditions, the sugars, in the idealized case, split off one by one, until an aldobionic or aldotrionic acid is obtained. The salt of the acid is obtained by adding the hydroxide or carbonate of an alkaline earth, removing the sulfate by filtration, and adding ethanol. Thus the salt can be separated from the sugars in solution. The same analyses are performed on the salt as on the free acid, and the sugars in the hydrolysate identified either by chromotography or derivatization. Concentration of the neutralized hydrolysate is usually desirable both before and after precipitation of the salt of the uronic acid to insure a complete precipitation with a low volume of ethanol. In recent years, investigators (14,15,16) have applied paper chromatography to the identification and separation of sugars. In many cases, this simple technique is the only good method of performing a complete qualitative analysis on the sugars.

The present investigation was undertaken to determine the kinds, relative amounts of the constituent sugars and uronic acids, and their relative positions in the complex polyuronide molecule. The application of paper chromatography to the identification of the sugars present was also investigated.

## DISCUSSION

The best method of isolation of the seed mucilage from big chia was found to be extraction with hot water. From several trials in which the volume of water used in the extraction was varied, the most suitable conditions were those in which the volume of water added was seven times the weight in grams of the seed. The seed was heated with the water on a water bath at 80° for three hours, and the mixture was allowed to stand overnight. Larger volumes would extract more of the mucilage, but the volume of alcohol needed for precipitation of the mucilage would be increased fourfold. Four volumes of 95 per cent ethanol seemed to be sufficient to precipitate almost all of the extracted mucilage in solution. In the preparation of the mucilage the greatest difficulty was in removing the mucilage from the seed. A considerable increase in yield was obtained by removing the mucilage by means of a seed press. This process proved to be tedious since the sieve would clog after a small amount of seed had been pressed.

The mucilage of the first 400 g. of seeds was removed by forcing it through muslin. The rest of the batches were prepared using a seed press. The weight of the seed which could be most conveniently handled at a time was 800 g. The dark brown color of the expressed mucilage was probably

caused by the absorption of water during the evaporation of the ether used in the last wash. The mucilage turned dark in the Buchner funnel during filtration and air drying, so a rubber dam was used in order to prevent air from being drawn through the mucilage. This prevented darkening. Later it was found that the mucilage could be filtered using a large cone funnel, and no darkening occurred.

One method of purification resulted in a separation into two fractions. As alcohol was added to a solution of crude mucilage which had been filtered through a Whatman #1 filter paper on a Buchner funnel, a gray precipitate formed. After a third of the alcohol had been added, the slurry was filtered, and the rest of the alcohol was added to the filtrate. The second portion of mucilage which precipitated was a light cream color. At first it was thought that the dark color of the more insoluble fraction was due to the higher concentration of water at the beginning of the precipitation, but this was later proved to be due to the difference in the metallic ion present.

The crude mucilage was purified in a different manner by dissolving the mucilage in a weak solution of hydrochloric acid. The purified mucilage was precipitated by adding a volume of 95 per cent ethanol equal to four times the volume of the mucilaginous solution. An almost white precipitate formed which settled with some difficulty. The purpose of the acid purification was to remove the

metallic ion.

The crude mucilage was tested for the presence of uronic acid, pentoses, methylpentoses, hexoses, nitrogen, and methyl groups. Spectroscopic analysis of the ash showed major constituents of both calcium and magnesium. All of these qualitative tests were run on (1) mesquite gum, (2) a hydrolyzed solution of the crude mucilage, and (3) the crude mucilage. Mesquite gum was used as a comparative standard because it is known to contain a uronic acid unit, arabinose, and galactose.(17) A test for hexuronic acid using napthoresorcinol(18) gave positive results for all three samples.

Bials orcinol test(19) showed pentoses to be present in the three samples used, but the aniline test(19) failed to give positive results for the crude mucilage. The hydrolyzing medium used in the test did not seem to be strong enough. The mesquite gum (1) gave a fair test while the hydrolyzed mucilage (2) gave a blood red color.

Rosenthaler's test for methylpentoses(19) gave doubtful results. The violet color, which shows the presence of methylpentose, was absent from all three tested samples. The hydrolyzed mucilage (2), however, gave a brown-orange color while the crude mucilage (3) and the mesquite gum (1) gave dark brown colors. If hexoses are present the violet color assumes a brownish tinge. Since the results of the analyses carried out later on the barium salts of the

hydrolyzed mucilage indicates that the hexose is the first product of hydrolysis and that the methylpentose is in the middle of the polyuronide molecule, the results of the test are not surprising.

The test for ketohexoses and aldohexoses (19) was doubtful for all three samples, probably due to the large amount of pentoses present. A test for the presence of nitrogen (20) on the crude mucilage also gave negative results.

A test for ether, ester, and glycosidic linked methoxyl groups has been described by von Fellenberg (21). He bases his test on the fact that sodium hydroxide will split an ester or glycosidic methoxyl group and will liberate methanol on heating. An ether-linked methoxyl group is split with 72 per cent sulfuric acid and methanol is also liberated. In both cases, Denige (22) has devised a method for testing the liberated methanol. The crude mucilage did not give a positive test for either type of methoxyl groups.

The crude mucilage, both of the fractions obtained by fractional precipitation, and the acid purified mucilage were analyzed quantitatively for moisture, ash, uronic acid, and pentosan. In addition, the purified mucilage was analyzed for methylpentosan and its specific rotation was measured.

Uronic acids, when boiled with 12 per cent hydrochloric acid, yield carbon dioxide. The uronic acid determination

depends upon the quantitative yield of one molecule of carbon dioxide for each molecule of uronic acid.(23) The carbon dioxide evolved is collected and measured quantitatively. The method used is a modification which was found to be best suited for this type of analysis.(24)

The pentosan determination is based upon the fact that pentose sugars give furfural when distilled with 12 per cent hydrochloric acid.(19) The furfural is driven off by distillation and precipitated as furfural phloroglucide. The yield of furfural is not, however, quantitative. Furthermore, different pentose sugars give different yields of furfural. Uronic acids and methylpentoses yield furfural and methylfurfural respectively. Further difficulty is encountered since the precipitation of furfural as furfural phloroglucide is not quantitative. By making the distillation and precipitation under carefully controlled conditions, it is possible to make a close approximation of the percentage pentosan in a sample containing only one kind of sugar by means of formulas or tables. It was later found by analysis of the hydrolysate from the mucilage that the mucilage contained both arabinose and xylose in addition to rhamnose. There was no way to determine the relative amounts of xylose and arabinose, so the pentose sugars were calculated as per cent pentosan.

A correction for the uronic acid present was made by dividing the weight of uronic acid present in a given sample

by 2.64 and subtracting this amount from the total weight of phloroglucide precipitate.

The per cent methylpentosan was determined by extracting the phloroglucide precipitate with hot 95 per cent ethanol.(19) The determination is based upon the solubility of methylfurfural phloroglucide and the insolubility of furfural phloroglucide in warm 95 per cent ethanol. However, it has been shown(19) that a small amount of furfural phloroglucide dissolves in the warm ethanol, but the amount dissolved is dependent upon the details of the manipulation because the procedure is entirely empirical. In the results shown on pages 31 and 36, no correction was made for the solubility of furfural phloroglucide. Therefore, the results of the analyses for pentosan and methylpentosan shown on these pages cannot be relied on for more than approximation. The percentage pentosan could be much higher or lower depending upon the relative amounts of xylose and arabinose. This fact also makes it difficult to predict the percentage hexose, since the amount of hexose is determined by difference.

The results of the analysis shown on page 31 for the crude mucilage would indicate that the amounts of uronic acid, pentose, and hexosan are present in the ratio of 1:2:2. Since the percentage methylpentosan was not determined, and the conversion factor for methylpentosan is high,

the amounts of uronic acid, pentosan, methylpentosan, and hexosan are probably present in the ratio of 1:3:1:1. This ratio gives an equivalent weight of 880.

The analysis of the ash from the crude mucilage, along with the results of the analysis of the two mucilage fractions obtained by fractional precipitation, indicated that these are uronic acid salts of calcium and magnesium. Fractions IIa and IIb have exactly the same equivalent weight when the results of the uronic acid determination shown on page 31 are calculated on an ash free basis. The percentage ash of the more insoluble gray fraction was considerably less than that of the light fraction. This might indicate that the gray fraction is a calcium salt. The percentage ash is approximately proportional to the molecular weights of the oxides of calcium and magnesium. The percentage pentosan does not show enough difference to postulate different ratios of pentose and hexose units. Another reason for the difference in solubility may be chain branching. Calculation and analysis involving methylpentose would indicate that the amounts of uronic acid, pentosan, methylpentosan, and hexosan present are probably in the ratio of 1:2:1:$\frac{1}{2}$.

The inorganic salts present in the crude mucilage were almost entirely removed in the acid purification. The results also indicate that a relatively large amount of the

hexose in the polyuronide was split from the molecule. This is one of the reasons for believing the hexose molecule is the last molecule in the polyuronide chain, and is farthest away from the uronic acid molecule or is in a side chain. The analyses of this purified mucilage show a 1:3:1:1/8 ratio for uronic acid, pentosan, methylpentosan, and hexosan. The calculated equivalent weight, 738, using this ratio, agrees with the equivalent weight of 772 listed on page 31.

The crude mucilage was then hydrolyzed in order to determine the sugars present, to find the relative positions of the sugars to each other in the polyuronide molecule, and to obtain a hexose free barium salt which could be used for the identification of the uronic acid.

The crude mucilage was hydrolyzed under three different sets of conditions increasing in severity of temperature and length of time. The most suitable method was found to be hydrolysis at about 80° with four per cent sulfuric acid. In the first and third hydrolyses, the mucilage was first triturated with water in a mortar until a thick paste was obtained. This paste was dissolved in more water and sulfuric acid was added until the solution was four percent with respect to sulfuric acid. In the second hydrolysis, the mucilage was dissolved directly in the four per cent sulfuric acid. The mucilaginous solution was hydrolyzed

for one and one-half hours at 80°, three hours at 80°, and three and one-half hours at 90°. In all cases, solid barium carbonate was added at the end of the hydrolysis period to neutralize the acid solution and to form the barium salt of the uronic acid. The barium sulfate was removed by filtration, and the solutions were decolorized with activated charcoal. After these solutions had been concentrated under reduced pressure, eight volumes of alcohol were added to precipitate the barium salt. The precipitates obtained were washed with alcohol and ether.

The filtrates from the barium salts were concentrated under reduced pressure either to very low volumes or to dryness.

The barium salts were analyzed quantitatively for moisture, ash, uronic acid, and pentosan. Two of the barium salts were analyzed for percent methylpentosan.

From the data given on page 36, the following conclusions can be made concerning the relative order in which the sugars are arranged in the polyuronide molecule. The hexose sugar is the most easily removed by hydrolysis, and most of it is split off even under the mildest hydrolysis conditions. Hydrolysis of the mucilage for three and one-half hours removes all of the hexose molecules. This fact is proven furtherrfrom the fact that the barium salt obtained from the hydrolysis under these conditions gave no test for hexose. (19)

This made it possible for a simple identification of the

uronic acid present. The hexose unit is therefore believed either to be farthest away from the uronic acid in the polyuronide molecule or present as a side chain. Since the ratio of methylpentosan units per uronic acid decreased from the mildest to the most severe conditions of hydrolysis, and since it decreases considerably in the hydrolysis of the mucilage, it might possibly be linked to the hexose molecule and be the second sugar removed. However, the ratio of pentosan units per uronic acid also decreases considerably in the mild hydrolysis of the mucilage so that a definite conclusion concerning the position of the methylpentosan molecule cannot be drawn. However, it is most likely near the end of the polyuronide molecule or in the same side chain with the hexose or a separate side chain. As previously mentioned, the results of the analyses for pentosan and methylpentosan cannot be expected to give more than a close approximation.

The barium salt III from the most severe hydrolysis conditions was used for the uronic acid identification, which depends upon the oxidation of the aldehyde group to form the dibasic acid.(19) The dibasic acid molecule is then split from the rest of the sugar molecule. Oxidation of galacturonic acid forms crystals of insoluble mucic acid, which are minute granular rhombic prisms. Glucuronic acid, if it is present, will oxidize to saccharic acid which is soluble in the aqueous solvent.

A method of analysis of sugars using horizontal paper chromatography as described by Rao and Beri [15,16] was used in the identification of the sugars from the hydrolyzed mucilage. This method affords a quick analysis of complex sugar mixtures with easy manipulation and simple apparatus. Whatman No. 1 circular filter papers were used for the development of the chromatogram. A tail was cut in center of the filter paper which was allowed to dip into a suitable irrigation solvent. The sugar solution was spotted and dried at the perpendicular bend that the tail made with the circular filter paper. On irrigation with the solvent the sugar mixture spreads into circular zones in about thirty minutes to three hours. The rate at which the solvent flows, and the distance the different sugars move outward from the center is dependent upon the solvent used and the temperature. The sugars may be identified by their characteristic circular $R_f$ values for any given solvent by calculating the ratio of the distances the sugar band and solvent has moved.

The sugar band is developed with aniline phthalate reagent [25]. Petri dishes were found to be the most suitable container for the solvent. Pentose sugars show pink colored rings with the developing reagent while the hexoses give brown bands. It was also noted that l-rhamnose gave yellow bands in all cases.

Four different solvents were tried, but only three were used in order to identify the sugars. Moist butanol gave good separations for d-galactose and l-rhamnose, but the pentoses present did not show separate bands. Moist p-cresol was used because the positions of the l-arabinose and l-xylose were inter-changed. It gave a good separation for l-arabinose, but d-galactose and l-xylose did not separate. A band could also be detected for l-rhamnose. Moist phenol showed a broad band of d-galactose, l-xylose, and l-arabinose. The l-arabinose band in some cases was detectable. The phenol was used because of the characteristic l-rhamnose band which had the highest $R_f$ value obtainable.

The $R_f$ values shown on page 40 leave little doubt that the hexose molecule in the polyuronide is d-galactose, the methylpentose is l-rhamnose, and the pentoses present are a mixture of l-arabinose and d-xylose. The bands for d-galactose were often very faint, but the controls which were run with the sugar mixtures also gave faint rings for d-galactose. Using moist p-cresol for a solvent, which was the only one which gave lines for l-arabinose, showed evidence that l-arabinose was present in a smaller concentration than l-xylose. The chromatographic bands for l-arabinose in the mixture of sugars from the mucilage hydrolysate were very faint while in the control, the rings were dark for l-arabinose. This might indicate that either the

relative amount of l-arabinose in the polyuronide molecule is less than that of l-xylose or that the l-arabinose is closer to the uronic acid and harder to remove by hydrolysis.

The measured $R_f$ values are lower than those reported, (15) which is probably due to the fact that the chromatograms were run at a temperature nearly ten degrees lower.

Further proof of the presence of l-arabinose and l-rhamnose was established by the preparation of l-arabinose α-benzylphenylhydrazone and l-rhamnose β-naphthylhydrazone.

To further establish the relative positions of the sugar molecules several more hydrolyses should be performed. More knowledge concerning the position of the l-rhamnose in the mucilage molecule could be obtained by hydrolysis at 80° for two hours. Another hydrolysis, which would cut the molecule to an aldobionic acid, would show which pentose sugar is linked to the uronic acid molecule. This type of hydrolysis would also tell more about the relative concentrations or positions of the arabinose and xylose molecules. However, conditions for this hydrolysis are so severe that the aldobionic acid is virtually destroyed.

In all the chromatograms made on the sugars from the various hydrolyses, arabinose, as previously mentioned, always gave faint bands while the other sugars, galactose, xylose, and rhamnose, gave strong bands. Therefore it is very possible that one or both of the pentosan units in

barium salt III are arabinose molecules, and that barium salt III is a straight chain salt of a polyuronide containing two pentose units per uronic acid, and a side chain rhamnosan unit which has been partially hydrolyzed from the straight chain portion. If the interpretation of the results of the analyses performed on the mucilage and the barium salts is correct, the following most probable structures for the mucilage can be postulated:

(1) galacturonic acid — pentose — pentose
rhamnose
xylose
galactose

(2) galacturonic acid — pentose — pentose
galactose rhamnose xylose

The side chain (1) or chains (2) could be linked in any combination to any sugar in the straight chain portion. The diagrams represent one equivalent weight of the polyuronide molecule which may be either a calcium or magnesium salt.

## EXPERIMENTAL

### Preparation of the Mucilage

Six different methods of extracting the mucilage were tried. In each case, 15 g. of seed was put through a 10 mesh sieve to remove the large particles. The dirt and smaller particles were separated with a 20 mesh sieve. Trial batches IA, IIA, and IIIA were heated in a boiling water bath for two hours and allowed to stand over-night. Trial batches IB, IIB, and IIIB were not heated but were allowed to remain in water over-night. In the case of trial batches IA, IIA, and IIIA, additional water was added as required to maintain a constant volume. The extract was filtered through muslin cloth and the mucilage expressed by squeezing.

Table I

MUCILAGE EXTRACTION

| Batch | Vol. water | Final volume | Vol. of 95% ethanol added |
|---|---|---|---|
| IA | 100 cc. | 70 cc. | 280 cc. |
| IIA | 200 cc. | 150 cc. | 600 cc. |
| IIIA | 300 cc. | 250 cc. | 1000 cc. |
| IB | 100 cc. | 50 cc. | 200 cc. |
| IIB | 200 cc. | 140 cc. | 560 cc. |
| IIB | 300 cc. | 230 cc. | 920 cc. |

It was found that boiling aided extraction. A small volume of water was desirable, since the volume of alcohol could be kept at a minimum. Batches IA, IIA, and IIIA gave more precipitate than IB, IIB, and IIIB, while IA gave the most

precipitate per volume of extract. The mucilage gave a positive naphthoresorcinol test for uronic acid.(18)

1. Preparation of Batch I

To 400 g. of the seeds, which was cleaned by sifting through 10 and 20 mesh sieves, was added 2 l. of water. The mixture was heated on a boiling water bath for two hours. The seeds were left in the water over-night and the mucilage was separated by squeezing through a cloth. To the 275 cc. of filtrate was added four volumes of 95% ethanol. A light yellow-brown precipitate formed. The seeds still appeared to contain a large amount of mucilage, so they were scraped from the cloth and heated with 1 liter of water on a boiling water bath for two hours. After standing over-night, 600 cc. of mucilaginous solution was separated from the seeds. To this extract was added four volumes of 95% ethanol. Both precipitates were left over-night. The supernatant liquid was separated by filtration and each precipitate was washed separately with 95% alcohol followed by ether. The mucilages I and IA were dried in a vacuum desiccator. A 1.45% yield, 5.8 g., was obtained from the first extraction, and a 0.75% yield, 3 g., was obtained from the second extraction. Both batches were dark brown.

2. Preparation of Batch II

The seeds for batch II were prepared as described for Batch I. To 200 g. of the cleaned seeds was added 1400 cc.

of water. The mixture was boiled for two hours and allowed to stand for 48 hours. The mixture was filtered and the mucilage separated from the seeds in a small seed press. To the 800 cc. of filtrate obtained was added four volumes of 95% ethanol. The precipitate which formed was removed by filtration, washed with alcohol followed by ether, and dried in a vacuum desiccator. A 3.7% yield, 7.4 g., was obtained. This mucilage had a light brown color.

3. Preparation of Batch III

The seeds for batch III were prepared as described for batch I. To 400 g. of seeds was added 2700 cc. of water and the mixture treated the same as in batch II. To the 1040 cc. of the filtrate obtained was added four volumes of alcohol. The flocculent precipitate was allowed to settle, and the supernatant liquid removed by siphon, leaving a thin layer of alcohol over the residue. The slurry of precipitate and ethanol was then centrifuged, and the ethanol siphoned from the precipitate, which was washed in the cups with 95% ethanol and recentrifuged. The ethanol was siphoned as before, the washing was repeated with ether, and the slurry was filtered through a hard-finish filter paper in a Buchner funnel. By using a rubber dam, most of the liquid was removed from the mucilage by suction. In this manner the precipitate was kept from the air as much as possible, and was partially dried in the Buchner funnel. The mucilage

was dried in a vacuum desiccator and a 2% yield, 8 g., of cream-white mucilage was obtained.

4. Preparation of Batch IV

Eight hundred grams of seeds was prepared exactly as described in batch III except a more efficient seed press was used. A 2.64% yield, 21.1 g., of light-colored mucilage was obtained.

5. Preparation of Batch V

Eight hundred grams of seeds was prepared exactly as described in batch IV. A 2.75% yield, 22 g., of light-colored mucilage was obtained.

## Purification of the Mucilage

1. Fractional Precipitation

The crude mucilage from batch II was dissolved in 350 cc. of water and warmed. The solution was filtered through a Whatman #1 filter paper in a Buchner funnel. After 390 cc. of 95% ethanol had been added, a gray precipitate formed. The solution was filtered, and the remaining 1160 cc. of 95% ethanol was added. A lighter colored precipitate which formed was allowed to settle and was then removed by filtration. Both precipitates were washed with 95% alcohol followed by ether. One and one-half grams of the gray fraction, IIb, and 1.7 grams of the light fraction, IIa, were obtained.

2. Acid Purification

In 350 cc. of 2% HCl was dissolved 3.8 g. of the

mucilage from batch III, and the mixture was warmed to 60°. After 30 minutes of standing at room temperature, the solution was filtered, and 1400 cc. of 95% ethanol was added. The supernatant liquid was drawn off, and the purified mucilage washed twice by centrifugation with 95% ethanol. The mucilage was then washed with ether, removed by filtration, and dried in a vacuum desiccator. A 44.7% yield, 1.7 g., of pure mucilage, IIIa, was obtained.

Qualitative Tests on the Mucilage

All the qualitative tests for the presence of sugars were run on (1) the crude mucilage, (2) mesquite gum which is known to contain arabinose, galactose, and uronic acid,[17] and (3) a solution of the crude mucilage which was hydrolyzed as follows: 0.5 g. of the crude mucilage was dissolved in 15 cc. of 3% sulfuric acid solution in a 50 ml. flask. A stopper was inserted and held in place by wiring. The solutions was heated on a boiling water bath for two hours.

1. Uronic Acid

The naphthorescorcinol test for hexuronic acid described by Tollens[18] was performed on the three samples. All gave positive violet-colored ether layers.

2. Pentoses

Bial's Orcinol Test[19] was performed on the three samples. All gave an immediate green color. The hydrolyzed mucilage and the crude mucilage gave a deep blue-green color on standing, and mesquite gum gave a deep blue color.

The Aniline Test[19] for pentoses in the presence of uronic acids was positive for both the hydrolyzed mucilage and the mesquite gum. The crude mucilage failed to give the test. The hydrolyzed mucilage and the mesquite gum gave a deep blood-red and a pink color respectively, and the crude mucilage gave a yellow color.

3. Methylpentose

Rosenthaler's test for methylpentoses[19] showed negative results. The hydrolyzed mucilage gave a brown-orange color, and both the crude mucilage and the mesquite gum gave dark brown colors. However, if hexoses are present in addition to methylpentoses and pentoses, the characteristic violet color should acquire a brownish tinge.

4. Hexose

Jordan and Pryde's test for ketohexoses[19] and aldohexoses was inconclusive. The hydrolyzed mucilage gave a light violet color at first and then changed to a dark brown. Both the mesquite gum and the crude mucilage gave dark brown colors. When the test solution was diluted, all three gave a brown color.

5. Nitrogen

A sodium fusion was done on the crude mucilage.[20] Both the benzidine and Prussian blue tests were negative.

6. Methoxyl Group

The test for ether, ester, and glycosidic methoxyl

groups was done according to the method of von Fellenberg. (21)

The test is based on the fact that methanol is liberated from an ester or glycosidic methoxyl group with 10% sodium hydroxide. An ether-linked methoxyl group is liberated as methanol by boiling 72% sulfuric acid. In both cases, the methanol is tested according to a method devised by Denige. (22) The crude mucilage, the only one tested, gave negative tests for both glycosidic methoxyl groups and ether-linked methoxyl groups.

7. Spectroscopic Analysis of the Ash

A photograph of the emission spectra of the ash from the mucilage showed intense lines for calcium and magnesium along with faint lines indicating traces of nine other elements.

## Methods of Analysis

1. Moisture

The percentage moisture was determined by drying a known weight of the sample under reduced pressure in an Abderhalden dryer, using boiling water as the heating medium. The samples were dried usually for a period of three hours.

2. Ash

The percentage ash was determined by igniting a weighed sample to constant weight with a Meker burner. The sample was placed in a small crucible, 1.5 cm. in diameter, which was then placed inside a larger one and ignited with

the cover off.

3. Uronic Acid Determination

The method used for the determination of uronic acid is described by Ledbetter.(22) Instead of a glycerin bath, a silicone bath with a heating element was used. Chromel wire was wrapped around a circular piece of pyrex glass rod and immersed in the silicone bath. The temperature was controlled with a Variac. The determination depends upon the evolution of one molecule of carbon dioxide from each uronic acid molecule when the uronic acid is boiled in a solution of 12% hydrochloric acid. The carbon dioxide is collected in an absorption tube and weighed. Semi-micro techniques were used in weighing and handling the absorption tubes.

4. Pentosan and Methylpentosan Determination

The percent pentosan was determined by heating the sample with 12% hydrochloric acid, distilling the furfural under controlled conditions, and precipitating the furfural with phloroglucinol. The correction for furfural phloroglucideformed from the uronic acid present was applied by dividing the weight of uronic acid present in the sample by 2.64 and subtracting this weight from the total weight of furfural phlorogluc ide. The percent pentosan was then calculated from the tables given. The percent methylpentosan was determined by extracting the precipitate with hot 95% alcohol. All of the methylfurfural phlorogluc ide

dissolves under these conditions. The percent methylpentosan can then be calculated from the loss in weight after extraction. No correction was applied for the solubility loss of furfural phloroglucide, since the amount which dissolves seems to be controversial. The amount dissolved in all cases was found to be small.

## Analysis of the Mucilage

The crude mucilage IIIA, reprecipitated fractions IIa and IIb, and the acid purified mucilage IIIA were analyzed for percent moisture, ash, uronic acid, and pentosan. The acid purified mucilage IIIA was also analyzed for methylpentosan. The results are shown in table II.

Table II

RESULTS OF THE ANALYSES OF THE MUCILAGE
(Corrected for Moisture and Ash)

| | III | IIa | IIb | IIIa |
|---|---|---|---|---|
| % Moisture | 10.46 | 6.32 | 6.60 | 6.05 |
| % Ash* | 10.20 | 7.63 | 4.37 | 0.67 |
| % Carbon Dioxide | 4.53 | 5.83 | 5.83 | 5.23 |
| % Uronic Acid | 19.78 | 25.46 | 25.46 | 22.84 |
| Total % Pentosan | 33.89 | 40.14 | 46.91 | 49.80 |
| % Pentosan | - | - | - | 46.51 |
| % Methylpentosan | - | - | - | 27.95 |
| % Hexosan (by difference) | 46.33 | 34.40 | 27.63 | 27.36 |
| % Hexosan**(by difference) | - | - | - | 2.60 |
| $[\alpha]_D^{25^\circ}$ | | | | +12° |
| Equivalent weight | 888 | 681 | 691 | 772 |
| Total Pentosan units per uronic acid | 2.3 | 2.2 | 2.5 | 2.9 |
| Pentosan units per Uronic acid | - | - | - | 2.7 |
| Methylpentosan units per uronic acid | - | - | - | 1.47 |
| Hexosan units per uronic acid | 2.6 | 1.5 | 1.2 | 1.3 |
| Hexosan units per uronic acid** | - | - | - | 0.12 |

*Calculated on a moisture free basis.

**Calculated from the difference of uronic acid, pentosan, and methylpentosan.

## Hydrolysis of the Mucilage

### 1. Preparation of Barium Salt I

Seven grams of the crude mucilage from batch IV was mixed in a mortar with water to a thick paste. Water was added a little at a time until a thick liquid was obtained, which was transferred to a 500 ml. breaker and dissolved in water. Four per cent sulfuric acid and enough concentrated sulfuric acid were mixed and added to the aqueous solution to make the final volume 250 cc. and the final concentration 4% with respect to $H_2SO_4$. The mucilage was hydrolyzed for one hour and thirty minutes at 80° by placing the breaker in a water bath. At the end of the hydrolysis period, the solution was neutralized with solid barium carbonate by adding a small portion at a time and removing the carbon dioxide by heating. The barium sulfate and occluded barium carbonate were removed by filtering the mixture through a Whatman #42 filter paper on a Buchner funnel. More barium carbonate was added to the filtrate until the supernatant liquid was just neutral to litmus, and the filtration process was repeated. The filtrate was decolorized with several grams of Nuchar by warming the mixture on a water bath. The carbon was removed by filtration through a Whatman #42 filter paper which contained a fine asbestos mat. This filtrate, which was now nearly colorless, was concentrated under reduced pressure to 100 cc. To the filtrate 800 cc.

of 95% ethanol was slowly added, and a white flocculent precipitate formed immediately. The slurry was centrifuged and the supernatant liquid was removed by siphon. The precipitate of barium salt was then washed with 95% ethanol and ether and dried in a vacuum desiccator over calcium chloride. A yield of 0.5 g. of the barium salt was obtained. The alcoholic supernatant liquid was then concentrated under reduced pressure to a volume of 5 cc. This solution was used for the identification of the sugars removed by hydrolysis.

2. Preparation of Barium Salt II

Ten grams of crude mucilage from batch IV was treated in the same general manner as that described for the preparation of barium salt I, except that the mucilage was dissolved directly in 4% sulfuric acid. The final volume of solution was 500 cc., and the mucilage was hydrolyzed for three hours at 80°. The neutralized hydrolysate was concentrated under reduced pressure to 90 cc. and 700 cc. of ethanol was added. The precipitate of barium salt was washed as described for barium salt I. A yield of 2.9 g. of a cream-colored barium salt was obtained. The barium salt was then purified by dissolving it in water and reprecipitating it by adding four volumes of alcohol. The light colored precipitate was washed with alcohol and ether, and dried in a vacuum desiccator. A recovery of 1.6 g.

resulted. The filtrate from this reprecipitation was concentrated under reduced pressure to 75 cc. To this solution was added 4 volumes of alcohol. The precipitate which separated was washed with alcohol, then ether, and dried in a vacuum desiccator. Five tenths gram of this fraction, IIx, was obtained. To 15 cc. of the concentrated filtrate from barium salt II was added five volumes of alcohol. A white flocculent precipitate separated which was washed with alcohol, then ether, and dried in a vacuum desiccator. A yield of 0.2 grams of this fraction, IIy, was obtained. The filtrate from this barium salt, which was concentrated under reduced pressure to 35 cc., was also used for the identification of sugars removed by hydrolysis.

3. Preparation of Barium Salt III

Fifteen grams of crude mucilage from batch V was treated in exactly the same manner as described for hydrolysis I. The total volume of the solution was made up to 500 cc. The mucilage was hydrolyzed for three and one-half hours at 90°. The neutralized hydrolysate was concentrated under reduced pressure to 130 cc., and 1040 cc. of 95% ethanol was added. The precipitate of barium salt was washed as described before, and a yield of 3.15 g. was obtained. The filtrate from this barium salt was evaporated under reduced pressure to dryness, and 3.2 g. of a yellow crystalline material was obtained, which was later shown to be a mixture

of l-rhamnose, l-arabinose, l-xylose and d-galactose.

## Analysis of the Barium Salts

### 1. Quantitative Analysis

The barium salts were analyzed for percentage moisture, ash, uronic acid, and pentosan. In addition, barium salts I and II were analyzed for methylpentosan. The same methods were used as those described for the analysis of the mucilage. The results are shown in table III.

### 2. Specific Rotation

The specific rotation of the barium salts was determined in an aqueous solution using a one decimeter tube.

Table III

RESULTS OF THE ANALYSES OF THE BARIUM SALTS
(Corrected for Moisture and Ash)

| | Barium Salt I | Barium Salt II | Barium Salt III |
|---|---|---|---|
| % Moisture | 8.08 | 8.48 | 12.62 |
| % Ash* | 13.74 | 14.86 | 10.78 |
| % Carbon Dioxide | 7.86 | 9.80 | 8.75 |
| % Uronic Acid | 34.32 | 42.79 | 38.21 |
| Total % Pentosan | 39.49 | 34.53 | 51.29 |
| % Pentosan | 34.46 | - | 47.61 |
| % Methylpentosan | 22.76 | - | 15.29 |
| % Hexosan (by difference) | 26.19 | 22.68 | 10.50 |
| % Hexosan** (by difference) | 8.46 | - | -1.11 |
| $[\alpha]_D^{25^\circ}$ | $-0.03^\circ$ | $+41^\circ$ | $-15^\circ$ |
| Equivalent weight | 513 | 411 | 461 |
| Total Pentosan units per Uronic Acid | 1.52 | 1.07 | 1.79 |
| Pentosan units per Uronic Acid | 1.35 | - | 1.66 |
| Methylpentosan units per Uronic Acid | 0.80 | - | 0.68 |
| Hexosan units per Uronic Acid | 0.83 | 0.58 | 0.30 |
| Hexosan units per Uronic Acid** | 0.27 | - | - |

* Corrected for moisture.
**Calculated from the difference of uronic acid, pentosan, and methylpentosan.

3. Identification of the Uronic Acid

Barium salt III was tested for hexose[19] by the method described on page27. The test was negative. The galacturonic acid was identified by oxidation to mucic acid as follows:[19] One gram of barium salt III was dissolved in 15 cc. of $HNO_3$ of sp. gr. 1.15, and the solution evaporated on a water bath to one-third its original volume. After three days, white crystals separated. The crystals were filtered from the solution, washed with cold water, and dried in a vacuum desiccator. They melted at 212°. Shriner and Fuson[20] report a melting point of 213°, and Browne and Zerban[19] report 213-214°.

Paper chromatography also showed evidence of barium galacturonate.[16] Two mixed solvents were used: butanol saturated with water and a solution of ethyl acetate and pyridine saturated with water. Controls of galacturonic acid, whose circular $R_f$ value is very close to one of those of the barium salt, were run with each trial.

## Identification of the Sugars

1. Chromatography

The methods for chromatographing the sugars were used as described by Rao and Beri.[15,16] In all cases, the tails on the filter paper were cut 2 mm. wide and 1 cm. in length. Three sugar solutions were used. The concentrated sugar solutions from hydrolyses I and II were used without dilution. Approximately 100 mg. of the crystalline sugar

product from hydrolysis III was dissolved in 1 cc. of water. These solutions were spotted on the filter paper with a capillary tubing drawn out to a fine tip. The size of the spot obtained was between three and four mm. in diameter. In all cases a control was run at the same time. The control was prepared by dissolving approximately 30 mg. each of d-galactose, l-arabinose, l-xylose, and l-rhamnose in 2 cc. of water. Three different solvents were used:

(1) The butanol-water solvent was prepared by shaking Baker's analyzed, chemically pure 1-butanol with excess water. The two layers were allowed to separate and the lower water-rich layer was drawn off. More consistant results were obtained when the butanol was shaken with water before each run. The butanol rich layer was poured into Petri dishes 15.5 cm. in diameter and enough butanol-saturated water was added so that the water layer just covered the bottom of the Petri dish. The wick was short enough so that it dipped into the butanol layer only. In this manner, the butanol remained saturated with water during the chromatographing period.

(2) The phenol-water solvent was prepared by mixing 450 gm. Baker and Adamson reagent grade phenol with 50 gm. of water.

(3) The p-cresol solvent used was prepared as follows: the red liquid was poured from Eastman Kodak practical grade p-cresol. The remaining crystals were

warmed to their melting point, and the liquid was poured into a 500 cc. Claisenn distilling flask. The fraction boiling between 95-96° (15 mm.) was collected. Two-thirds of the p-cresol distilled within this temperature range and the distillate was colorless. The p-cresol was shaken with an excess of water, and the layers were allowed to separate. The lower p-cresol rich layer was pipetted into the Petri dishes, taking care to prevent any water from accompaning the p-cresol, since the water would remain above the p-cresol rich layer and interfere with the chromatographic process. All the runs were made between 26 and 27°. The developing aniline phthalate reagent used was prepared as described by Partrich.(25) A mixture of 0.191 g. of aniline and 1.66 g. of phthalic acid was dissolved in 200 cc. of butanol saturated with water. After drying the filter papers for five minutes in an oven at 110°, they were sprayed with the reagent, and then heated in an oven at 110° for five minutes.

The results of the chromatograms are shown in table IV. Galactose and arabinose gave the faintest bands in all the sugar hydrolysates, while xylose and rhamnose gave strong bands which were considerably wider. The center of the bands was used in calculating the circular $R_f$ values.

Table IV

RESULTS OF CHROMATOGRAMS

| | Control | Butanol-Water I | II | III | Rao & Beri |
|---|---|---|---|---|---|
| d-galactose | 0.22 | 0.23 | 0.22 | 0.23* | 0.26 |
| l-arabinose | 0.28 to | 0.33 | 0.33 | 0.34 | 0.33 |
| l-xylose | 0.33 | | | | 0.37 |
| l-rhamnose | 0.44 | 0.46 | 0.45 | 0.44 | 0.48 |

| | Control | p-Cresol-Water I | II | III | Rao & Beri |
|---|---|---|---|---|---|
| d-galactose | 0.34 | 0.35 | 0.38 | 0.36 | 0.37 |
| l-xylose | | | | | 0.39 |
| l-arabinose | 0.42 | 0.43* | 0.45* | 0.43* | 0.49 |
| l-rhamnose | 0.49 | 0.52 | 0.53 | 0.53 | 0.53 |

| | Control | Phenol I | II | III | Rao & Beri |
|---|---|---|---|---|---|
| d-galactose | 0.43 | 0.49 | 0.50 | 0.49 | 0.60 |
| l-xylose | | | | | 0.67 |
| l-arabinose | 0.53 | | | | 0.68 |
| l-rhamnose | 0.64 | 0.64 | 0.64 | 0.64 | 0.76 |

*Very faint lines.

2. Derivatives for the Sugars

l-arabinose. A derivative for l-arabinose was prepared as follows:[19] 0.5 g. of the crystalline sugar product from hydrolysis III was warmed slightly in 4 cc. of 75% ethanol with 0.7 g. of α-benzylphenylhydrazine hydrochloride and 0.25 g. of sodium acetate. An orange oil immediately separated and after one-half hour, flowers of white crystals separated on the sides of the tube. Soon the whole mass became solid.

The crystals were purified by recrystallization from 75% ethanol.[19] They melted at 168°. Browne and Zerban report a melting point of 174°.

l-rhamnose. A derivative of l-rhamnose was prepared as follows: 0.5 g. of the crystalline sugar product from hydrolysis III was warmed slightly in 6 cc. of 95% ethanol with 0.9 g. of β-naphthylhydrazine hydrochloride and 0.25 g. of sodium acetate. Light orange crystals separated upon cooling. The β-naphthylhydrazine of l-rhamnose, which was recrystallized from 95% ethanol, melted at 191°. Browne and Zerban[19] report a melting point of 192°.

## SUMMARY

The crude mucilage from the seed of the chia grande, Hyptis suaveolens, is best extracted with water and precipitated with 95% ethanol. The unpurified mucilage is the calcium or magnesium salt of a complex polyuronide and is composed of a mixture of two mucilage fractions with different solubilities but with the same ash free equivalent weight. This complex polyuronide contains about three pentosan units, one methylpentosan unit, and one hexosan unit per uronic acid.

Preparation of the free acid from the crude mucilage indicates that the hexosan unit is the first sugar molecule to be removed by hydrolysis.

The mucilage was hydrolyzed in four per cent sulfuric acid for varying lengths of time and under various temperature conditions. The analysis of the barium salts offers further proof that the hexosan unit is the first sugar unit to be removed by hydrolysis, and indicates that the methylpentosan sugar molecule is near the end of the uronic acid molecule or in a side chain.

Horizontal paper chromatography showed the sugars in the mucilage to be d-arabinose, l-xylose, and l-rhamnose.

The barium salt obtained from hydrolysis under the most stringent conditions showed no hexose to be present.

This barium salt was used for the identification of the uronic acid by oxidation to mucic acid, which proved the presence of galacturonic acid.

## BIBLIOGRAPHY


1. Gentry, "Rio Mayo Plants", Carnegie Institution of Washington, Washington, D. C., 1942, p. 228.

2. Anderson, E., and Sands, L., "Advances in Carbohydrate Chemistry", Vol. I, Academic Press, Inc., New York, 1943, pp. 329-344.

3. Abderhalden, "Biochemisches Handlexikon", Springer, J, Vienna, 1932.

4. Whistler and Smart, "Polysaccharide Chemistry", Academic Press, Inc., New York, 1953, pp. 4, 327.

5. Norman, "Biochemistry of Cellulose, The Polyuronides, Lignin, etc.", Clarendon Press, Oxford, 1937, p. 134.

6. Harvey, "Plant Physiological Chemistry", Century Co., New York, 1930, p. 168.

7. Bailey, K., Biochem J., 26, 2477 (1935).

8. Bailey, K., and Norris, F. W., Biochem J., 26, 1609 (1932).

9. Anderson, E., and Crowder, J. A., J. Am. Chem. Soc., 52, 3711 (1930).

10. Anderson, E., and Lowe, H. J., J. Biol. Chem., 148, 289 (1947).

11. Renfrew, A. G., and Cretchner, L. H., J. Biol. Chem., 97, 503 (1932).

12. Anderson E., and Fireman, M., J. Biol. Chem., 109, 437 (1935).

13. Anderson, E., Gillette, L. A., and Seeley, M. G., J. Biol. Chem., 140, 569 (1941).

14. Rao, P. S., and Beri, R. M., Current Science, 20, 99 (1951).

BIBLIOGRAPHY
(cont)

15. Rao, P. S., and Beri, R. M., Proc. Indian Acad. Sci., 33A, 368 (1951).

16. Rao, P. S., and Beri, R. M., Proc. Indian Acad. Sci., 34A, 236 (1951).

17. Anderson, E., and Otis, L., J. Am. Chem. Soc., 52, 4461 (1930).

18. Tollens, B., Ber., 41, 1783 (1908).

19. Browne, C. A., and Zerban, F. W., "Sugar Analysis", 3rd Ed., John Wiley and Sons, New York, 1941.

20. Shriner, R. L. and Fuson, R. C., "Identification of Organic Compounds", 3rd Ed., John Wiley and Sons, New York, Chapmons and Hall, London, 1948, pp. 52-54.

21. von Fellenberg, T., Biochem. Z., 85, 44, (1918).

22. Deniges, M. G., Comp. rend., 150, 529, (1910).

23. Von der Haar, A. W., "Anleitungzum Nachweis, zur Trennung und Bestimmungder Monosaccharide und Aldehydesauren", Gebruder Borntraeger, Berlin, 1920, p. 71.

24. Ledbetter, H. D., Master's Thesis, University of Arizona, (1950).

25. Partrich, S. M., Nature, 164, 443 (1949).