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STUDIES ON THE HOLOCELLULOSE OF SITKA SPRUCE

I. DILUTE ACID HYDROLYSIS

by

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ABSTRACT

Holocellulose has been prepared from Sitka spruce heartwood. This material has been subjected to acid hydrolysis for increasing periods of time. The residues have been examined for residual pentosan and alpha-cellulose. The hydrolyzates have been examined for free sugars by means of paper partition chromatography. Glucose, galactose, mannose, arabinose and xylose have been identified and the presence of a methoxyuronic acid inferred. A procedure has been devised for the quantitative determination of these sugars after their separation on paper chromatograms.

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TABLE OF CONTENTS

INTRODUCTION	Page No. 1
OBJECT OF STUDY	13
EXPERIMENTAL	
I. Preparation of Holocellulose	14
II. Hydrolysis of Holocellulose	15
III. Examination of Hydrolyzates	
A. Preliminary experiments on the chromatography of sugars	19
B. Qualitative identification of hydrolysis products	21
C. Estimation of sugars in hydrolyzates	23
DISCUSSION	29
SUMMARY	36
BIBLIOGRAPHY	38
APPENDIX	

FIGURES

Figure 1. Components of extractive-free wood Page No. 7

Figure 2. Micro-pipette 24

Figure 3. Micro-scale volumetric flask 24

Figure 4. Weight loss on hydrolysis of holocellulose with 1.021 N H_2SO_4 at 93° C Appendix I

Figure 5. Residual pentosan after hydrolysis of holocellulose with 1.021 N H_2SO_4 at 93° C Appendix II

Figure 6. Alpha-cellulose in residues after hydrolysis of holocellulose with 1.021 N H_2SO_4 at 93° C Appendix III

TABLES

Table 1. Analysis of holocellulose Page No. 15

Table 2. Residues after hydrolysis with 1.005 N H_2SO_4 at 102° C 16

Table 3. Pentosan in residues after hydrolysis with 1.005 N H_2SO_4 at 192° C 16

Table 4. Residues after hydrolysis with 1.021 N H_2SO_4 at 93° C 17

Table 5. Pentosan in residues after hydrolysis with 1.021 N H_2SO_4 at 93° C 18

Table 6. Alpha-cellulose remaining after hydrolysis with 1.021 N H_2SO_4 at 93°C 18

Table 7. Comparative distance travelled (in cm.) by components of hydrolyzate and by standard sugars in 22 hours irrigation with pyridine-water-ethyl acetate (1-2-2). 22

Table 8. Comparative distance travelled (in cm) by components of hydrolyzate and by standard sugars in 29.5 hours irrigation with butanol-water-acetic acid (4-1-5). 23

Table 9. Per cent sugar in hydrolyzates after hydrolysis with 1.021 N H_2SO_4 at 93° C 28

PLATES

Plate I. Effect of uneven temperature on direction of solvent flow — Appendix IV

Plate II. Disposition of sugars on chromatogram after 22 hours irrigation with pyridine-water-ethyl acetate (1-2-2) — Appendix V

Plate III. Disposition of sugars on chromatogram after 29.5 hours irrigation with butanol-water-acetic acid (4-1-5) — Appendix VI

Wood Components

The cell walls of woody tissue are composed of a material containing carbohydrates and lignin. The carbohydrate portion is composed largely of cellulose, a polysaccharide of known structure, and also contains a group of polysaccharides of uncertain structure known as the hemicelluloses. Lignin is an organic material, rich in aromatic rings, which may be a polymer having a phenyl propane radical as the basic unit. In addition, wood contains a group of extraneous substances not an integral part of the cell wall, which include volatile oils, fats, waxes, resenes, monosaccharides and acid salts. These may be removed by extraction with neutral solvents, hence, are classed as "extractives". There may also be present difficultly soluble extraneous substances such as starch and calcium pectate. Lignin and the carbohydrate fractions are found in all woods, but the extractives vary from species to species and often serve for taxonomic classification.

The present work is concerned with the carbohydrate material, hence, lignin and the extractives will not be considered further. A more detailed discussion of cellulose and the hemicelluloses is in order, however.

Heuser and Boedeker (1) identified wood cellulose with cotton cellulose which has been shown to be a polymeric substance consisting of linear chains of glucose residues joined by β1,4 linkages (2). The number of glucose anhydride units per molecule may vary over a wide range, natural celluloses having a degree of polymerization of 1500-2500. These chains are oriented to give a fiber which is essentially crystalline but possesses amorphous areas (2).

The term hemicellulose is applied to those carbohydrates of the wood which are alkali-soluble and hydrolyzable by dilute acids to give free sugars. The definition includes polysaccharides of two different types which Norman (3) prefers to term separately "cellulosans" and "polyuronide hemicelluloses". The cellulosans are oriented short-chain polysaccharides which participate in the cellulose micelle and crystallites in the same way as do the individual cellulose chains. The cellulosan fraction of hardwoods appears to be predominantly a xylose polymer, or xylan. In addition, there may be present some glucosan which probably represents very short cellulose chains. In softwoods this fraction has usually a much lower xylan content, and contains mannans in addition to glucosans. The polyuronide hemicelluloses are non-oriented polysaccharides situated in the amorphous regions of the cellulose fiber. They contain glucuronic or galacturonic acid or their mono-methoxy derivatives, accompanied by hexoses or pentoses or both.

Very small amounts of pectin, a polysaccharide consisting almost exclusively of galacturonic acid units may also be present in the hemicellulose fraction (4).

Isolation of the Carbohydrates

The removal of lignin is prerequisite to the study of the carbohydrates of the cell wall. The earliest and best known method for the removal of lignin is that of Cross and Bevin (5) in which extractive free wood is treated first with one per cent sodium hydroxide then with moist chlorine gas followed by sodium sulfite and finally sulfurous acid to remove the chlorinated lignin. Many minor modifications of the Cross and Bevin method have been introduced, and in 1934 Ritter and Mitchell (6) published a procedure which combines the most desirable features of these. Essentially the process consists in alternate treatment of extractive-free wood with chlorine and with sulfurous acid and sodium sulfite.

The object of the Cross and Bevin method was the quantitative isolation of cellulose rather than the removal of lignin. The result is, however, a compromise in that the isolated material contains cellulose and the cellulosans, the polyuronide hemicelluloses having been removed with the lignin. Norman and Jenkins (7) devised a procedure in which wood is treated alternately with sodium hypochlorite (15 per cent available chlorine) and sodium sulfite. The product is identical with Cross and Bevin cellulose but the procedure has the advantage that gaseous chlorine is not required.

In 1920, Schmidt and Graumann (8), by treating wood

alternately with chlorine dioxide and sodium sulfite solution were successful in removing lignin without affecting the carbohydrate fraction. The material thus isolated they termed "Skelettsubstanz". Schmidt and co-workers (9) later modified the procedure by replacing the sodium sulfite solution with aqueous pyridine. The chief objection to the procedure is that approximately one month is required for complete removal of lignin. Ritter and Kurth (10) improved the procedure by employing an alcoholic solution of pyridine as a solvent for chlorinated lignin. Repeated chlorination and extraction with this solvent reduced the time required to two days and yielded a product similar to "Skelettsubstanz" which they designated "Holocellulose".

Van Bechum and Ritter (11) modified the procedure by replacing pyridine with monoethanolamine as a solvent for chlorolignin. This reagent has the added advantage that it gives a color reaction with chlorolignin which permits the observation of the end point in the delignification process. It was found later (12,13) that approximately two per cent nitrogen was retained by the holocellulose due perhaps to reaction of the monoethanolamine with some portion of the hemicelluloses.

Jayme (14), in 1942, described a method for the preparation of holocellulose by treatment of wood with aqueous sodium chlorite and acetic acid. The chlorite treatment was carried out at 60° for 12 hours then the

residue was filtered and washed. The procedure was repeated until three successive treatments of 12, 8 and 8 hours duration had been carried out. The final white residue still contained 2.8 to 3.5 per cent lignin which could not be removed without loss of carbohydrate. The method was studied by Wise et. al. (13) who showed that the intermediate washing and filtering was unnecessary and that the delignification was almost complete in four hours if the treatment was carried out at 70 to 80 degrees. Jayme's findings that complete removal of lignin caused a marked loss of hemicellulose were substantiated.

Fractionation of the Carbohydrates

In general, the approach to hemicellulose isolation has been an alkaline extraction of the hemicellulose from wood or holocellulose. It was mentioned previously that the material thus isolated contains both the cellulosans and the polyuronide hemicelluloses. Cellulosans may be obtained directly by alkaline extraction of Cross and Bevin cellulose, but no direct procedure exists for the isolation of the polyuronides. Van Beckum and Ritter (15), by hydrolysis of chlorite holocellulose for two hours with 1.3 per cent sulfuric acid, obtained a material which proved almost identical with Cross and Bevin cellulose. It should, therefore, be possible to isolate the cellulosans from hydrolyzed chlorite holocellulose.

Extraction of holocellulose or Cross and Bevin cellulose with 17.5 per cent sodium hydroxide leaves a residue which is

termed "alpha-cellulose". Acidification of the extract precipitates a "beta-cellulose" fraction, leaving "gamma-cellulose" in solution. The alpha-cellulose fraction approaches true cellulose in composition but a part of the associated cellulosans is retained (16). The separation into alpha-, beta-, and gamma-cellulose fractions depends more on chain length than on actual chemical differences in the materials but the determinations are of considerable value in commercial pulping control work. Wise and Ratliff (16) have shown that when chlorite holocellulose is extracted with gradually increasing concentrations (from 2 to 24 per cent) of aqueous potassium hydroxide under nitrogen a point of diminishing returns is reached beyond which practically nothing more is extractable. The residue is somewhat less than that obtained with 17.5 per cent sodium hydroxide but still contains some mannan and xylan.

The relationship and composition of some of the fractions mentioned is shown in the following diagram which is a composite of similar schemes presented by Norman (2) and by Wise (16)

Figure 1: Components of extractive-free wood

Holocellulose			
	Hemicellulose		
Cross and Bevin Cellulose			
Alpha-cellulose			
"True" Cellulose	Cellulosans	Polyuronide Hemicelluloses	Lignin
Long chains of -1-4, anhydro-glucose units	xylan glucosan mannan	Shorter chains containing uronic or methoxy uronic acids xylose arabinose glucose galactose	The non-carbo-hydrate material of the cell wall
Oriented	Oriented	Amorphous	Amorphous

O'Dweyer (17) fractionated a hemicellulose preparation prepared by extracting wood with 4 per cent alkali. The precipitate obtained by acidifying the alkaline extract she termed "hemicellulose A" and that obtained by the addition of alcohol to the acid filtrate she called "hemicellulose B". Anderson, (18) and Sands and Gary (19) carried out similar fractionations using increasing proportions of alcohol. No fractions were obtained entirely free of polyuronides although concentration of xylan and mannan was effected in some cases. Norman (2) points out that the system of differential precipitation will give chemically distinct fractions only if it happens that these differences are also correlated with differences in molecular size.

Lignin Carbohydrate Complex

Although the hemicelluloses are defined as being alkali-soluble, they are not completely removed by alkali treatment unless the wood is previously or concurrently delignified. Once isolated, however, they are readily soluble in dilute alkali and often in water. This behavior is difficult to explain without assuming linkage to some other cell-wall constituent. In the case of the polyuronide hemicelluloses, evidence has accumulated which points to linkage with the lignin. Norman and Shrikhande (20) showed that in the Cross and Bevin procedure for cellulose, chlorination is as important for the removal of the polyuronides as for the removal of lignin. Little lignin or hemicellulose is removed by sulfite treatment alone, but chlorination, followed by sulfite treatment, removes them at approximately equal rate. Harris, et. al. (21) report that whereas isolated lignin may be methylated readily, additional methoxyl groups cannot be introduced into lignin in situ unless preceded by an acid treatment capable of hydrolyzing associated polysaccharides. The observation of Jayme (14), and of Wise et.al. (13), that removal of the last 3 per cent of lignin from chlorite holocellulose is accompanied by a loss of hemicellulose, points to a lignin-hemicellulose linkage. Brauns (22) isolated a compound from wood which showed no reducing action but which, when hydrolyzed, gave a substance resembling lignin and a solution which gave a strong Fehlings test for reducing sugar. This may have been a lignin-

carbohydrate fraction. No direct evidence of such a linkage has been found, but Brauns (23) has discussed the possible ways in which lignin and cell-wall carbohydrates could be chemically combined.

Determination of Sugars

The constitutional study of a polysaccharide involves the acid hydrolysis of the isolated material followed by qualitative identification of the component sugars and sugar acids. The quantitative determination of the components gives an indication of the ratio of the various sugars present in the material. Methylation of the polysaccharide followed by hydrolysis and determination of the methylated sugars provides an insight into the manner of their linkage in the original molecule.

These methods may be applied to the constitutional study of the hemicelluloses, but with greater difficulty. The balance between liberation and destruction of pentoses and uronic acids in the hydrolysis of hemicelluloses is a delicate one, therefore, results may often be difficult to interpret. Link and Niemann (24) observed that concentrations of hydrochloric or sulfuric acid above 5 per cent are very effective in decarboxylating uronic acids, but that hydrolysis proceeds so slowly with acid concentrations of 2 per cent or less that free uronic acid is destroyed almost as rapidly as it is liberated. Aldobionic acids exhibiting marked resistance to hydrolysis have been encountered. Anderson and Kinsman (25) isolated an acid formed by one

xylose unit linked to glucuronic acid which resisted boiling 4.5 per cent sulfuric acid for 17 hours.

Identification of sugars and sugar acids, or their methylated derivatives in a mixture is frequently difficult. The classical methods of identification require the preparation of crystalline derivatives whose physical properties can be determined for comparison with the physical properties of reference compounds. Glucose, galactose, and their uronic acids are identified by oxidation to saccharic and mucic acid respectively. Mannose may be characterized as the phenylhydrazone, arabinose as the diphenylhydrazone and xylose as the p-bromophenylosazone. Mannose and the pentoses may be quantitatively determined as the derivatives mentioned, but glucose and galactose are normally determined through selective fermentation by yeasts. Methylated sugars are separated by fractional distillation, and may be identified and approximately determined as derivatives, such as amides or anilides, after oxidation.

Paper partition chromatography, developed in 1943 by Consden, Gorden and Martin (26) for the separation and identification of amino acids, was adapted to the resolution of sugars by Partridge (27), in 1948. The procedure consists in placing a spot of a sugar solution near the top of a strip of filter paper, the top of which is immersed in a trough. The trough and paper are placed in an air tight chamber so that the atmosphere becomes saturated with water vapour and solvent vapour. Absorbed water provides a stationary phase

on the paper, and the sugars are separated by virtue of their differences in partition between this and the organic phase moving down the paper. The separated sugars are detected by spraying the paper with suitable reagents (28, 29, 30) and are identified by the rate at which they move under standard conditions or by reference to standards run on the same paper. Hirst, Jones, et. al. (31), have applied the method to the separation of methylated sugars and have tabulated observed values for the rate of travel of 38 sugars. The method has been adapted to the quantitative separation and estimation of the sugars (32, 33, 34). The method has the tremendous advantage that selective reagents are not required. Once separated, an oxidizing agent that will deal with micro quantities serves for the determination of all reducing sugars.

Paper partition chromatography is useful in the identification of sugars but differentiation between D and L stereo-isomers is not possible. It may still, therefore, be necessary to isolate the individual sugars at least on a semi-micro scale. This may be accomplished, using the partition principle, by replacing filter paper strips with columns of powdered cellulose (35) or silicates (36). Chromatographic adsorption of sugars on columns of solid adsorbents such as calcium carbonate, magnesium oxide, or alumina, may also be employed in the isolation of the sugars (37). In these last mentioned techniques the solvent is passed through the column continuously so that

the resolved sugars are swept through the column and are collected in the eluate by means of an automatic fraction collector which changes the receiver at intervals.

OBJECT OF STUDY

The chlorite procedure for holocellulose now permits the rapid isolation of the total carbohydrate fraction of wood and thus provides an excellent source of material for constitutional studies. The program of study in this laboratory is directed toward the elucidation of the structure of and the relationship between the various components of holocellulose. The present work involves a preliminary investigation into the behavior of Sitka spruce holocellulose under the hydrolytic influence of dilute acids, and the identification of the products of hydrolysis. Other experiments dealing with the alkylation and hydrolysis of the methylation derivatives of holocellulose are being carried out concurrently with the present investigation (38). Partition chromatography promises to be the most satisfactory method of separating complex mixtures of methylated sugars in such hydrolyzates. It seemed advisable, therefore, to apply this technique to the identification of the simple sugars resulting from the hydrolysis of holocellulose. The development of a procedure for the quantitative determination of free sugars on the paper chromatogram was also envisioned.

EXPERIMENTAL

1. Preparation of Holocellulose.

A board identified as Sitka spruce (Picea sitchensis) heartwood was obtained from the Forest Products Laboratory of the Department of Resources and Development at Vancouver. The wood was cut into small blocks and ground in a Wiley mill to pass a 40 mesh screen. The sawdust was then screened to remove all material finer than 50 mesh size. The extractives were removed by continuous extraction for 8 hours in a soxhlet extractor with methyl alcohol-benzene (1-2). The sawdust was treated in 15 gram batches until 600 grams of extractive-free sawdust had been prepared.

The wood was then delignified by the following procedure (13):

100 g. of the extractive free sawdust was suspended in 3200mls. of water, 20 ml. of glacial acetic acid were added followed by 30 g. of sodium chlorite, $NaClO_2$. The mixture was heated for one hour at 70° to $80^{\circ}C$, another 20 ml. of acetic acid and 30 g. of $NaClO_2$ were added at the end of the first hour, and the heating continued for another hour. The process was repeated until four additions had been made, and the mixture had been heated for four hours. The mixture was cooled in an ice bath, filtered through a Buchner funnel, the residue washed with ice water and finally with

acetone. The resulting holocellulose was snow white in color and retained the structure of the original sawdust. When 300 g. of the holocellulose had been prepared the air dried material was analysed for lignin, alpha-cellulose, pentosan, ash and uronic acid (as carbon dioxide) by the standard T.A.P.P.I. methods (39).

Table 1.: Analysis of Holocellulose

	I	II	III	average
ash	0.45	0.47	0.48	0.47
Lignin	2.6	3.0	2.7	2.8
alpha-cellulose	62.5	62.2	62.7	62.5
pentosan	5.8	6.0	6.2	6.0
carbon dioxide	1.2			
methoxyl[1]	1.4	1.5	-	1.5

1 Determined by A.J.Martin

II. Hydrolysis of Holocellulose

Experiment 1.

Two gram samples of holocellulose were accurately weighed into 250 ml. erlenmeyers, 50 ml. of 1.005 N sulfuric acid added to each flask and the mixtures refluxed at the boiling point on a hot plate. At the end of each hour a flask was removed, cooled under running water, and the contents filtered through a tared sintered glass crucible. When five samples had been treated, the residues were oven dried and weighed to determine the per cent loss in weight.

The oven dried residues were then analysed for residual pentosan. The hydrolyzates were saved for future study.

Table 2: Residues after hydrolysis with 1.005 N H_2SO_4 at 102°C

Sample	Hours	Per cent solids	Per cent loss
1	1	77.0	23.0
2	2	74.4	25.6
3	3	69.2	30.8
4	---	----	----
5	5	69.6	30.4

Table 3: Pentosan in residues after hydrolysis with 1.005 N H_2SO_4 at 102°C

Sample	Hours	Residual Pentosan*	Loss as Pentosan
1	1	2.3	3.7
2	2	1.3	4.7
3	3	1.4	4.6
4	---	---	--
5	5	0.7	5.3

* Percentages based on holocellulose

Excessive bumping during refluxing resulted in the loss of material from one of the samples during this experiment. Bumping could not be eliminated by the use of glass beads, boiling chips, or boiling tubes, because of the blanketing effect of the sawdust.

Experiment 2.

A second series of samples were hydrolyzed in flasks which were suspended in a water bath consisting of a 12" evaporating dish heated by a Meker burner. Analysis of the resulting residues yielded inconsistent results indicating

that uneven heating had occured due to poor circulation of the bath water. The water bath was then modified to include a stirrer and a water inlet tube so that a constant level could be maintained by dripping warm tap water into the bath.

Experiment 3.

Two gram samples of holocellulose were weighed into 250 ml. erlenmeyer flasks and heated on the water bath with 50 ml. of 1.021 N H_2SO_4 which had been brought to bath temperature (93°C) before being added. Flasks were removed at intervals, until seven samples had been treated cooled and filtered. The per cent loss in weight was determined and the residues were analysed for residual pentosan.

Table 4: Residues after Hydrolysis with 1.021 N H_2SO_4 at 93°C

Sample	Hours	Percent solids	Percent loss
1	1/2	91.5	8.5
2	1	86.1	13.9
3	2	73.5	26.5
4	3	74.2	25.8
5	4	75.6	24.4
6	5	75.0	25.0
7	6	74.0	26.0

Table 5: Pentosan in residue after hydrolysis with 1.021N H_2SO_4 at 93°C

Sample	Hours	Residual Pentosan	Loss as Pentosan
1	1/2	3.8	2.2
2	1	3.0	3.0
3	2	2.4	3.6
4	3	2.5	3.5
5	4	2.0	4.0
6	5	1.9	4.1
7	6	1.9	4.1

Experiment 4.

A fourth series of samples was treated in the same manner and under the same conditions as in Experiment No. 3. The resulting residues were analysed for alpha-cellulose which was calculated as a per cent of the original sample.

Table 6: alpha-cellulose remaining after hydrolysis with 1.021N H_2SO_4 at 93°C

Sample	Hours	Residual alpha-cellulose
1	1/2	52.3
2	1	45.5
3	2	45.9
4	3	43.5
5	4	40.1
6	5	39.8
7	6	32.7

In all cases it was observed that a fine silt settled out when the hydrolyzates were permitted to stand overnight after filtration. This material, which did not exceed 1% of the original samples, was presumed to be a part of the residues and was not examined.

III. Examination of the Hydrolyzates

A. Preliminary experiments on the chromatography of sugars.

Standard samples of glucose, galactose, mannose, xylose, arabinose, glucuronic acid and galacturonic acid containing 10 mg. of sugar per ml. were prepared. Chromatograms were formed by depositing a drop of each standard solution along a line drawn 7 cm. from one end of an 18 x 57 cm. sheet of Whatman No. 1 filter paper. The paper strips were suspended from a glass trough enclosed in a 12" x 24" cylindrical glass chamber having a plate glass lid which could be sealed with vaseline. The chromatograms were developed by filling the trough with solvent which siphoned over and down the paper irrigating the deposited sugars. After treatment the papers were removed and air dried. The sugars were detected by spraying the paper with the aniline-hydrogen-phthalate reagent of Partridge (29). The sprayed papers were air dried then heated in the oven at 105° for 10 to 15 minutes whereupon the pentose sugars were revealed as brown spots and the hexoses and uronic acids as pink spots.

Several of the solvent mixtures reported in the literature were examined to determine which would give the best resolution of all of the sugars in a single mixture. The solvents found most satisfactory were butanol-water-acetic acid (4-1-5 $^{v}/v$) (27), and pyridine-water-ethyl acetate (1-2-2 $^{v}/v$) (40). The separations were most effective when the sugars had travelled about 3/4 the length of the paper. This required from 16 to 24 hours with the pyridine mixture, and from 36 to 40 hours with the butanol-water-acetic acid. The Rf values (ratio of distance travelled by sugar to distance travelled by solvent front) are very low with these solvents, so that the sugars have travelled only a few centimeters when the solvent has reached the bottom of the paper. This difficulty was overcome by placing a pad of absorbent cotton at the base of the hanging paper and allowing the solvent to run off into the cotton. The rate of descent of the solvent appeared to decrease if it was permitted to buildup and drip off the lower edge of the paper.

It was observed that the properties of a solvent mixture were markedly altered if small amounts of a previously used mixture were left in the bottom of the chamber. It was, therefore, necessary to wash and dry the chamber before a new solvent was used if reproducible results were desired. The chromatographic chamber had to be shielded from draughts and kept away from radiators since it was found that temperature gradients caused the solvent front to angle down

the paper. Plate No. I in the appendix shows the effect of a Bunsen burner standing three feet from the chamber.

B. Qualitative Identification of Hydrolysis Products

The hydrolyzates from hydrolysis No. 1 were treated with saturated barium hydroxide solution until basic to phenolphthalein and the excess barium removed by back titrating with 1 N sulfuric acid. The resulting solutions were found to be too dilute for chromatographic work and were therefore concentrated by vacuum distillation. Filter paper chromatograms were attempted during various stages of the concentration. Faint sugar spots could be detected on the paper at volumes up to 100 ml. but no satisfactory chromatograms were obtained until the volume had been reduced to approximately 1 ml.

With pyridine-water-ethyl acetate as solvent, galactose, glucose, and xylose were readily identified by reference to the standards. Mannose and arabinose travelled the same distance on the chromatogram so that the adjacent spot from the hydrolyzate could not be identified definitely. A very faint spot and a well marked spot were detected, neither of which corresponded to standards. Galacturonic acid did not appear on the chromatogram, and no spot corresponding to the glucuronic acid standard was evident. The well defined spot of unknown material travelled only a few centimeters down the paper while the bulk of the sugars travelled a considerable distance. It was, therefore, suspected that the material was the barium salt of a uronic acid. Half quantities of

barium hydroxide were added to the uronic acid standards so that the barium salts and the free acids would appear on the chromatograms. It was then found that the glucuronic acid standard yielded a spot corresponding to the slow moving unknown material. The galacturonic acid standard again failed to show up on the paper. In the following table the distances travelled by the various components of the hydrolyzate are compared to the distances travelled by the standard sugars on the same chromatogram.

Table 7: Comparative distance travelled (in cm) by components of hydrolyzate and by standard sugars in 22 hours irrigation with pyridine-water-ethyl acetate (1-2-2)

Components of Hydrolyzate	3.2	8.7	25.6	28.7	32.9	38.1	--
Standard sugars:							
glucose	-	-	-	29.5	-	-	-
galactose	-	-	25.9	-	-	-	-
mannose	-	-	-	-	33.5	-	-
xylose	-	-	-	-	-	38.1	-
arabinose	-	-	-	-	32.7	-	-
glucuronic acid	-	-	-	-	-	-	50
Ba glucuronate	3.5	-	-	-	-	-	-

When butanol-water-acetic acid was employed as the solvent, mannose was well separated from the other sugars and could be detected in the hydrolyzate. Arabinose travelled almost the same distance as xylose so that it was again difficult to detect arabinose among the components. The galactose and glucose standards were likewise poorly separated.

Only one unidentifiable spot appeared among the hydrolytic components, and no slow moving material was apparent. The glucuronic acid standard also failed to produce a slow moving spot but did yield two spots, neither of which corresponded to components of the hydrolyzate. The distances travelled by the standard sugars are compared to those travelled by the components of the hydrolyzate, in the following table.

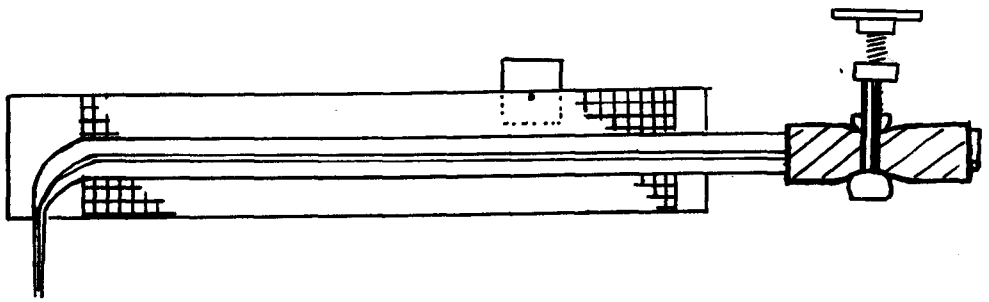
Table 8: Comparative distance travelled (in cm) by components of hydrolyzate and by standard sugars in 29.5 hours irrigation with butanol-water-acetic acid (4-1-5)

Components of Hydrolyzate	12.2	20.8	21.3	23.9	27.2	28.6	-	-
Standard sugars:								
glucose	-	-	20.9	-	-	-	-	-
galactose	-	20.1	-	-	-	-	-	-
mannose	-	-	-	24.2	-	-	-	-
xylose	-	-	-	-	-	28.4	-	-
arabinose	-	-	-	-	27.5	-	-	-
glucuronic acid	-	-	-	-	-	-	-	32.8
Ba glucuronate	-	-	-	-	-	-	16.2	-

C. Estimation of Sugars in Hydrolyzates

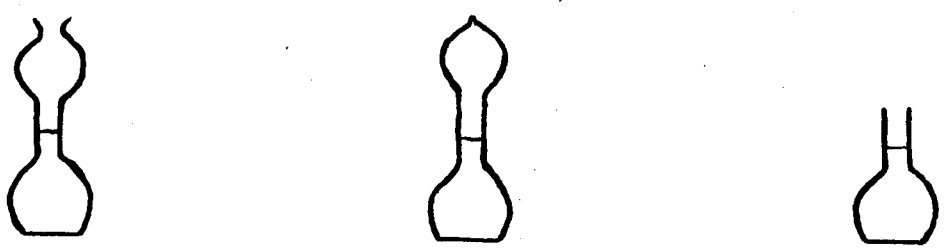
A micro-pipette for the transfer of solution on to the chromatograms was prepared from 1 mm. capillary tubing, and was calibrated with mercury to deliver up to 90 micro-liters.

Figure 2 : Micro-pipette.



A number of one ml. volumetric flasks were also prepared and calibrated. The flasks, which were blown from 4 mm. pyrex tubing, were provided with a bulb part way up the neck to facilitate mixing of the contents. The flasks could be sealed in a flame after filling, the contents mixed by shaking from bulb to bulb, and the upper bulb then cut off to leave a micro-florence flask which could be stoppered by slipping a rubber policeman over the neck.

Figure 3 : Micro-scale volumetric flask



flask open flask sealed bulb removed

Standard solutions of glucose and xylose were prepared and were used to check the reagents and instrument to be used in the quantitative determinations. The method selected is a photometric adaptation (41) of the colorimetric method of Folin and Malmros (42), using a Klett-Summerson

photoelectric colorimeter. The details of the procedure are as follows:

1.6 ml. of sample is measured into a pyrex colorimeter tube and 0.80 ml. of 0.4 per cent potassium ferricyanide is added, followed by 0.4 ml. of cyanide-carbonate reagent (8g. of anhydrous sodium carbonate in 40-50 ml. of water, plus 15 ml. of 1 per cent sodium cyanide, the whole diluted to 500 ml.). The solutions are mixed, heated in a boiling water bath for 8 minutes, cooled 1-2 minutes, and 3.0 ml. of ferric iron reagent added (20 g. gum ghatti dissolved in 1 liter of water, plus 5 g. of ferric sulfate, 75 ml. of 85 per cent phosphoric acid, 100 ml. water, and 15 ml. of 1 per cent potassium permanganate). The volume is made to 10 ml. with distilled water and the color read against a reagent blank at 520 mμ. The range is 4-40 micrograms of glucose with a precison of ±0.4 micrograms.

The experimental values agreed with the known values for the xylose and glucose standards, and Beer's Law was obeyed.

The filtrates from hydrolysis No. 3 were treated with solid barium hydroxide until slightly basic. The barium sulfate precipitate was filtered out and excess barium ion was removed by passing carbon dioxide through the solution then filtering out the barium carbonate. The hydrolyzates were then concentrated under reduced pressure. In all cases a precipitate, which tested for carbonate, settled

out as the volume was reduced. It was necessary to decant the hydrolyzates, wash the precipitate, and reconcentrate the hydrolyzates and washings. When the solutions had been concentrated almost to dryness they were transferred to the small volumetric flasks by means of a micro-pipette. The distilling flasks were washed repeatedly with drops of water and the washings added to the volumetric flasks until the volume had been made up to 1 ml. A chromatogram containing a sample of each of the hydrolyzates was prepared with the pyridine-water-ethyl acetate solvent and it was found that all samples, with the exception of the four hour hydrolyzate, were too dilute to yield satisfactory chromatograms. Further reduction in volume of the hydrolyzates would have required the preparation and calibration of smaller volumetric flasks. Insufficient time remained for this undertaking, or for the repetition of the hydrolysis using larger samples. It was therefore decided to carry on with the existing solutions in order to investigate the techniques involved in the remainder of the procedure. A chromatogram was prepared from each hydrolyzate as follows:

A 3 cm. margin was ruled down each side of an 18 x 57 cm. strip of filter paper and a third line was drawn across the strip 7 cm. from one end. Sample was delivered onto the paper in a series of spots along the last mentioned line between the margins. The volume was accurately read from the micro burette with the aid of a hand lens.

A spot from the 4 hour hydrolyzate was centered on the starting line in each margin. When the spots were dry the papers were suspended in the Chamber and irrigated with pyridene-water-ethyl acetate for 24 hours. The papers were then removed and air dried. The margins were cut from the papers, sprayed with aniline-phthalic acid, and heated in the oven at 105°C until colored spots appeared. The margins were then matched up with the main body of each chromatogram and the zones containing the individual sugars located by ruling lines between the spots revealed in the margins.

Several chromatograms were unsatisfactory due to uneven temperature conditions which caused deviations in the direction of flow of the solvent. This effect was evidenced by the appearance of colored zones in the margins. The zones containing xylose, galactose and glucose were labelled and cut from the satisfactory chromatograms. Mannose and arabinose were not separated by the solvent used, therefore, were disregarded. A blank strip, equal in area to the zones containing sugar, was also cut from each paper. A blank strip and the strips containing xylose were rolled up and each placed in the cylinder of a micro soxhlet extractor. The papers were extracted for 6 hours with sufficient water to just fill the extractor when 2 ml. of water remained in the bottom. The resulting solutions were then transferred to colorimeter tubes and analysed, together with a standard xylose solution, by the colorimetric method previously described. The galactose samples were similarly extracted

and determined together with a glucose standard. The colorimeter ceased to function during the determination of glucose, hence this sugar was not determined. The amount of each sugar present was determined from the proportion

$$\frac{\text{micrograms of unknown}}{\text{transmittance of unknown}} = \frac{\text{micrograms of standard}}{\text{transmittance of standard}}$$

The values obtained were calculated back as a percentage of the holocellulose samples.

Table 9: Percentage of sugars in hydrolyzates after hydrolysis with 1.021 N H_2SO_4 at 93°C

Sample	Hours	Galactose	Xylose
1	1/2	-	0.02
2	1	-	-
3	2	0.03	-
4	3	-	0.08
5	4	0.05	0.07
6	5	-	-
7	6	0.05	-

The concentrate from sample 6 was poured into an excess of alcohol and the heavy white precipitate filtered out, dried and weighed.

Yield : 15 per cent of sample weight

DISCUSSION

Comparison of the present analytical data with similar data recorded in the literature is difficult due to species differences. No data on Sitka spruce holocellulose, and very little on Sitka spruce wood could be found. In addition, all figures in the present investigation are based on holocellulose. Some of the recent literature contains data on this basis, but all of the older data is based on whole wood. This investigator did not determine the holocellulose yield, but Hawley and Campbell (43) report 29.3 per cent lignin and 9.9 per cent pentosan in Sitka spruce. Holocellulose containing 3 per cent lignin should then constitute 73.5 per cent of the wood. This amount is in approximate agreement with the values given by Hajny and Ritter (44) for White spruce (72.9 per cent) and Red spruce (73.3 per cent) holocellulose. Pentosan based on holocellulose would then be 13.4 per cent which again is close to the figures given for Red and White spruce, loc cit. The pentosan values obtained in the present investigation fall far short of this value. The difference may be explained, in part, by the fact that corrections for furfural loss and for the presence of hydroxymethyl furfural from cellulose were applied inthe present calculations. The older method employing Krober's tables would give results approximately 1.5 per cent higher. Other factors are the relative ages of the trees sampled, the height at which the sample was taken, and the question

of whether the samples were heartwood or sapwood. In addition values for pentosan in wood are slightly high due to the liberation of formaldehyde by lignin (45). The picture is further confused by analytical data given by Jayme and Fink (46) for chlorite holocellulose from spruce (species not given). The material constituted 74.06 per cent of the wood and contained 6.71 per cent pentosan and 3.05 per cent lignin, in fair agreement with the present values. Alpha-cellulose, methoxyl, and carbon dioxide values agree well with those reported for Red and White spruce holocellulose.

Hydrolysis of holocellulose with 1 N sulfuric acid at 93° C appears to remove material rapidly during the first three hours, after which there is no further loss. Increase of temperature to 102° C causes a more rapid and somewhat greater loss of material, but again there is little loss after three hours. Miller and Swanson (47) report a similar phenomenon when White spruce is hydrolyzed with increasing concentrations of hydrochloric acid for six hours at 96° C. The loss in weight increased decidedly until an acid concentration of 0.75 per cent was reached. From that point on the loss in weight became almost constant. They concluded that an acid concentration of 0.75 per cent was the point of differentiation between two parts of the carbohydrate material with respect to hydrolysis resistance. In the present work it is possible that all linkages sensitive to 1 N sulfuric acid at 93° C have been attacked during the first three hours and then an additional type or types of

linkages have been severed in this time at 102° C. It might be profitable to carry out three separate series of hydrolysis on holocellulose in which time, temperature and acid concentration, respectively are varied. It is possible that some correlation could be found between the points of differentiation in each case.

The material resistant to 1 N acid at 93° C is equal to 75 per cent of the holocellulose. This is in excess of the alpha-cellulose determined (65.8 per cent, not corrected for ash and lignin) but lower than Cross and Bevin cellulose (85 per cent calculated from the data of Hawley and Campbell (43)). Assuming that Cross and Bevin cellulose is predominantly cellulose and cellulosan, it is apparent that some fraction of the cellulosans has been removed.

The curve for alpha-cellulose in the residues suggests that some of the material removed may normally be associated with the alpha-cellulose fraction. Sherrard, (48) and others (16) have shown that alpha-cellulose retains considerable mannan and xylan. It is, therefore, more probable that some of the longer chain cellulosans have been sufficiently degraded during hydrolysis to become alkali soluble. These shorter chains remain insoluble in 1 N sulfuric acid but are removed by the 17.5 per cent sodium hydroxide treatment during the alpha-cellulose determination. Curves of this nature, showing a rapid loss followed by a plateau, are well known

in cellulose chemistry. Their significance was discussed by Heuser and others at the 1948 T.A.P.P.I. Symposium (49). It was concluded that the point at which the curve flattens out and becomes linear represents the point at which amorphous cellulose and hemicellulose have been removed and at which only drystalline cellulose remains.

The fine material which settled out after filtration of the hydrolyzates is probably holocellulose flour, which has passed through the filter. Hand screening does not completely remove all material of small particle size, therefore, mechanical shaking should be employed in screening of the wood before preparing holocellulose.

The sugars identified by the paper chromatographic method are xylose, arabinose, glucose, galactose and mannose. The intensity of the sugar spots on the chromatograms indicate that arabinose and galactose were present in very low concentration, with xylose somewhat higher. Glucose and mannose were present in much higher concentration. Such a range might be expected in the light of Jayme's (46) analysis of spruce holocellulose: pentosan 6.71; mannan 9.85; galactan 0.25.

The compound which corresponded to barium glucuronate in the pyridine solvent, but not in the butanol-water-acetic acid mixture is probably a mono-methoxyuronic acid. The per cent carbon dioxide and methoxyl obtained on analysis of the holocellulose are in agreement with this view. The compound parallels the behavior of barium glucuronate in the basic

solvent but is probably converted to the free acid and possibly the lactone by acidic solvents. The elliptical tails which precede the barium salts on the chromatogram may be due to free acid liberated by ion exchange of barium with the paper. Relatively few of the methoxy uronic acids in nature have been characterized due to the resistance of the aldobionic acids to hydrolysis and to the lability of the free acid. Characterization of this compound through chromatography, therefore, offers an interesting avenue of research. The other unidentified compound on the chromatogram may be a disaccharide fragment. It has been shown (27) that disaccharides travel satisfactorily on the chromatogram.

The large proportion of alcohol insoluble material precipitated from sample 5 indicates that a true picture of the hydrolysis can be obtained only by rehydrolysis of the extracts. Two sets of determinations should be made on each hydrolyzate. The first, after hydrolysis, to determine the composition of the most sensitive material. The second, after rehydrolysis of the extracts, to determine the composition of the material solubilized by the first hydrolysis.

It is unfortunate that only those sugars present in low concentration were determined in the present investigation. The validity of the values obtained for galactose and xylose is extremely uncertain since the analytical method was not checked with standards. The low values obtained can be reconciled to some extent, however. The total galactose is low according to Jayme's (46) figure. Xylose is rapidly

decomposed in acid solution therefore might not increase in concentration as the hydrolysis proceeds. The procedure for the quantitative determination of the sugars appears to be practical if somewhat larger samples are used. The solvent mixtures employed in this investigation permit the isolation of allthe sugars. Glucose, galactose, xylose and uronic acid may be determined after separation with pyridine-water-ethyl acetate. Mannose and arabinose may be determined together from this chromatogram. Mannose may then be isolated from a chromatogram prepared with butanol-water-ethyl acetate solvent, and arabinose calculated by difference. However, a solvent which would accomplish a complete separation on a single chromatogram would be advantageous. The most serious difficulty in quantitative work is loss of sample due to drift into the margins of the chromatogram. Insulation of the chamber would eliminate this trouble but would prevent the observation of the chromatograms while running. Since in some cases it may be desirable to observe the chromatograms without removing them from the chamber, it would be more advantageous to have a thermostatically controlled constant-temperature room of sufficient size to contain a number of chambers and to permit movement of the operator. Alternatively a large incubator cabinet could be used. Temperature would then become a controllable variable which might be used to advantage. Alteration of temperature would change the rate of travel of compounds on the chromatogram by altering their solubilities in the solvents.

The most satisfactory chromatograms are obtained from neutral sugar solutions, free of inorganic ions (27). It is therefore necessary that the anion of the hydrolyzing acid be removed. This is normally accomplished by the addition of the hydroxide or carbonate of a metal which forms an insoluble salt. The use of barium hydroxide for the removal of sulfate, followed by treatment of the solution with carbon dioxide to remove excess barium, is not to be recommended. Reedy (50) states that if an excess of carbon dioxide is passed into an alkaline earth hydroxide, at first the carbonate is precipitated, but later as the concentration of hydrogen and bicarbonate ions increase, the precipitate will dissolve. During the present investigation the dissolved barium carbonate was reprecipitated during the concentration of the hydrolyzates. This necessitated filtration at a stage where the solution was highly concentrated, introducing the possibility of sugar loss. The use of lead carbonate followed by precipitation of excess lead with hydrogen sulfide is probably preferable. An investigation into the use of ion exchange resins for the neutralization of hydrolyzates might prove fruitful.

SUMMARY

1. Holocellulose is rapidly attacked during the first three hours of hydrolysis with 1N sulfuric acid at 93° C. After three hours little material is removed. Two thirds of the pentosan pass into the hydrolyzate in this time, and the alpha-cellulose fraction is somewhat degraded. Only a small fraction of the solubilized material appears as free sugars, therefore, rehydrolysis of the extracts is required before quantitative determination of the individual sugars.

2. Glucose, galactose, mannose, xylose and arabinose have been identified in the hydrolyzates by the chromatographic method. The presence of a methoxy uronic acid was indicated.

3. A procedure for the quantitative determination of the sugars present has been given. Glucose, galactose, xylose and the uronic acid are determined after separation on a chromatogram using pyridine-water-ethyl acetate (1-2-2 v/v). Mannose and arabinose are not separated on this chromatogram but may be determined together. Mannose is determined after separation on a second chromatogram prepared with butanol-water-acetic acid (4-1-5 v/v) as solvent. Arabinose is determined by difference.

4. The use of a constant temperature cabinet or room for use during the preparation of chromatograms for quantitative work is recommended. Constant temperature conditions will eliminate losses due to irregular movement of solvent.

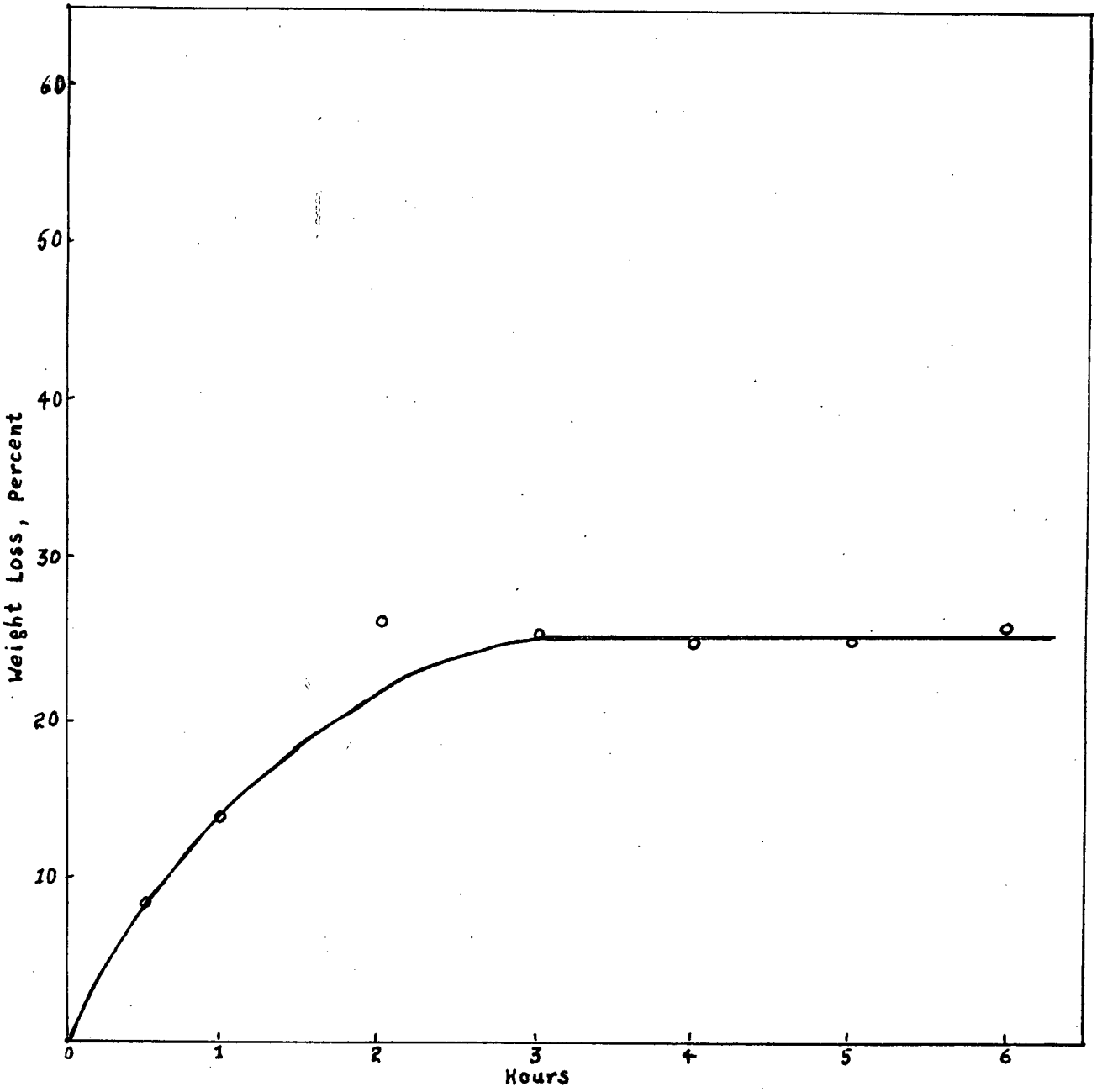


Figure 4: Weight loss on hydrolysis of holocellulose with 1.021 N H_2SO_4 at 93°C

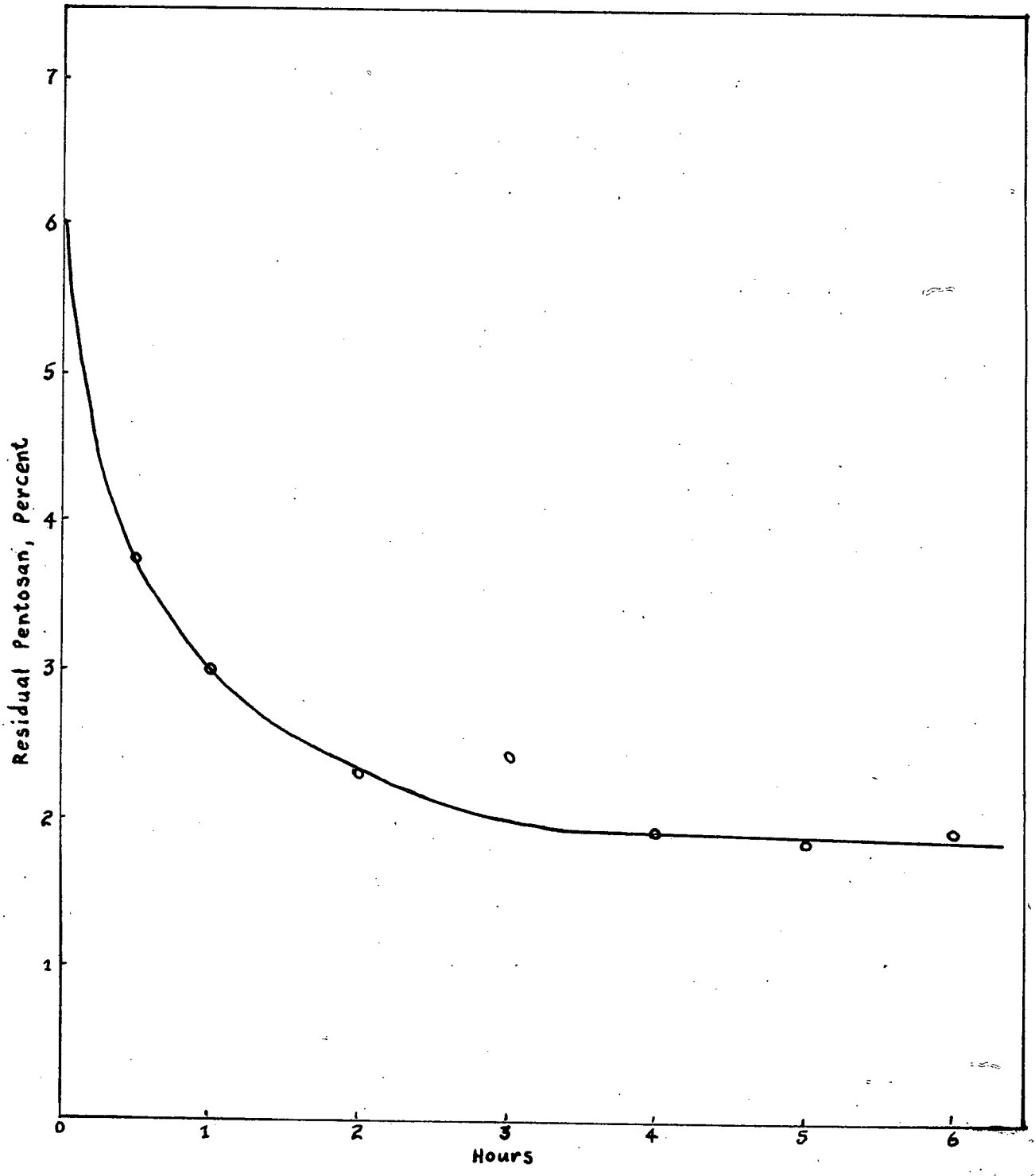


Figure 5: Residual pentosan after hydrolysis of holocellulose with 1.021 N H_2SO_4 at 93°C

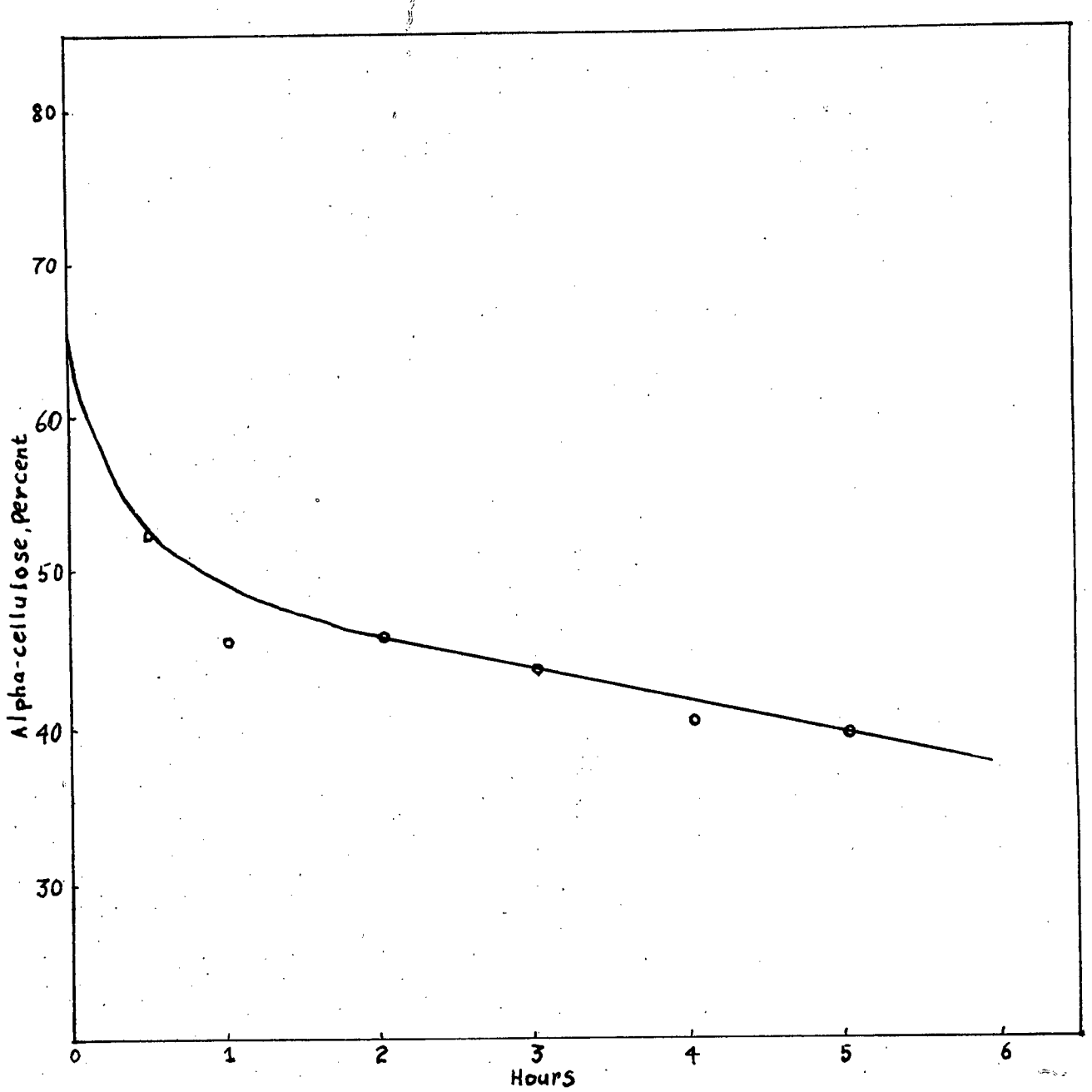


Figure 6: Alpha-cellulose in residues after hydrolysis of holocellulose with 1.021 N H_2SO_4 at 93°C

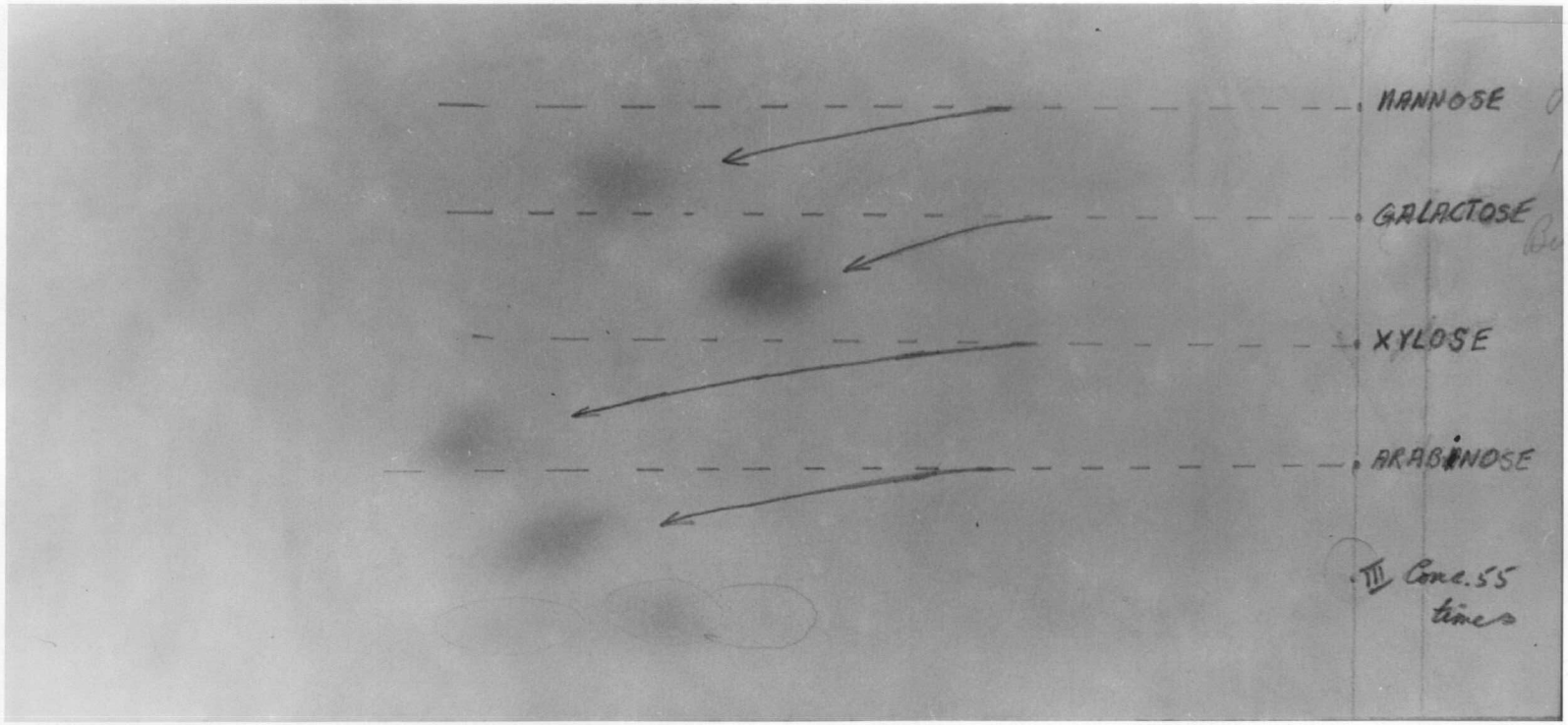


Plate I. Effect of uneven temperature on direction of solvent flow.

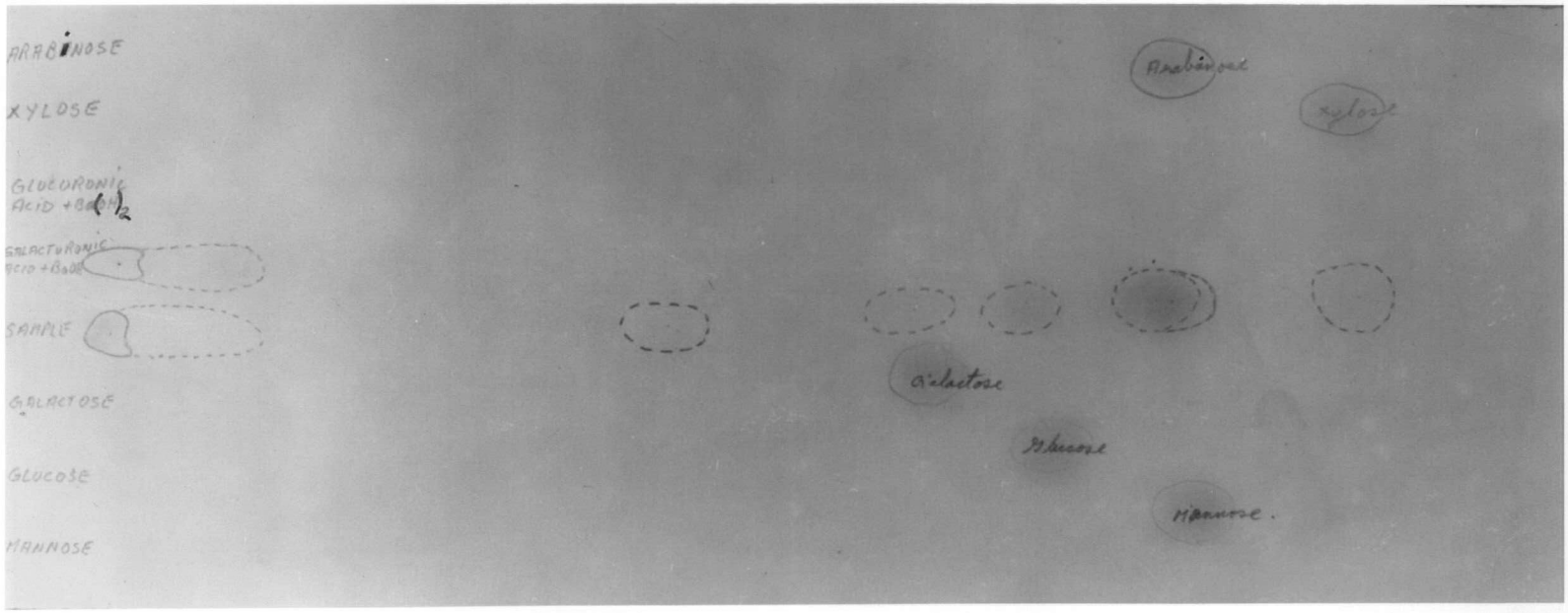


Plate II. Disposition of sugars on chromatogram after 22 hours irrigation with pyridine-water-ethyl acetate (1-2-2 v/v).

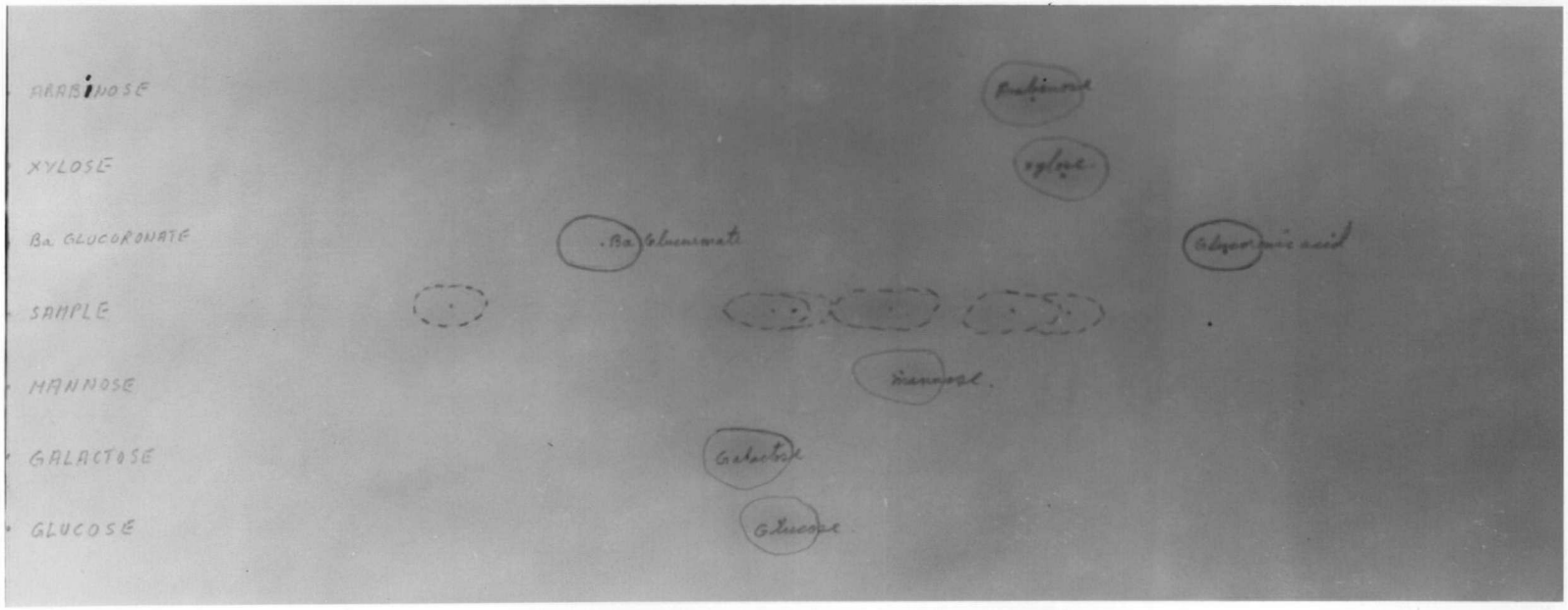


Plate III. Disposition of sugars on chromatogram after 29.5 hours irrigation with butanol-water-acetic acid (4-1-5 v/v).

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