

## A Study of Methods for Preparing Oligosaccharides from Cellulose

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Oligosaccharides are prepared from acid hydrolyzates of cellulose by ethanol-water gradient elution from chromatographic columns composed of stearic acid-treated mixtures of charcoal and Celite.

### INTRODUCTION

A study of methods for preparing oligosaccharides of degree of polymerization of 2-7 from cellulose is presented in this paper. The procedures which were investigated consisted of partial breakdown of cellulose by acetolysis and deacetylation (1, 2), or by hydrolysis with fuming hydrochloric acid (3, 4), followed by ethanol-water gradient elution of the product on chromatographic columns composed of various stearic acid-treated mixtures of charcoal and Celite (5).

### EXPERIMENTAL

#### CELLULOSE ACETOLYSIS AND DEACETYLATION

Based on the procedures of Wolfrom *et al.* (1, 2), 180 g. of Whatman standard grade cellulose powder was added in portions during a 1-hr. period to a mixture of 700 ml. of glacial acetic acid, 700 ml. of acetic anhydride, and 75 ml. of conc. sulfuric acid. The mixture was warmed to 50° at the beginning of the reaction, was kept from rising above this temperature by external cooling with ice water, and was stirred continuously. After the last of the cellulose powder was added, the mixture was stirred at 50° for an additional 2 hr. It was then poured into 10 l. of vigorously stirred water and cracked ice, which precipitated the mixed acetylated oligosaccharides. Excess acid present was neutralized to Congo red by the addition of sodium carbonate, and the mixture was allowed to stand overnight. The precipitate was then removed by filtration and was washed on the filter until the filtrate was just acid to litmus. Excessive washing was avoided because of a tendency for the precipitate to be-

come colloidal. The product was dried *in vacuo* over sodium hydroxide. The yield was 315 g.

To dispose of undesired cellobiose octaacetate and possibly also of oligosaccharide acetates of very high degrees of polymerization, advantage was taken of their low solubility in methanol. The 315 g. of crude acetates was mixed with 1500 ml. of anhydrous methanol and stirred with the aid of a Waring blender. The suspension was filtered, and the material on the filter was washed by resuspending in 750 ml. of anhydrous methanol and refiltering. The insoluble material, amounting to 215 g. after drying, was discarded.

The filtrate and washing were combined, reduced to 400 ml. by means of a rotating vacuum evaporator, and poured into 1500 ml. of water. To coagulate the colloidal product which resulted, 200 ml. of 10% sodium chloride was added and the suspension was allowed to stand overnight in the refrigerator. The product was then collected by centrifugation, and the precipitates were transferred to a porcelain dish and dried *in vacuo* over phosphorus pentoxide. The yield was 56 g.

The 56 g. of fractionated oligosaccharide acetate mixture was next suspended in 300 ml. of absolute methanol, and small amounts of insoluble material were removed by centrifugation in capped bottles. Following the procedure of Braun (6) for deacetylation of cellobiose octaacetate, 50 ml. of methanol in which 0.70 g. of sodium was dissolved was then added to the clarified solution of the acetates. The container was stoppered and was shaken from time to time during a 2-hr. period. The precipitate of free oligosaccharides which formed during this period was collected by filtration, washed thoroughly with methanol, and dried in a vacuum desiccator over phosphorus pentoxide. The yield of yellowish powder was 24.5 g.

For chromatographic fractionation, 10 g. of the powder was extracted four times with 40-ml. quantities of water. Gelatinous material which failed to dissolve was separated by centrifugation and discarded.

### CELLULOSE HYDROLYSIS

In a modification of the procedures of Zechmeister and Tóth (3) and of Jermyn (4), 10 g. of cellulose powder was first suspended in 100 ml. of conc. hydrochloric acid (sp. gr., 1.19) in a 250-ml. Erlenmeyer flask at room temperature. One hundred milliliters of ice-cold fuming hydrochloric acid (sp. gr., 1.21) was then added in one portion, and the mixture was stirred for about 1 min. to dissolve the cellulose. The procedure of suspending the cellulose in concentrated hydrochloric acid at room temperature served to wet and disperse the cellulose particles, thus facilitating the ultimate dissolving of the cellulose by the fuming hydrochloric acid. The clear, heavily viscous solution which resulted was warmed and was allowed to stand at 25°. The time of hydrolysis used in different tests was 2-3 hr. At the end of this time the mixture, which had become much less viscous and had turned yellow, was poured into 600 ml. of ice-cold water and was brought to pH 4-5 by careful addition of 210-220 g. of sodium bicarbonate. If this neutralization step was not carried out, the oligosaccharides which were subsequently isolated were unstable, due to contamination with the hydrochloric acid. Gelatinous material which separated was removed by centrifugation and was discarded. The clear supernatant solution was used for chromatographic fractionation.

For determination of the rate of hydrolysis of cellulose, a run was carried out on one-tenth the scale described above, and 2-ml. samples of the reaction mixture were withdrawn at intervals of  $\frac{1}{2}$ , 1, 2, 3, and 4 hr. The samples were each diluted with 6 ml. of distilled water and were neutralized with 2 g. sodium bicarbonate. The precipitates which formed were centrifuged, washed repeatedly to remove adherent sodium chloride, dried *in vacuo* at 40°, and weighed. Two-tenth-milliliter aliquots of the supernatant fluids were used for analysis of oligosaccharide content by a micro column chromatographic procedure (7).

### ADSORBENT PREPARATION

Preparation of adsorbent was based on the directions of Alm (5). In the present work, two variations in method of preparation were used, as follows:

Adsorbent A was prepared by mixing 400 g. of

charcoal Darco G-60<sup>1</sup> and 400 g. Celite<sup>2</sup> with 3200 ml. of 1% stearic acid (technical) in absolute ethanol for one-half hour, filtering, resuspending the adsorbent in 3200 ml. of 50% ethanol, refiltering, and finally resuspending the adsorbent in 2000 ml. of 10% ethanol to give a uniform slurry.

Adsorbent B was prepared by the same procedure as adsorbent A except that it was treated with 2½% stearic acid in absolute ethanol, washed with 50% ethanol which was saturated with stearic acid, and finally suspended in water.

### CHROMATOGRAPHIC FRACTIONATION

Slurry of adsorbent was poured into a glass tube 90 cm. long, 5 cm. inside diameter, fitted at the bottom with a medium porosity fritted-glass plate and drawn down to an 8-mm. delivery tube below the plate. The liquid was pressed through with the aid of air pressure of 4 lb./sq. in. applied at the top of the column, care being taken to prevent the level of liquid from going below the upper surface of the adsorbent. A space of approximately 6 cm. was left unfilled.

The column was washed with 4 l. or more of water, and the hydrolyzate was introduced into the column. The column was again washed with 4 l. or more of water to remove salt and glucose. Oligosaccharides were then separated by gradient elution with ethanol-water, as follows: The top of the column was connected by way of rubber stoppers and glass and rubber tubing to the bottom outlet of a 4-l. aspirator bottle used as a mixing vessel. The mixing vessel was filled with water, was operated with a magnetic stirrer, and was fed at the top from a reservoir containing 60% ethanol (v/v) when adsorbent A was used, or 45% ethanol when adsorbent B was used. Air pressure, amounting to 5-6 lb./sq. in. was applied to the system by way of an inlet tube to the reservoir, to give an initial rate of elution of about 4 ml./min. With time the elution rate slowed to about 2 ml./min. A volume-type fraction collector was used to collect the eluate, each fraction amounting to about 23 ml.

Aliquots of the different eluate fractions were analyzed for carbohydrate content by a colorimetric procedure described below. On the basis of the data obtained, the fractions were combined to give separate pools of the different oligosaccharides. The pools were concentrated with the aid of a rotating vacuum evaporator to about 30-40 ml. for the lower oligosaccharides, to about 70-100 ml.

<sup>1</sup> An activated carbon preparation manufactured by Atlas Powder Company, Wilmington, Delaware.

<sup>2</sup> A siliceous filter-aid manufactured by Johns-Manville Co., New York, N. Y.

for the higher. The concentrates were then clarified, if necessary, by centrifugation, and were finally reduced to the solid state by lyophilization.

When eluates containing cellobiose or celloheptaose were allowed to stand for a day or so before being concentrated, some precipitation of the oligosaccharides occurred. The precipitate obtained from the cellobiose fraction was crystalline and redissolved fairly readily in water after the alcohol was removed. The precipitate obtained from eluates containing celloheptaose was probably microcrystalline; its solubility in water was very low.

Purity of the oligosaccharide preparations was ascertained by iodometric (8) and micro-column chromatographic (7) procedures. Moisture contents were determined by drying to constant weight at 56°; ash, by burning at 600°.

#### COLORIMETRIC ANALYSIS OF CARBOHYDRATE IN ELUATES

To 0.05-ml. aliquots of eluates were added 3-ml. aliquots of color reagent composed of 0.2% orcinol in 70% (v/v) sulfuric acid. The samples and reagent were mixed by inverting five times, were heated at 100° for 20 min., and cooled. Absorbances were measured at 550  $m\mu$  in a colorimeter.

#### RESULTS AND DISCUSSION

When oligosaccharide mixtures which were obtained by the acetolysis-deacetylation procedure were separated on chromatographic columns containing adsorbent A, the distribution of components in eluate fractions was that shown by the top curve in Fig. 1. When mixtures obtained by 3-hr. acid hydrolysis were separated on adsorbent A, the distribution was that shown by the middle curve. With 2-hr. acid hydrolysis and adsorbent B, the results shown by the bottom curve were obtained. The degrees of polymerization of the different components are indicated alongside the corresponding peaks in the patterns. The results show, first of all, that the proportion of cellobiose obtained with the acetolysis procedure was lower than that obtained by the hydrolysis procedure. This is explained by the fact that most of the cellobiose was removed as methanol-insoluble cellobiose octaacetate in the former procedure. The results also show that elution of oligosaccharides takes place with a shallower ethanol-water gradient when adsorbent B is used than when ad-

adsorbent A is used. Finally, the results show better yields of most of the oligosaccharides, judging by sizes of peaks, with a 3-hr. hydrolyzate than with a 2-hr. hydrolyzate. The lower yield from the 2-hr. hydrolyzate was attributable to a considerable degree, however, to loss by occlusion on the greater bulk of insoluble material which resulted with the shorter period of hydrolysis.

In additional tests, not shown, it was found that with steep gradients, produced by placing higher concentrations of alcohol in the reservoir, the oligosaccharides were eluted more rapidly; and vice versa with shallow gradients. For example, when 70% alcohol was used as feed solution in combination with adsorbent A, or when 50% alcohol was used with adsorbent B, zones of oligosaccharides in the eluates were so close together that they overlapped. On the other hand, when 45% alcohol was used in combination with adsorbent A, separation of oligosaccharides only up to and including pentaose had taken place by the 600th tube.

Through experience with the two different adsorbents, it was found that the chromatographic properties of adsorbent treated with 2½% stearic acid were generally more reproducible than those of adsorbent treated with only 1% stearic acid. However, reuse of columns prepared with either adsorbent A or adsorbent B could not be depended upon to produce satisfactory fractionation of oligosaccharide components.

Octaose and nonaose, detectable in cellulose hydrolyzates when the small columns of the micro chromatographic method were used (see Fig. 3, below), were not isolated with the larger preparative columns. It appeared at first that this might have been due to the fact that the lower concentrations of ethanol used in the reservoirs for the preparative columns, compared to the absolute ethanol used in reservoirs for micro columns, led to too early a flattening of the ethanol-water gradients. Provision for maintenance of steepness of gradient by substituting 65% ethanol for 45% ethanol after the 200th tube, and absolute ethanol after the 350th tube, succeeded, however, in producing only traces of components presumed to be octaose and nonaose. Possibly, precipitation of these

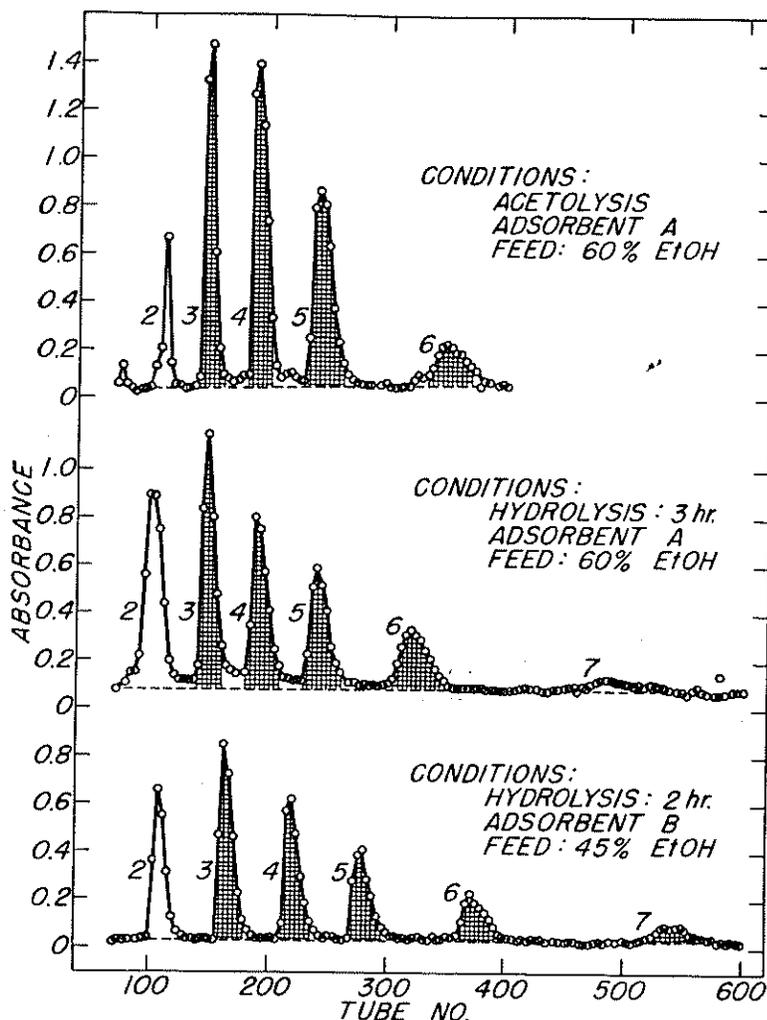


FIG. 1. Distribution of oligosaccharides in eluate fractions from preparative columns.

TABLE I  
YIELDS OF OLIGOSACCHARIDES

Fraction	Acetol.- deacetyl. (Adsorbent A)		3-hr. hydrol- ysis (Adsorbent A)		2-hr. hydrol- ysis (Adsorbent B)	
	gm.	%	gm.	%	gm.	%
Cellotriose	1.43	1.9	1.20	12.0	0.72	7.2
Cellotetraose	1.21	1.7	0.92	9.2	0.66	6.6
Cellopentaose	1.43	1.9	0.72	7.2	0.55	5.5
Cellohexaose	0.32	0.4	0.45	4.5	0.39	3.9
Celloheptaose					0.08	0.8

components within the preparative columns was responsible for their failure to be separated in the expected proportions.

Cross-hatched areas on the curves in Fig.

1 enclose the fractions selected for the pools. Yields obtained for individual oligosaccharides in the different experiments are summarized in Table I. It can be concluded from the data that under the various conditions tested, the preparation of oligosaccharides by direct hydrolysis with fuming hydrochloric acid was much more efficient than preparation by acetolysis-deacetylation. Furthermore, it can be judged from the relative amounts of labor involved in the two procedures that the method using hydrolysis was also the more convenient. It is possible, however, that the period of time used for the acetolysis of cellulose may not have been optimal.

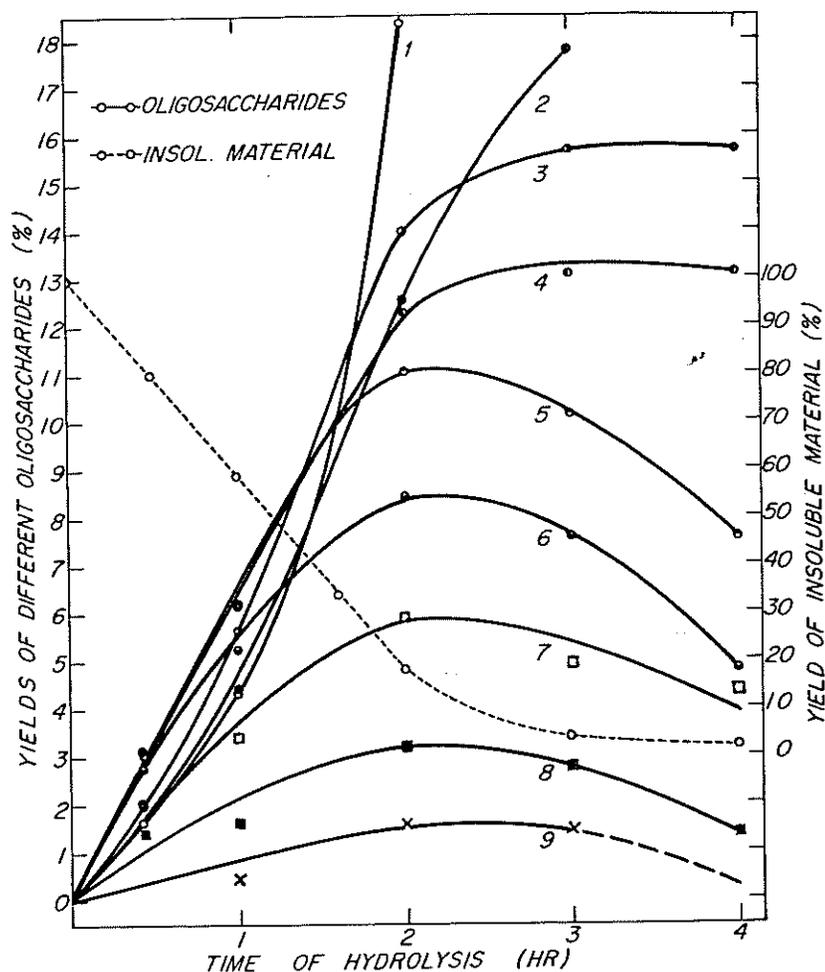


FIG. 2. Rate of hydrolysis of cellulose. Numbers alongside curves indicate degrees of polymerization of different products.

Yields afforded by the procedure involving direct acid hydrolysis followed by gradient-elution chromatographic fractionation also appear to advantage when comparisons are made with findings of earlier investigators. For example, yields reported by Zechmeister and Tóth (3), using hydrolysis by fuming hydrochloric acid followed by repeated fractionation with ethanol were only 0.5% cellotriose, 1.2% -tetraose, and 0.5% -hexaose [probably -pentaose (1)]. Yields reported by Wolfrom and co-workers (1, 2) using acetylation, chromatographic fractionation of acetates, and deacetylation, tended to be low also, due in part to losses sustained during the repeated fractionations which were carried out to insure maximum purity.

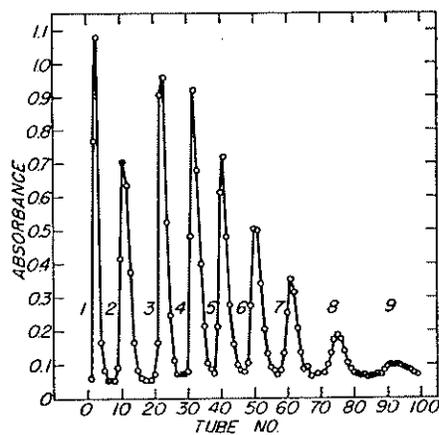


FIG. 3. Distribution of oligosaccharides in eluate fractions from analytical column.

Yields reported by Whitaker (9), using acetolysis and deacetylation followed by chromatographic fractionation from untreated carbon with stepwise, increasing concentrations of ethanol were similar to those in the present work in which acetolysis was used, except for the lower amounts of -triose and higher amounts of -hexaose obtained by Whitaker. Jermyn (4), using hydrolysis with fuming hydrochloric acid followed by gradient elution from untreated carbon with butanol-water, did not report exact yields.

Moisture contents of oligosaccharides described in the present work were 0-6%; ash, 0-0.2%. As measured by the iodometric method (8), preparations of cellotriose, -tetraose, and -pentaose were 95-98% pure, after correction for moisture and ash; cellohexaose and -heptaose preparations, 93-94% pure. Contamination by homologs, shown in a separate investigation (7) by the micro-column chromatographic test, was only 1-2% and was due principally to homologs next lower to the oligosaccharides being tested. Rechromatographic treatment of the oligosaccharide preparations should be highly effective for further purification.

The rate of hydrolysis of cellulose, as revealed by data obtained with the aid of the micro-column chromatographic method, is shown in Fig. 2. A similar set of curves, showing yields of corresponding acetates during acetolysis, has been presented by Wolfrom *et al.* (2). From a preparative standpoint, optimal yields for most of the oligosaccharides were obtained at 2 hr. With shorter periods the amounts of all oligosaccharides decreased; with longer periods, yields of shorter chain oligosaccharides increased at the expense of the

longer chains. The maximal amounts obtained for each oligosaccharide are in fair agreement with those calculated on the assumption of random splitting of linkages within cellulose molecules (10). Also given in Fig. 2 are data for the quantities of water-insoluble product which were obtained at different periods of hydrolysis. In separate tests, it was found that the effect of time of hydrolysis on yields of oligosaccharides was essentially the same when fuming hydrochloric acid alone was used.

In Fig. 3 is shown the distribution of oligosaccharides obtained in the micro-column chromatographic analysis of the 2-hr. hydrolyzate. The occurrence of what are assumed to be cellooctaose and -nonaose in the hydrolyzate has not heretofore been reported.

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