

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF THE UNIVERSITY OF DETROIT]

The Separation of Some Methylated Sugars by Chromatographic Adsorption of their Azoyl Esters

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One of the conventional procedures for determining the constitution of polysaccharides includes methylation, hydrolysis and subsequent distillation at reduced pressure to separate the mixture of methylated sugars. The difficulties inherent in the distillation step such as pyrolysis, demethylation and non-quantitative recovery would be avoided by a process which would allow quantitative separation at room temperature. By incorporation of such a refinement into present procedures constitutional investigations of cellulose, starch and glycogen could be established on a more secure basis and could be greatly extended. Chromatographic adsorption methods appear to offer a promising physical tool for the quantitative separation at room temperature of mixtures of methylated sugars.

It has been shown by Reich¹ that mixtures of certain monosaccharides, in the form of the colored *p*-phenylazobenzoyl esters, can be conveniently separated by the chromatographic adsorption method. The yield upon esterification of glucose was 80% whereas 95% recovery was reported after adsorption and elution of a mixture of glucose and fructose esters. The chromatographic method has been extended to the separation of other monosaccharides and disaccharides by the investigations of Coleman and co-workers² who reported 94–98% recovery after elution.

The object of the present investigation was the application of the method to artificial mixtures of methylated sugars. Of particular interest was the separation of the ternary mixture consisting of the esters of 2,3-dimethylglucose, 2,3,6-trimethylglucose and 2,3,4,6-tetramethylglucose compounded to simulate the type of mixture obtainable by hydrolysis and esterification of fully methylated starch. It should be pointed out that the presence of impurities in the hydrolyzate from a methylated polysaccharide may render the present method not directly applicable without certain modifications.

Using silica as the adsorbent several binary and ternary mixtures of methylated glucose azoyl esters were separated. Two of these separations are reported in this paper.

Experimental

Preparation of the Azoyl Esters.—The pyridine used in the esterification must be absolute and was obtained by twice distilling the analytical reagent from calcium oxide which had been ignited at red heat for one hour. The product distilled over the range 114.5–115.5°. The ester-

ification procedure was a modification of that used by Freudenberg.³ The dried sugar derivative, 0.5 to 2.0 g. depending on the number of hydroxyl groups to be esterified, was dissolved in 20–25 ml. of absolute pyridine and cooled to 0°. After the addition of *p*-phenylazobenzoyl chloride (azoyl chloride) in 15% excess of the theoretical amount,⁴ the mixture was allowed to stand three days at 0°, followed by two days at 30°, and three days at 0°. The pyridine was removed *in vacuo*, the residue washed thoroughly with water and dried *in vacuo*. The material was dissolved in chloroform, filtered to remove the undissolved *p*-phenylazobenzoyl acid, and passed through a small column filled with alumina⁵ to a height of 4–5 cm. The latter treatment serves to remove any dissolved *p*-phenylazobenzoyl acid. The percolate was filtered and the chloroform removed *in vacuo*, the crude azoyl ester remaining. The yield of crude product was 95% or better in all instances. The azoyl esters of glucose, 3-methylglucose, and 2,3-dimethylglucose were further purified by precipitation from chloroform solution by the addition of ethanol, and the ester of 2,3,4,6-tetramethylglucose by recrystallization from ethanol. Though difficulty was encountered in attempting to purify the ester of 2,3,6-trimethylglucose by the ordinary methods, a pure sample was obtained by the chromatographic method of purification using silica as the adsorbent.

Preparation of the Silica.—In preliminary experiments several adsorbents, including permutit, silica, calcium carbonate, alumina and magnesia, were investigated. While magnesia decomposed the esters, the best results were obtained with silica prepared from sodium silicate. Sodium silicate (40% solution) was acidified with hydrochloric acid, and the resulting gel dried and ignited over a Meker burner. When cool, all lumps were broken and the powder was stirred with a large quantity of water for two hours. The silica was filtered off, dried at 110° for twelve hours, thoroughly pulverized in a mortar and again stirred with water. After a final filtration, the silica was washed until the washings no longer gave a positive chloride test, then thoroughly dried (twenty-four hours at 110°) and pulverized.

Chromatographic Adsorption.—The column (35 × 2.2 cm.) was most suitably and symmetrically packed by the wet method.⁶ About 50 g. of silica was suspended in the solvent mixture of equal volumes of chloroform, benzene and petroleum ether (65–110°), and poured into the column. By means of suction excess solvent was removed. To prevent channeling as the solvent was drawn from the top layer of adsorbent, a firm pressure was maintained on this layer by means of a plunger. A quantity of solvent was washed through and this followed by a solution containing the mixture of esters. The chromatogram was developed with the same solvent mixture until the zones were sufficiently separated to allow sectioning. The column was then allowed to run partially dry, the adsorbent removed, and the zones separated by sectioning.

Elution and Quantitative Determination.—Elution was accomplished with a 20% solution of ethanol in chloroform. After a soaking period of one hour the silica was removed by filtration (Whatman no. 50 paper) and washed with 5-

(3) K. Freudenberg and co-workers, *Ber.*, **73**, 609, 621 (1940).

(4) For one gram of 2,3,4,6-tetramethylglucose 1.20 g. of azoyl chloride was used; the amounts of azoyl chloride per gram of trimethylglucose and dimethylglucose were, respectively, 2.53 and 4.05 g.

(5) Merck C. P. Alumina, activated at 300° for four hours.

(6) Capacity of the column was approximately 100 mg. of azoyl esters.

(1) W. S. Reich, *Compt. rend.*, **208**, 589, 748 (1939); *Biochem. J.*, **33**, 1000 (1939).

(2) G. H. Coleman, A. G. Farnham and A. Miller, *THIS JOURNAL*, **64**, 1501 (1942).

ml. portions of the eluant solution until the washings no longer showed traces of color. For determination of physical constants of the component of each zone, most of the solvent was removed by distillation, and the last few ml. removed *in vacuo*. Direct weighing was not satisfactory for the determination of the quantities in each zone because of the presence of colloidal silica. Colorimetric means were therefore employed. The eluted material was dissolved in an accurately measured volume of the eluant solution and compared with at least two different standards prepared from the corresponding pure compounds. Because of the volatility of the solvents a fair amount of speed was essential in this operation.

Individual Separations.—The separation of the esters of 2,3,4,6-tetramethylglucose and 3-methylglucose was typical of the separation of a given pair of methylated glucose esters. This binary mixture was chromatographed according to the above procedure. When development was complete there was observed an upper yellow zone separated from a lower red zone by an almost colorless region. The results of this separation are shown in Table I.

TABLE I

SEPARATION OF A BINARY MIXTURE OF METHYLATED SUGARS BY CHROMATOGRAPHIC ADSORPTION OF THEIR AZOYL ESTERS

Components of mixture	Charged to adsorption column		
	Mg.	M. p., °C. (obs.)	
Azoyl ester of 2,3,4,6-tetramethylglucose	13.4	116-118	
Azoyl ester of 3-methylglucose	58.6	222-224	
Total	72.0		
Position after development of chromatogram	Eluted from column		
	Mg.	M. p., °C. (obs.)	M. p., °C. (lit.) ¹
Upper zone	16.2	115-117	116
Lower zone	55.0	221-223	220
Total	71.2		

A mixture of the azoyl esters of 2,3,4,6-tetramethylglucose, 2,3,6-trimethylglucose and 2,3-dimethylglucose was chromatographed and showed four distinct zones upon completion of development. The two lower light yellow zones gave eluted materials which corresponded to the two forms of the dimethylglucose ester reported by Freudenberg.⁴ The data are presented in Table II.

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TABLE II

SEPARATION OF A TERNARY MIXTURE OF METHYLATED SUGARS BY CHROMATOGRAPHIC ADSORPTION OF THEIR AZOYL ESTERS

Components of mixture	Charged to adsorption column		
	Mg.	M. p., °C. (obs.)	
Azoyl ester of 2,3,4,6-tetramethylglucose	5.9	116-118	
Azoyl ester of 2,3,6-trimethylglucose	27.2	169-171	
Azoyl ester of 2,3-dimethylglucose (Mixture of forms) ⁷	5.8	187-190	
Total	38.9		
Position after development of chromatogram	Eluted from column		
	Mg.	M. p., °C. (obs.)	M. p., °C. (lit.) ¹
Top zone	5.5	115	116
Second zone	27.1	170-172	172
Third zone (Form I)	3.4	185-187	185, 189
Fourth zone (Form II)	3.2	207-208	207, 209
Total	39.2		

suggestions and interest were largely responsible for initiation of the work.

Summary

1. The *p*-phenylazobenzoyl esters of several methylated glucoses have been prepared according to a modification of the Freudenberg procedure with yields of 95% or better.

2. Separations of mixtures of these compounds were accomplished by the chromatographic adsorption method with nearly quantitative results. Of particular concern was the separation of the ternary mixture of the esters of 2,3-dimethylglucose, 2,3,6-trimethylglucose, and 2,3,4,6-tetramethylglucose.

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(7) Fractional crystallization yielded one fraction of m. p. 185-187° and one fraction melting at 207-209°.