CXXIII. A METHOD FOR THE SEPARATION OF SUGARS BY THE CHROMATOGRAPHIC ADSORPTION OF THEIR COLOURED ESTERS

I. SEPARATION OF GLUCOSE AND FRUCTOSE

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IN spite of the very large amount of work which has been done on the separation of individual carbohydrates from their mixtures the methods in general use are not very satisfactory if only small quantities of the substance are available. These methods depend ultimately on differences in solubilities or boiling-points of the carbohydrates or of their derivatives. The recent development of the method of chromatographic adsorption, has shown that the degree of affinity of adsorption may vary to a much greater extent with the molecular structure, than does the boiling-point or solubility. Further, the method of chromatographic adsorption can readily be adapted for microchemical work which is frequently essential in biochemical research.

From a large number of chromatographic tests carried out during the present investigation with sugars and their derivatives the best results were obtained with two groups of arylated derivatives, namely the azobenzene-*p*-benzene-sulphonyl and azobenzene-*p*-benzoyl esters. This latter group has the advantage that the sugars are more easily separated by saponification after the chromatographic separation has been effected.

For brevity azobenzene-*p*-benzoyl chloride will be termed azoyl chloride, and the corresponding esters, azoyl esters.

The azoyl chloride reacts readily in pyridine solution with the hydroxyl groups of sugars. After reaction is complete the excess of azoyl chloride is converted into the methyl ester by the addition of methanol. The methyl ester can then be readily eliminated owing to its great solubility in most organic solvents.

The azoyl esters are coloured substances varying from orange to dark red. They are adsorbed from their solutions in organic solvents by many of the adsorbents commonly employed for chromatographic adsorption. Brockmann's Al_2O_3 , treated with methanol for three days at 37° and dried in the air, possesses good adsorbent qualities for these esters but subsequent elution is not quantitative. Better results are obtained with silica (pure precip. B.D.H.) as the adsorbent. In this case the elution of the ester can easily be made quantitative.

The high molecular weight of the azoyl group and the fact that it is possible, in small adsorption columns, to carry out the separation of a few mg. of substances, makes the use of this method possible for microchemical work. For example, using a column 7 mm. in diameter and 150 mm. high it has been possible to carry out the separation of 10 mg. of glucopyranose ester and 10 mg. of fructopyranose ester which are equivalent to less than 2 mg. of each sugar. A preliminary account of this work has already been published [Reich, 1939].

EXPERIMENTAL

1. *Preparation of the azobenzene-p-benzoic acid*

This substance was prepared by Angeli & Valori [1913] by condensation of *p*-aminobenzoic acid and nitrosobenzene in glacial acetic acid. The authors give m.p. 241°. The following method has been found to give better yields. 69 g. of dry *p*-aminobenzoic acid are dissolved in 450 ml. of hot absolute alcohol and 80 ml. of glacial acetic acid are added to the hot solution. The solution is cooled to room temperature, 60 g. of thoroughly pulverized dry nitrosobenzene are added and the mixture is shaken until the latter has dissolved. The mixture is then kept for 60 hr. at room temperature, followed by 12 hr. at 0°. Most of the azobenzene-*p*-benzoic acid formed separates from the solution in crystals which are filtered off, well washed with water, dried *in vacuo* over CaCl_2 and recrystallized from boiling absolute alcohol, the concentrated solution being cooled slowly to 0°. The substance crystallizes as mats of red needles with a golden glint. A further portion of the pure substance can be obtained from the mother liquors by precipitation with water, and recrystallization from absolute alcohol. Yield about 70 % of the theoretical.

2. *Preparation of the azoyl chloride*

50 g. of the dry acid are mixed with 150 ml. of pure dry benzene. A solution of 40 g. of SOCl_2 in 100 ml. of benzene is then added and the mixture is heated under reflux for 1 hr. on a boiling water bath. The dark red solution is evaporated *in vacuo* and the dry residue redissolved in benzene. The benzene is again evaporated *in vacuo* and the operation repeated a third time. Finally the residue is dissolved in the minimum of boiling light petroleum (b.p. 60–80°), the hot solution is filtered through a heated Jena glass filter and the clear solution slowly cooled to 0°. Bright red crystals are obtained. Recrystallization may also be carried out from boiling benzene. In this case, beautiful dark red crystals with a purple metallic glint are obtained, m.p. 93°. The yield is quantitative. (Found: C, 64.04; H, 3.91; N, 11.8; Cl, 14.33 %. $\text{C}_{13}\text{H}_9\text{ON}_2\text{Cl}$ requires C, 63.8; H, 3.71; N, 11.5; Cl, 14.5 %.)

Recently Ladenburg *et al.* [1938] published a method for the preparation of azobenzene-*p*-benzoyl chloride. These authors state that they had difficulty in preparing the chloride from the acid, but were successful in preparing it by the action of a large excess of boiling thionyl chloride on a mixture of the acid with $2\frac{1}{2}$ times its weight of Na_2CO_3 . They give m.p. 93–94° for the chloride. During the course of my work I have prepared several hundred g. of the chloride from the acid by the method described in the present paper and have always obtained a quantitative yield.

3. *Esterification of glucose; preparation of α -penta-azoyl-d-glucopyranose*

8.2 g. of well pulverized dry azoyl chloride are added to 50 ml. of pure pyridine (twice distilled over barium oxide) and cooled to -20° . The mixture is shaken for 30 min. at -20° and 1 g. of thoroughly pulverized dry glucose (6 hr. at 80° over P_2O_5 *in vacuo*) is added. The mixture is kept with frequent shaking for 4 days at 12° . It is then cooled to -20° , and 4 ml. of absolute methanol (dried over barium oxide and twice distilled), are added. The mixture is kept for 3 hr. at -20° , with frequent shaking and then for 12 hr. at 0° . The solid which separates is collected, dissolved in chloroform, precipitated with absolute ethanol, again collected and washed with absolute ethanol. The red powder thus

obtained is recrystallized from dioxane. Red crystals are obtained, m.p. 234–236°. Yield about 80 % of the theoretical. (Found: C, 69.32; H, 4.14; N, 11.36 %. $C_{71}H_{52}O_{11}N_{10}$ requires C, 69.8; H, 4.29; N, 11.47 %.) $[\alpha]_{D}^{20} = +193^\circ$ (chloroform; $c = 1$).

4. Esterification of fructose

8.2 g. of well powdered azoyl chloride are added to 50 ml. of pure dry pyridine, previously cooled to -20° ; the mixture is shaken for 30 min. at -20° , and 1 g. of well powdered dry fructose which had been dried in a very thin layer for 24 hr. over P_2O_5 *in vacuo* is added. The mixture is shaken for 4 hr. at -20° , and then left for 4 days at -12° , being frequently shaken during this time. It is then cooled to -20° , and 4 ml. of absolute methanol added. The mixture is kept for 3 hr. at -20° and then for 12 hr. at 0° . The solution is filtered and the filtrate evaporated *in vacuo* at room temperature in an apparatus designed by the author.¹ The residue is dissolved in the minimum of hot benzene and precipitated with absolute ethanol. The substance is recrystallized from CCl_4 and is obtained in the form of red crystals, m.p. 135–136°. (Found: C, 69.32; H, 4.47; N, 11.25 %. $C_{71}H_{52}O_{11}N_{10}$ requires C, 69.8; H, 4.29; N, 11.47 %.) $[\alpha]_{D}^{20} = -345^\circ$ (chloroform; $c = 1$).

5. Chromatographic separation of a mixture of azoyl esters of glucose and fructose

(a) *Preparation of the adsorption column.* 70 g. of silica (pure precip. B.D.H.) are mixed with 300 ml. of a solution of 25 % benzene (pure dry) in dry light petroleum (b.p. 60–80°); the mixture is kept for about 30 min. The adsorption column is prepared with this mixture in the following way: a part of the mixture is poured into a tube 30 mm. in diameter and 400 mm. high. The bottom of the tube is closed by a wad of cotton-wool, on which is placed a perforated disc of porcelain. Establishing a pressure by compressed nitrogen from the top of the tube, the liquid is filtered through the column of silica, until only a few mm. of liquid remain above the surface of the silica. At this stage a fresh quantity of the mixture is poured into the tube and the filtration continued. This is repeated until the column acquires a height of about 300 mm. when a second perforated porcelain disc is placed at the top of the column, and about 100 ml. of the benzene-petroleum mixture (25 : 75) is filtered by pressure through the column, so that the latter is made compact and homogeneous before beginning the chromatographic adsorption. During this operation, as during the entire chromatographic adsorption, the column must never become dry. The pressure of the nitrogen is regulated during the formation of the column so that the liquid filters through the column at a speed of 6–10 ml. per min. A speed of 3–4 ml. per min. is used during the adsorption and development of the chromatogram.

(b) *Adsorption.* In a typical experiment a solution of azoyl esters was prepared in the following way: 200 mg. of the glucose ester and 200 mg. of the fructose ester were dissolved in 40 ml. of chloroform, 80 ml. of benzene and 80 ml. of petroleum are added and the solution was filtered through the column. During the operation the formation of two red layers, separated by an orange layer was observed at the top of the column. When filtration of the solution of esters was nearly com-

Fig. 1.

¹ The apparatus is now obtainable through Gallenkamp & Co. Ltd.

plete, so that only a few mm. remained above the column, the chromatogram shown in Fig. 1 was observed. The numbers indicate the thickness of the layers in mm.

For the development of the chromatogram, 800–900 ml. of the benzene-petroleum mixture (25:75) were filtered through the column, the current of nitrogen being maintained at the end of the filtration until the column was completely dry. The distribution of the chromatogram at the end of the procedure is shown in Fig. 2.

40	Dark orange	Layer A	After elution 172 mg. of substance				[α] _{D430} ^{20°} = -323° (theor. -345°)
65	Nearly colourless	„ x	„	„	17	„	
2	Orange	} „ B	„	„	16	„	„
6	Nearly colourless						
30	Dark orange	„ C	„	„	166	„	[α] _{D430} ^{20°} = +171° (theor. +193°)
105	Nearly colourless	„ y	„	„	9	„	
2	Orange	„ D	„	„	1	„	
	Colourless				381 mg. (theor. =400 mg.)		

Fig. 2.

Fig. 2.

(c) *Elution*. For the separation of the different layers, the tube was held horizontally and, beginning with the upper layer, the column was scraped with a spatula, each layer being taken out separately and placed in a flask. For the elution of the adsorbed substance the powder was suspended in a mixture of methanol and chloroform (2:8), left for 12 hr. at room temperature and treated in the following way. Each mixture was filtered through a Jena glass filter No. G 3 (diameter 25 mm. height 150 mm.), a mixture of methanol and chloroform being drawn slowly through the powder on the filter until this became colourless. The clear filtrate was then evaporated *in vacuo*. The residue was dissolved in a small quantity of CCl₄, the solution filtered and the filtrate precipitated with light petroleum (B.P. 40–60°). After collecting the precipitate it was dried *in vacuo*. The yields of the substances eluted in this way are indicated in Fig. 2. One can see from the values of the specific rotation that, after a single adsorption, the substances are nearly pure. A second adsorption, carried out on the individual fractions, leads to the isolation of the pure substances.

Work upon the separation of other sugars and polysaccharides by similar methods is in progress.

SUMMARY

1. By the action of azobenzene-*p*-benzoyl chloride (azoyl chloride) on glucose or fructose, in pyridine solution, coloured azoyl esters are obtained.
2. These esters can be adsorbed from their solution in organic solvents by various adsorbents from which they can be recovered by elution.
3. The adsorption is selective, allowing the separation of mixtures of these esters into their components by chromatographic adsorption.
4. The yields of pure substances, recovered from such mixtures are nearly quantitative.

5. The separation of a very small quantity (for example 20 mg.) of the sugar azoyl esters, can be executed by this method.

I wish to take this opportunity of thanking the Governing Body of the Lister Institute, for the hospitality of its laboratories and to Prof. R. Robison for the kindly interest he has shown in this investigation. I am indebted also to Dr Kenyon who placed at my disposal a cadmium lamp for polarimetric measurements.

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