THE SEPARATION OF GLYCOSIDES ON A STRONGLY BASIC ION-EXCHANGE RESIN: AN INTERPRETATION IN TERMS OF ACIDITY

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ABSTRACT

The anomeric methyl glycopyranosides of D-glucose, D-mannose, D-galactose, 2-amino-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-mannose, and 2-acetamido-2-deoxy-D-galactose were separated on a preparative scale, in gram amounts and in a good yield, by using Dowex-1 columns in the hydroxide form. The separation of these glycosides on the basic column is assumed to be due to an ion-exchange process involving ionisation of one of the hydroxyl groups of the glycosides. The acidity of the glycosides is explained in terms of the anomeric effect and other forms of dipole interaction.

INTRODUCTION

The preparation of pure glycosides has been accomplished, apart from methods involving fractional crystallisation, by the use of columns of powdered cellulose[1], Fuller's earth[2], charcoal–Celite[3], and starch[4]. In 1963, Austin *et al.*[5] and Matsushima *et al.*[6] introduced strongly basic ion-exchange resins in the hydroxide form for the separation of glycosides. An advantage of this method is that the resin retains any free sugars present in the reaction mixture, an observation made earlier by several authors[7].

Recent work in this laboratory has been concerned with the inhibitory effect of some 2-acylamido-2-deoxy-D-glucopyranosides on the activity of hen's egg-white lysozyme[8]. For this purpose, the preparation of pure anomers was carried out with a column of Dowex-1 (HO^-) resin.

This report presents the conditions and some of the results obtained with this method of separation. In addition, some preliminary observations are made on the quantitative aspects of retention of these glycosides on a strongly basic ion-exchange resin in the hydroxide form.

EXPERIMENTAL

Melting points were recorded on a Kofler block and are uncorrected. Optical rotations were determined on a Perkin–Elmer 141 polarimeter at 25° in a 1-dcm cell.

Absorbance was recorded on a Unicam SP 700 spectrophotometer. Dowex-1 resin (2% cross-linked, 200–400 mesh) was obtained from Sigma Chemical Co.

Glycosidation. — The alkyl glycosides were prepared[9] with Dowex-50 (H^+) and the appropriate alcohol. The reaction mixture was usually refluxed for 3 h unless otherwise stated; the resin was then filtered off, and the filtrate was evaporated to dryness under reduced pressure. The methyl 2-amino-2-deoxy-D-glucopyranosides were prepared by glycosidation of 2-benzyloxycarbonylamino-2-deoxy-D-glucose and removal of the protecting group by hydrogenolysis[10]. Excess hydrochloric acid was removed over sodium hydroxide pellets, under high vacuum. In some cases, it was more convenient to separate the methyl 2-amino-2-deoxy-D-glucopyranosides and then carry out acylation; one example of this route is reported.

Separation of glycosides. — Dowex-1 resin (450 g; 2% cross-linked, 200–400 mesh) was recycled twice between the hydroxide and chloride forms with 2M sodium hydroxide and 2M hydrochloric acid. The resin was finally washed in an acrylic column (60 × 3 cm, 0.1-ml dead space) with deionised, distilled, carbon dioxide-free water. The mixture to be separated was placed on the column with the aid of a three-way tap and syringe with Luer fittings. The sample was normally dissolved in 3 ml of water and washed onto the column with 1 ml. The column was then eluted with deionised, distilled, carbon dioxide-free water from a reservoir fitted with a soda-lime trap. The flow rate was controlled with a peristaltic pump (LKB) at a rate of 27–30 ml/h. The column was eluted with 400 ml of water, and then 5-ml fractions were collected. The fractions were monitored by optical rotation and, in the case of the methyl 2-acylamido-2-deoxy-D-hexopyranosides, by absorbance at 225–230 nm. The fractions containing the separated anomers were combined and evaporated to dryness at 40° under reduced pressure. The solid residues were normally recrystallised from methanol or ethanol, unless otherwise stated. Difficulty was experienced in crystallising some of the separated anomers; this was due to the tendency of the concentrated alcoholic solutions to set into gels. This was usually overcome by warming the solution and adding a drop of water before cooling the solution again.

In most of the examples presented, the column loading was approximately 1 g, but good resolution has been obtained on the column with loadings up to 3 g. Reducing sugars retained on the resin undergo considerable degradation, as other authors have noted[11], and tend to destroy the integrity of the column. Any hydrochloride present in reaction products will decrease the capacity of the resin. Hence, when separations of reaction mixtures were carried out, reducing sugars and hydrochloric acid, in the case of the methyl 2-amino-2-deoxy-D-glucopyranosides, were removed by passing the reaction mixture through a small column (25 × 2.5 cm) of Dowex-1 (HO^-) resin under the same conditions. With these precautions, the resolving capacity of the main column was maintained over at least ten separations.

Elution volume. — This was taken as the volume, in ml of water, at which the maximal concentration of the anomer was observed. It was of interest to determine whether the elution volumes of a pair of anomers were reasonably constant and did not depend on the amount of elutant passed through the resin. An initial separation of the methyl D-glucopyranosides was carried out before a series of separations was embarked upon. This separation was repeated after six runs had been carried out. The methyl D-glucopyranosides were eluted at 710 and 925 ml in the first run, and at 695 and 900 ml for the second run.

Methyl 2-deoxy-2-formamido-D-glucopyranosides. — Amounts (0.5 g) of the methyl 2-amino-2-deoxy-D-glucopyranoside anomers, previously separated on Dowex-1 (HO^-), were separately dissolved in 5 ml of formic acid, and the solution was cooled to 0°. Acetic anhydride (1.86 ml) was added slowly, and the mixture was allowed to stand at room temperature for 3 h. It was then evaporated at room temperature under high vacuum, and the syrupy residue was dissolved in water (5 ml). The pH of the solution was adjusted to 10 with 2M sodium hydroxide and maintained at that pH for 1 h at room temperature. Each solution was passed through Dowex-50 (H^+) resin, and the filtrate was evaporated to dryness. The residues were recrystallised from hot ethanol to give the α-D anomer (530 mg, 92%), m.p. 142–144°, $[\alpha]_D^{25}$ +143.4° (*c* 1, water) (Found: C, 43.45; H, 7.02; N, 6.30. $C_8H_{15}NO_6$ calc.: C, 43.43; H, 6.85; N, 6.33%); and the β-D anomer (524 mg, 91%), m.p. 190–192°, $[\alpha]_D^{25}$ −45.2° (*c* 1, water) (Found: C, 43.63; H, 6.99; N, 6.34%); lit.[12] m.p. 204–205°, $[\alpha]_D$ −47.2°.

Ethyl 2-deoxy-2-propionamido-D-glucopyranosides. — The glycosides were prepared from 2-deoxy-2-propionamido-α-D-glucopyranose (m.p. 185–187°, $[\alpha]_D^{25}$ +57 → +37° (*c* 1, water). A portion (2.3 g) of the reaction mixture, after preliminary treatment with Dowex-1 (HO^-) resin, was dissolved in water and placed on the column; the α anomer was eluted at 492 ml. The α anomer was recrystallised from hot ethanol and the β anomer from warm acetone: α anomer (2.05 g), m.p. 172–174°, $[\alpha]_D^{25}$ +138.5° (*c* 1.05, water), +154.1° (*c* 1, ethanol) (Found: C, 50.20; H, 8.16; N, 5.23. $C_{11}H_{21}NO_6$ calc.: C, 50.19; H, 7.98; N, 5.32%); β anomer (0.033 g), m.p. 186–188° $[\alpha]_D^{25}$ −31.1° (*c* 1.03, water), −34.6° (*c* 0.5, ethanol) (Found: C, 49.78; H, 7.90; N, 5.15%)

Unless otherwise stated, the following separations were carried out by placing together on the column weighed amounts of the previously separated anomers.

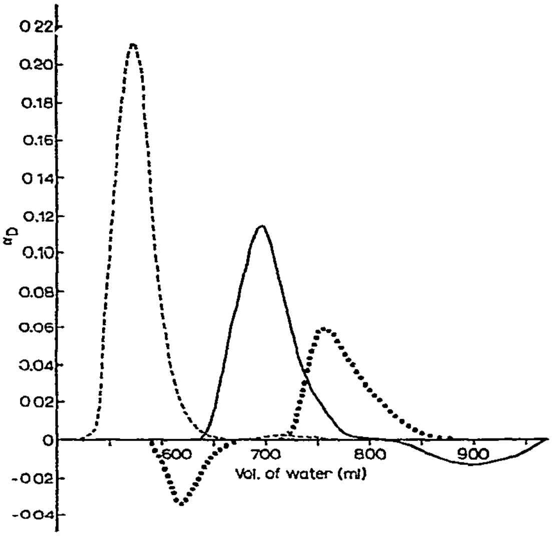
Methyl D-glucopyranosides. — Amounts of 0.5 g of each anomer were separated (Fig. 1).

Methyl-D-galactopyranosides. — The glycosidation product (2.3 g) was passed through Dowex-1 (HO^-) resin, and the residue obtained was placed on the column. Only a small amount of the β anomer was isolated. The yield of the α anomer was 0.51 g. The low yields of some of the glycosides could have been due to the formation of furanosides. These would have been retained on the resin[5].

Methyl D-mannopyranosides. — Amounts of 0.5 g of the α anomer and 0.36 g of the β anomer were separated (Fig. 1). The β anomer, when recrystallised from isopropyl alcohol and dried over phosphorus pentoxide at 50° under vacuum, had $[\alpha]_D^{25}$ −64.3° (*c* 1, water). The same treatment at room temperature gave a product having $[\alpha]_D^{25}$ −50.8° (*c* 1.5, water). The melting point is given for material dried by the latter treatment.

*Methyl 2-amino-2-deoxy-*D*-glucopyranosides.* — Amounts of 0.5 g of each anomer were separated (Fig. 2).

*Methyl 2-acetamido-2-deoxy-*D*-glucopyranosides.* — Amounts of 0.58 g of the α anomer and 0.30 g of the β anomer were separated (Fig. 2).



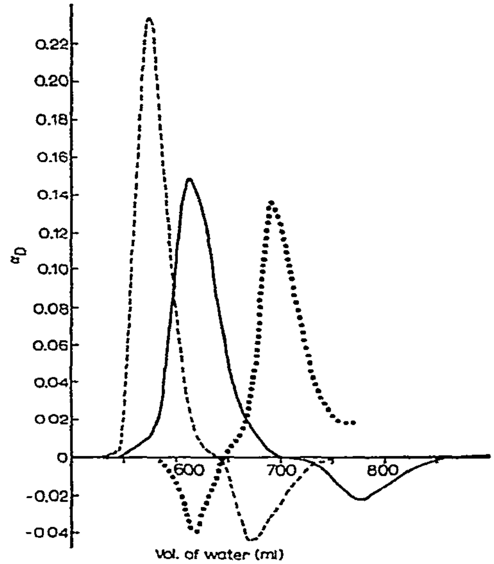


Fig. 1. Elution patterns of methyl D-galactopyranosides (– – – –), methyl D-glucopyranosides (———) and methyl D-mannopyranosides (····). Optical rotations recorded in a 0.1-dm cell.

Fig. 2. Elution patterns of methyl 2-amino-2-deoxy-D-glucopyranosides (– – – –), methyl 2-acetamido-2-deoxy-D-glucopyranosides (———), and methyl 2-acetamido-2-deoxy-D-mannopyranosides (····). Optical rotations recorded in a 0.1-dm cell.

*Methyl 2-acetamido-2-deoxy-*D*-mannopyranosides.* — Amounts of 0.5 g of 2-acetamido-2-deoxy-D-mannose (m.p. 103–105 °C, $[\alpha]_D^{25}$ +9.5°) were converted into the methyl glycosides. The reaction product was treated with Dowex-1 (HO^-) resin, and the filtrate was evaporated to a small volume and placed on the column (Fig. 2). Attempts to crystallise these anomers failed. The specific rotations were obtained by drying the syrup over phosphorus pentoxide at 78.5° under vacuum and dissolving the dried syrup in 1 ml of water.

*Methyl 2-acetamido-2-deoxy-*D*-galactopyranosides.* — An amount of 0.5 g of 2-acetamido-2-deoxy-D-galactose, $[\alpha]_D^{25}$ $+114.8 \rightarrow +79.2°$ (*c* 1, water), was glycosidated at 5° for one week. The reaction product was treated with Dowex-1 (HO^-) resin, and the filtrate was evaporated to a small volume and placed on the column. The total yield was 28% (33 mg of α anomer and 115 mg of β anomer). The β anomer had $[\alpha]_D$ −11° (*c* 1, methanol); lit.[13] $[\alpha]_D$ −12° (methanol).

The remarkable consistency of this method of separation and the extended life of the resin column for preparative purposes deserve emphasis. Austin *et al.*[5] used this method by loading (1.9 g of reaction material) onto a 25 × 1.7 cm column and obtained good resolution, but used the resin once only. Matsushima *et al.*[6], on the other hand, loaded 50-g amounts onto a 75×8 cm column and obtained relatively poor resolution. However, the present work demonstrates that, provided precautions are taken with the operation of the column, repeated separations of gram amounts on the same column can be obtained, as was reported by Evans *et al.*[14] who used 1-propanol–water (3:1 v/v) to elute the glycosides from the resin.

DISCUSSION

The separation of glycosides described here and in papers quoted in the Introduction depends on the use of the strongly basic ion-exchange resin Dowex-1 in the hydroxide form. Dowex-2 (8% cross-linked) gives only a partial separation of methyl D-glucopyranosides[5], and the chloride form of Dowex-1 was found to separate three closely related methyl D-glycopyranosides differing at C-6, without apparently distinguishing between the α and β anomers[14]. The requirement for high basicity of the resin is most readily explained by assuming that the adsorption is an ion-exchange process involving the loss of a proton from one of the hydroxyl groups of the glycoside. Dissociation constants of the order of 10^{-13} to 10^{-14} have indeed been reported for non-reducing carbohydrates[20], and the pH on the surface of Dowex resin particles in the hydroxide form is almost certainly sufficiently high to make the required ionisation possible. Dekker[21] has shown that the order of elution of related nucleosides from a Dowex-1 column is that to be expected from the dissociation constants of the hydroxyl groups of the sugar moiety as measured by a spectrophotometric method[22]. It is thus possible that the elution volumes reported here give an indication of the relative acidities of the various glycosides investigated.

As already mentioned, glycosides behave in water as weak acids, but the ionisation constants have not been measured with great accuracy. Derevitskaya *et al.*[23] have determined the comparative acidities of hydroxyl groups of various carbohydrates, including methyl α- and β-D-glucoside, in ethylenediamine by electrometric titration and have found that all hydroxyl groups can ionise on addition of sufficient amounts of strong base. Glycosides thus can behave as polybasic acids, but we may assume that in water we are dealing mainly with one ionisation step. This first ionisation in a glycoside may be assumed to be largely associated with HO-2, the higher acidity of which is due to the proximity and inductive effect of the hemiacetal function at C-1. This interpretation, which may be extended to other glycopyranosides, is also in accordance with the relatively high reactivity of HO-2 observed in glucosides and cellulose under certain conditions[24]. In 2-acetamido-2-deoxypyranosides, the most acidic hydroxyl group is likely to be at C-3, which is adjacent to the acetamido group, whereas, with the 2-amino-2-deoxypyranosides, the choice is more open.

For the seven pairs of glycosides in Table I, the higher retention volume is found for that anomer which has the bulky substituents at the C-1 and C-2 positions in *trans* relationship. Following the interpretation adopted above, this means that the acidity of the β anomer exceeds that of the α anomer in the glucose and galactose series, but not in the mannose series, where the α anomer is the more acidic. The acidity of the equatorial HO-2 in the glucosides and galactosides is determined to a large extent by the number and proximity of other polar substituents in the molecule. Also important are the relative angles of the various dipoles to each other and to the hydroxyl group which ionises. In methyl β-D-glucopyranoside and methyl β-D-galactopyranoside, the dipole moments of the ring oxygen atom and of the glycosidic oxygen bond largely re-inforce one another, whereas, in the corresponding α anomers, they cancel one another to a significant extent. Indeed, this is the accepted explanation[25] for the "anomeric effect". Thus, we would expect that the combined effects of two dipoles will make the β-D-glucoside and β-D-galactoside more acidic than the corresponding α anomers. This dipole interaction is almost certainly the reason why the protonated 2-amino-2-deoxy-β-D-glucopyranose has a higher ionisation constant than the α anomer[26]. This effect will also operate in the galactosides, but in this case the change of configuration in C-4 will produce a lowering of acidity in both anomers owing to a change of direction of the C-4 dipole. In the mannosides, the situation is different. In methyl α-D-mannopyranoside, the dipole of the bond formed between C-1 and the glycosidic oxygen atom is at an angle of 180° with the C-2–OH bond and thus will markedly facilitate ionisation in the latter position. The orbitals of the ring oxygen atom will, to some extent, weaken the effect of this dipole, but in mannosides the effects of the two adjacent axial dipoles in the 1- and 2-positions overshadow the influence of the "anomeric effect". Thus, with mannosides, the α-anomer is the stronger acid. This particular dipole interaction, possibly together with steric factors, is likely to be the basis of the $\Delta 2$ effect.

A more difficult problem is presented by the 2-amino-2-deoxy- and the 2-acetamido-2-deoxy-pyranosides. The assumption, which was stated above, that in these compounds it is HO-3 which is mainly ionised in a strongly alkaline medium, together

TABLE I

PHYSICAL CONSTANTS OF SEPARATED GLYCOSIDES

Configuration	*Aglycon and anomer*	*Group at C-2*	*M.p. (degrees)*	$[\alpha]_D^{25}$ *(degrees) (c 1, water)*	*Elution volume (ml)*	*Ref.*
Gluco-	α-CH_3	OH	166–168	+152.5	710	15
	β-CH_3	OH	111–113	−31.8[c]	925	
Manno-	α-CH_3	OH	193–195	+77.9	756	16
	β-CH_3	OH	65–69	−50.8	618	
Galacto-	α-CH_3	OH	116–118	+195.2	576	17
	β-CH_3[e]	OH	[a]	[a]	715	
Gluco-	α-CH_3	NH_2	148–152	+154.6	575	18
	β-CH_3	NH_2	126–130	−44.0	672	
Gluco-	α-CH_3	NHAc[g]	190–193	+128.2	614	3, 18
	β-CH_3	NHAc[g]	196–199	+43.5	775	
Manno-	α-CH_3	NHAc[g]	[b]	+45.2[h]	692	
	β-CH_3	NHAc[g]	[b]	−45.7[h]	618	
Galacto-	α-CH_3	NHAc[g]	216–220	+179.8	582	19
	β-CH_3	NHAc[g]	235–237	+8.4[c,f]	664	13

[a]Not determined; [b]syrup; [c](*c* 2, water); [d]60 × 3 cm column; [e]recrystallised from isopropyl alcohol; [f]$[\alpha]_D^{25}$ −11.0° (*c* 1.1, methanol); [g]*c* 7, water; [h]*c* 1.1, water.

with the proposition that the dipole interaction responsible for the anomeric effect affects ionisation in the equatorial position in C-3 in the same way as it does in C-2, explains why in the amino- and acetamido-glucosides and galactosides the β anomer is more acidic than the α anomer. With 2-acetamido-2-deoxymannosides, the amide dipole in C-2 has to be included in any explanation suggested and as its direction in relation to the pyranose ring is unknown, no estimate of its effect can be given. However, there is the possibility that ionisation in the 2-acetamido-2-deoxyglycoside consists of a removal of a proton from the imino group. The acidic ionisation constant (removal of proton) in acetamide has been stated[27] to be 8.3×10^{-16}, but is likely to be slightly lower, *i.e.*, similar to that of an aliphatic hydroxyl group, *i.e.*, with a pK of ~ 16. In a 2-acetamido-2-deoxy-glycoside, the imino group has on adjacent carbon atoms, on the one side a hemiacetal structure and on the other a hydroxyl group. This and other substituents might reduce the pK to values of 13.4 to 14.0. On this basis, the behaviour of all anomers in Table I may be rationalised.

REFERENCES

1 D. F. Mowery, Jr., *Methods Carbohyd. Chem.*, 2 (1963) 328; I. Augestad and E. Berner, *Acta Chem. Scand.*, 8 (1954) 251.
2 D. F. Mowery, Jr. and G. R. Ferrante, *J. Amer. Chem. Soc.*, 76 (1954) 4103.
3 F. Zilliken, C. S. Rose, G. A. Braun, and P. Gyorgy, *Arch. Biochem. Biophys.*, 54 (1955) 392.
4 D. F. Mowery, Jr., *Anal. Chem.*, 31 (1959) 1911.
5 P. W. Austin, F. E. Hardy, J. G. Buchanan, and J. Baddiley, *J. Chem. Soc.*, (1963) 5350.
6 Y. Matsushima, T. Miyazaki, and J. T. Park, *J. Biochem.* (Tokyo), 54 (1963) 109.
7 F. H. Vorston, *Pulp Paper Mag. Can.*, 50 (1948) 108; L. I. Woolf, *Nature*, 171 (1953) 841; S. Roseman, R. H. Abeles, and A. Dorfman, *Arch. Biochem. Biophys.*, 36 (1952) 232.
8 A. Neuberger and B. M. Wilson, *Biochim. Biophys. Acta*, 147 (1967) 473.
9 G. R. Dean and R. E. Pyle, U. S. Patent, 2,606,186 (1952); *Chem. Abstr.*, 47 (1952) 147; *British Patent*, 670,480 (1952); *Chem. Abstr.*, 46 (1952) 9332; J. F. Cadotte, F. Smith, and D. Spriestersbach, *J. Amer. Chem. Soc.*, 74 (1952) 1501.
10 E. Chargaff and M. Bovarnick, *J. Biol. Chem.*, 118 (1937) 421.
11 Y. Matsushima and T. Miyazaki, *J. Biochem.* (Tokyo), 55 (1964) 464; J. D. Phillips and A. Pollard, *Nature*, 171 (1953) 42.
12 A. Neuberger and R. P. Rivers, *Biochem. J.*, 33 (1939) 1580.
13 Z. Tarasiejska and R. W. Jeanloz, *J. Amer. Chem. Soc.*, 80 (1958) 6325.
14 M. E. Evans, L. Long, Jr., and F. W. Parrish, *J. Chromatogr.*, 32 (1968) 602.
15 T. S. Patterson and J. Robertson, *J. Chem. Soc.*, (1924) 300; H. S. Isbell and H. L. Frush, *J. Res. Nat. Bur. Stand.*, 24 (1940) 125.
16 B. Helferich and G. Duve, *Ber.*, 91 (1958) 1790.
17 J. K. Dale and C. S. Hudson, *J. Amer. Chem. Soc.*, 52 (1930) 2534.
18 Y. Matsushima and T. Miyazaki, *Bull. Soc. Chem. Japan*, 38 (1965) 1325.
19 M. Stacey, *J. Chem. Soc.*, (1944) 272.
20 L. Michaelis and P. Rona, *Biochem. Z.*, 49 (1913) 232; P. Hirsch and R. Schlags, *Z. Physik. Chem.*, *A.* 141 (1929) 387; F. Urban and P. A. Shaffer, *J. Biol. Chem.*, 94 (1931) 697.
21 C. A. Dekker, *J. Amer. Chem. Soc.*, 87 (1965) 4027.
22 J. J. Fox and D. Shugar, *Biochim. Biophys. Acta*, 9 (1952) 369.
23 V. A. Derevitskaya, G. S. Smirnova, and Z. A. Rogovin, *Dokl. Akad. Nauk SSSR*, 141 (1961) 1090; *Proc. Acad. Sci. USSR* (*Chemistry*), 141 (1961) 1254.
24 J. M. Sugihara, *Advan. Carbohyd. Chem.*, 8 (1953) 1.
25 J. T. Edward, *Chem. Ind.* (London), (1955) 1102.
26 A. P. Fletcher and A. Neuberger, *Carbohyd. Res.*, 17 (1971) 79.
27 G. E. Branch and J. D. Clayton, *J. Amer. Chem. Soc.*, 50 (1928) 1680.