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Acid and Enzymic Hydrolysis of β-Glucosides. III

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Scheiber [1] examined the acid and the enzymic hydrolysis of different aryl-β-D-glucosides in order to determine the influence of different substituents in the aromatic nucleus on the velocity of hydrolysis. It was found that the acid hydrolysis was not much influenced by the introduction of a single methyl group or by two methyl groups, one in *o*-, the other in *p*-position to the hydroxyl group, but the introduction of two methyl groups in *o,o'*-position caused an increase of the velocity constant to 4 times the value for the unsubstituted or the monosubstituted phenol-β-D-glucoside.

For the enzymic hydrolysis, on the other hand, the effect of the introduction of methyl groups was quite different. With sweet almond emulsin as enzyme the velocity constant for *o*-cresol-β-D-glucoside was found 13 times as great, for *p*-cresol-β-D-glucoside only 1/3 as great as for phenol-β-D-glucoside, and the value for *o,p*-xylenol-β-D-glucoside is only 1/3 of the value for *o*-cresol-β-D-glucoside. The effect of two methyl groups in *o*-position to the glucoside linkage was a retardation of the velocity of hydrolysis, the constant being only 1/3—1/4 of the value for phenol-β-D-glucoside, slightly inferior to the value for *p*-cresol-β-D-glucoside.

This effect reminds of the effect of methyl groups on the hydrolysability of alkyl-β-D-glucosides, examined by Veibel and Frederiksen [2] and by Veibel and Lillelund [3]. For the acid hydrolysis it was found that the constants for methyl- and propyl-β-D-glucoside were nearly equal. The velocity constant for ethyl-β-D-glucoside will presumably be very similar to the two other constants. For isopropyl-β-D-glucoside the velocity constant is about twice as great as for methyl and propyl-glucoside, whereas the constant for trimethylcarbinol-

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β-D-glucoside is superior to the other constants by 2—3 orders of magnitude. For the enzymic hydrolysis, on the other hand, the velocity constants for ethyl-, isopropyl-, and trimethylcarbinol-β-D-glucoside were $5.3 \cdot 10^{-2}$, $16.9 \cdot 10^{-2}$, and $0.02 \cdot 10^{-2}$ respectively, *i.e.* an increase by a factor 3 for one methyl group and a decrease by 2—3 orders of magnitude for two methyl groups.

Scheiber does not mention the hydrolysis of mesitol-β-D-glucoside in which the phenol is substituted by two methyl groups in *o*-position and one in *p*-position. In order to see if the retarding influence of the *p*-methyl group is sufficient to cause a decrease in velocity of hydrolysis of mesitol-β-D-glucoside as compared with *o,o'*-xylenol-β-D-glucoside we prepared the two glucosides and examined the acid and the enzymic hydrolysis.

Scheiber indicates that he has used 0.052 *M* solutions of the glucosides investigated both for the acid and for the enzymic hydrolysis. The mesitol-glucoside is, however, so slightly soluble in water that a 0.007 *M* solution is nearly saturated at 30°. For the enzymic hydrolysis this solution, therefore, means the upper limit of concentration and in order to obtain comparable results we used this solution for the experiments with acid hydrolysis too.

The result of the investigation is that the velocity constants for the acid hydrolysis of the two glucosides are very nearly equal, that for mesitol-glucoside being slightly greater than that for the other. For the enzymic hydrolysis the inverse is found. In both instances are the differences, however, hardly greater than the experimental error.

EXPERIMENTAL

Substrates. *o,o'*-Xylenol-β-D-glucoside was prepared as indicated by Helferich and Scheiber [1] from *o,o'*-xylenol. This latter substance may be prepared easily according to Carlin and Landerl [4].

Mesitol-β-D-glucoside: On a steam bath a mixture of 20 g of β-pentaacetylglucose and 14 g of mesitol was melted, and 0.26 g of *p*-toluenesulfonic acid was added. The homogenous melt was heated on the steam bath for 5 hours, the resulting dark brown sirup was dissolved in 250 ml of benzene, mesitol and *p*-toluenesulfonic acid was removed by washing the refrigerated solution with 2 *N* sodium hydroxide and with water. After drying over calcium chloride the benzene solution was evaporated in vacuo and the crystalline residue was freed from a sirupous substance by recrystallising it from methanol till the m.p. remained constant. Tetraacetyl-mesitol-β-D-glucoside, m.p. 164.5—165°, $[\alpha]_{20}^{D}$ −14.8° (chloroform, $c = 1.016$, $l = 2$, $\alpha = -0.30°$), was obtained as colourless needles in a yield of 65%.

$C_{23}H_{30}O_{10}$ (466.5)	Calc.	C	58.88	H	6.02
	Found	»	59.21	»	6.48

Deacetylation produced the mesitol-β-D-glucoside, colourless prisms recrystallised from water. M.p. 222.5—223.5°, $[\alpha]_{20}^{D}$ —6.07° (water, $c = 0.989$, $l = 2$, $\alpha = -0.12°$).

$C_{15}H_{22}O_6$ (298.3)	Calc.	C	60.37	H	7.44
	Found	»	60.70	»	6.95

Enzyme. The β-glucosidase used was prepared from sweet almonds. 1 kg of sweet almonds were pressed in a hydraulic press, the dry presscake (about 500 g) was milled to a coarse powder from which the β-glucosidase was extracted essentially according to the indications of Helferich [5]. During the extraction the solution was stirred mechanically. For all centrifugations a refrigerated centrifuge from *International Equipment Co., Boston, Mass.*, was used. We take the opportunity to thank the *Carlsberg Foundation* for a grant which enabled one of us (S.V.) to obtain the centrifuge. Yield of β-glucosidase 5.26 g with a Sal.f. of 0.60 (β-glucosidase value 2.0), somewhat higher than indicated by Helferich (β-glucosidase value 1.25). The standardisation of the β-glucosidase was made with o-cresol-β-D-glucoside as substrate (Veibel and Lillelund [6–8]). Table 1 gives the result.

Table 1. Standardisation of β-glucosidase.

o-Cresol-β-D-glucoside 0.0400 *M*. $\alpha_{beg.} = -1.325°$, $\alpha_{end} = +0.620°$. Emulsin: 2 ml of a solution containing 0.0074 g in 20 ml dissolved to 50 ml. $e = 0.00074$. $\alpha_{Emulsin} = 0°$. Phosphate-citrate buffer pH 4.4. 30°.

$$K' = K_m/K_{m_1} + K_m/K_{m_2} - 1 = -0.28$$

t min	α	c—x	$10^4 \cdot k_{obs}$	$K' \cdot x$	$10^2 \cdot k_3$/Sal.f.
0	—1.325	1.945	—	—	—
10	—1.220	1.840	24.1	0.0003	(17.2)
20	—1.100	1.720	29.3	0.0010	20.6
30	—0.970	1.590	34.1	0.0017	23.6
40	—0.840	1.460	37.0	0.0024	25.3
60	—0.620	1.240	35.5	0.0034	23.7
90	—0.360	0.980	34.1	0.0048	22.2
120	—0.170	0.790	31.5	0.0060	20.0
				mean value	22.6

For k_3 Veibel and Lillelund [6] found $37.5 \cdot 10^{-2}$ (phosphate-citrate, pH 4.4). Sal.f., therefore, is $\frac{22.6}{37.5} = 0.60$ and β-glucosidase value $= \frac{\text{Sal.f.}}{\log 2} = 2.0$.

Methods. In previous investigations in this laboratory the polarimetrical method for following the hydrolysis was used. The mesitol-β-D-glucoside is, however, so slightly soluble in water that at 30° the most concentrated solution is about 0.007 *M*. The total change of rotation for complete hydrolysis being only 0.11—0.12° we had to use another method than the polarimetrical, and the Hagedorn-Jensen method for determination of glucose, as modified by Hanes [9], was considered the most reliable.

The enzymic hydrolysis was followed by taking samples of 5 ml at intervals, suppressing the enzymic action by adding the sample to 1 ml of 20% potassium carbonate and

determining the glucose content in 5 ml of the mixture. For the acid hydrolysis the method described by Veibel and Frederiksen [2] was used. Here, too, the hydrolysis was followed by Hagedorn-Jensen-determination of the glucose content of the samples, not polarimetrically. The acid hydrolysis was interrupted by adding 5 ml samples to 2 ml 20% potassium carbonate in experiments with *N* acid, to 2 ml 10% potassium carbonate in experiments with 0.5 *N* acid. The results are given in Tables 2—4. It is seen from the tables that there is no significant difference between the velocity constants for the two glucosides.

Table 2. Enzymic hydrolysis.

Glucoside 0.007 *M*. Emulsin 0.0043 g in 50 ml. Sal.f. = 0.60. Phosphate-citrate buffer pH 4.4. 30°. Use of 0.0129 *N* $Na_2S_2O_3$ by complete hydrolysis 11.71 ml ~ 5.25 mg glucose.

A. *o,o'*-Xylenol-glucoside				B. Mesitol-glucoside			
t min	ml $Na_2S_2O_3$	$c_{end} - c_t$ mg glucose	$k \cdot 10^4$	*t* min	ml $Na_2S_2O_3$	$c_{end} - c_t$ mg glucose	$k \cdot 10^4$
0	—	5.25	—	0	—	5.25	—
15	0.20	5.16	5.00	15	0.18	5.17	4.47
30	0.33	5.10	4.20	30	0.29	5.12	3.63
60	0.65	4.94	4.42	60	0.60	4.97	3.97
90	0.76	4.89	3.46	90	0.73	4.91	3.23
135	1.12	4.74	3.29	135	1.09	4.75	3.22
195	1.73	4.48	3.53	195	1.69	4.50	3.44
285	2.42	4.17	3.51	285	2.37	4.19	3.44
		mean value	3.9			mean value	3.6
	k/e (Sal.f.) $= 15 \cdot 10^{-2}$				k/e (Sal.f.) $= 14 \cdot 10^{-2}$		

Table 3. Acid hydrolysis. 1.0 N HCl.

Glucoside 0.007 *M*. 60°. Complete hydrolysis 10.22 ml $Na_2S_2O_3$ ~ 4.50 mg glucose

A. *o,o'*-Xylenol-glucoside				B. Mesitol-glucoside			
t min	ml $Na_2S_2O_3$	$c_{end} - c_t$ mg glucose	$k \cdot 10^4$	*t* min	ml $Na_2S_2O_3$	$c_{end} - c_t$ mg glucose	$k \cdot 10^4$
0	—	4.50	—	0	—	4.50	—
15	0.50	4.28	14.5	15	0.51	4.27	15.2
30	1.04	4.03	16.0	30	1.23	3.95	18.9
50	1.80	3.70	17.0	50	1.94	3.63	18.7
80	1.96	3.63	11.7	80	3.13	3.10	20.2
120	2.47	3.40	10.0	120	3.28	3.03	14.3
180	2.90	3.21	(8.0)	180	3.73	2.83	(11.2)
		mean value	13.8			mean value	17.5

Table 4. Acid hydrolysis. 0.5 N HCl.

Glucoside 0.007 *M*. 60°. Complete hydrolysis 10.22 ml $Na_2S_2O_3 \sim 4.50$ mg glucose.

A. *o, o'*-Xylenol-glucoside				B. Mesitol-glucoside			
t min	ml $Na_2S_2O_3$	$c_{end} - c_t$ mg glucose	$k \cdot 10^4$	*t* min	ml $Na_2S_2O_3$	$c_{end} - c_t$ mg glucose	$k \cdot 10^4$
0	—	4.50	—	0	—	4.50	—
15	0.23	4.40	6.5	15	0.29	4.37	8.5
30	0.47	4.29	6.9	30	0.51	4.27	7.6
60	0.86	4.12	6.4	60	0.98	4.06	7.5
90	1.17	3.98	5.9	90	1.24	3.95	6.3
150	1.87	3.66	6.0	150	1.97	3.62	6.3
210	2.65	3.32	6.3	210	2.58	3.26	6.7
300	3.16	3.09	5.4	300	3.04	3.14	5.2
		mean value	6.2			mean value	6.9

In Table 5 we have collected the velocity constants found by Scheiber [1], recalculated by us so that they are comparable with the constants found here and in previous papers by Veibel *et al.* [7,10,11]. Scheibers constants for the acid hydrolysis are determined at 61.2° (boiling chloroform). Veibel *et al.* have determined the velocity constants at different temperatures and have calculated the heat of activation, so that the values for phenol- and *o*-cresol-*β*-D-glucoside at 61.2° may be calculated. For *o,o'*-xylenol- and mesitol-*β*-D-glucoside, too, we have calculated the velocity constants at 61.2°, using the same heat of activation as found for phenol- and *o*-cresol-glucoside.

Scheibers constants for the enzymic hydrolysis are determined at 30° in acetate buffered solution at pH 5.0. The constant for *o*-cresolglucoside is directly comparable with the constant determined by Veibel and Lillelund [7], as the value found by them for a 0.040 *M* solution may be recalculated to the concentration 0.052 *M* used by Scheiber by application of the formula $k(K_m + c) = \text{const.}$, K_m being the Michaelis-constant, 0.027 (acetate, pH 5.0). Veibel, Møller and Wangel [10] have found the value $18.1 \cdot 10^{-2}$ for the velocity constant of hydrolysis for phenolglucoside, using a phosphate-citrate buffer at pH 6.0. According to the findings of Veibel and Lillelund [7] the velocity constant in acetate buffer at pH 5.0 is some 30 % higher than in phosphate-citrate buffer at pH 6.0. Veibel and Hjorth [11], on the other hand, found for 0.0400 *M* solutions in phosphate-citrate buffer at pH 4.4 $k = 43.6 \cdot 10^{-2}$, $K_m = 0.10$, from which is calculated $k = 40.0 \cdot 10^{-2}$ for 0.052 *M* solutions.

Table 5. Velocity constants for the hydrolysis of different β-D-glucosides.

Aglucon	Enzymic, 30°		Acid		
	Scheiber	Veibel	Scheiber 61.2°	Veibel 60°	Veibel recalc. 61.2°
	$10^2 \cdot k/e$ (Sal.f.)		$10^4 \cdot k$	$10^4 \cdot k$	$10^4 \cdot k$
Phenol	26.2	$24<k<40$	9.4	7.5	8.7
o-Cresol	343	567 (0.052 *M*) 1320 (0.007 *M*)	7.8	6.6	7.8
m-Cresol	—	—	8.3	—	—
p-Cresol	8.6	—	8.3	—	—
o,o'-Xylenol	7.4	15.3	35.3	13.8 6.2	16.5 1.0 *N* HCl 7.4 0.5 *N* HCl
o,p-Xylenol	113	—	8.1	—	—
o,o', p-Mesitol	—	14.3	— —	17.5 6.9	20.6 1.0 *N* HCl 8.2 0.5 *N* HCl

The velocity constant in acetate buffer at pH 5.0 is practically identical with the velocity constant in phosphate-citrate buffer at pH 4.4 (Veibel and Lillelund [7]). We, therefore estimate the value to be compared with Scheibers value to be somewhere between $24 \cdot 10^{-2}$ and $40 \cdot 10^{-2}$, the latter value being the more probable of the two.

Table 5 shows that when our values for the enzymic hydrolysis of *o*-cresol- and *o,o'*-xylenol-β-D-glucoside are compared with Scheibers, approximately the same ratio is found, even if the single values found by us are nearly twice as great as Scheibers values. If, however, we calculate a $k_{o\text{-cresol}}$-value for 0.007 *M* solution we find $k = 1320 \cdot 10^{-2}$ (for 0.0100 *M* solution Veibel and Lillelund [7] found experimentally $k = 1139 \cdot 10^{-2}$), and then we find the ratio *o*-cresol/*o,o'*-xylenol nearly twice as great as that found by Scheiber. In both cases a significant retarding effect of the second methyl group is found, a retarding effect more pronounced than the accelerating effect of the first methyl group ($k_{\text{phenol}} > k_{o,o'\text{-xylenol}}$).

The value for mesitol-β-D-glucoside is within the limit of error identical with the value for *o,o'*-xylenolglucoside. The retarding effect of a methyl group in *p*-position found by Scheiber for *o*-cresol-/*o,p*-xylenol-glucoside is not found for the couple *o,o'*-xylenol-/mesitol-glucoside, but on the other hand no trace of the accelerating effect found in the couple ethyl-/isopropyl-glucoside seems to exist.

For the acid hydrolysis the values for phenol- and *o*-cresol-β-D-glucoside found by Scheiber and by Veibel and Hjorth [11] are identical within the limit

of error. For *o,o'*-xylenol-β-D-glucoside Scheiber finds the velocity constant twice as great as found by us. Here, too, Scheiber has used the concentration 0.052 *M*. Veibel and Hjorth have, in experiments with 0.05 *M* and 0.1 *M* glucoside solutions, found the velocity of acid hydrolysis proportional to the concentration of glucoside, but minor deviations from the proportionality may be responsible for the difference found for 0.052 *M* and 0.007 *M* solutions.

The acid hydrolysis is found proportional to the concentration of hydrogen ions, the constants calculated for 0.5 *M* HCl as indicated by Veibel and Hjorth[11] being 6.0 and 6.9 respectively, as compared with the experimentally found values 6.2 and 6.9.

The accelerating effect of a second *o*-methyl group found by Scheiber is to a minor degree found by us, too. Only a slight retarding influence of a methyl group in *p*-position is found for the couples phenol-/*p*-cresol-, *o*-cresol-/*o,p*-xylenol-, and *o,o'*-xylenol-/mesitol-glucoside.

In the aliphatic series the effect of the second methyl group is much greater than found here in the aromatic series.

In the first paper in this series [2] we have discussed the possibility of steric hindrance being responsible for the slow enzymic hydrolysis of glucosides of tertiary alcohols. The voluminous enzyme molecules cannot attach themselves to the glucoside linkage when three alkyl groups are linked to the carbon atom responsible for the glucoside linkage. The acid hydrolysis, on the other hand, is catalysed by hydrogen ions which on account of their smaller volume are not being hindered in attacking the glucoside linkage.

A determination of the constant *B* in the Arrhenius-equation did not, however, support this idea. We, therefore, have not continued the investigation in this direction.

Lack of affinity between enzyme and substrate might be responsible for the slow enzymic hydrolysis of *o,o'*-xylenol-β-D-glucoside. For trimethylcarbinol-β-D-glucoside Veibel [12] found that the extremely slow hydrolysis was partially due to failing affinity, the Michaelis-constant being 1.46. For *o*-cresol-β-D-glucoside we have, as indicated above, found the value 0.027 for the Michaelis-constant. A value for the Michaelis-constant for *o,o'*-xylenol-glucoside may be estimated from the velocity constants for 0.052 *M* (Scheiber) and 0.007 *M* solutions. From the equation $k(K_m + c) = \text{const.}$ the value for K_m is calculated to 0.036, *i.e.* an affinity nearly as great as that between the enzyme and *o*-cresolglucoside. Evidently, lack of affinity is not responsible for the slow hydrolysis of *o,o'*-xylenolglucoside.

Further investigations seem necessary before the question of the mechanism of hydrolysis of glucosides can be solved.

SUMMARY

The acid and the enzymic hydrolysis of *o,o'*-xylenol- and mesitol-β-D-glucoside have been investigated and the results compared with results obtained previously with phenol-, *o*-, *m*-, *p*-cresol-, *o,o'*- and *o,p*-xylenol-β-D-glucoside.

In experiments with enzymic hydrolysis an accelerating effect of one methyl group in *o*-position to the glucosidic linkage is found, but if two methyl groups are introduced in *o*-position to the glucosidic linkage the acceleration is transformed into a retardation.

In experiments with acid hydrolysis the effect of one methyl group is a slight retardation, that of two methyl groups in *o*-position to the glucosidic linkage an acceleration of the velocity of hydrolysis.

The effect of a methyl group in *p*-position is for enzymic hydrolysis a retardation of the velocity of hydrolysis. For acid hydrolysis a hardly discernable retardation is found.

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