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A NON-HYDROGEN-BONDING ROLE FOR THE 4-HYDROXYL GROUP OF D-GALACTOSE IN ITS REACTION WITH D-GALACTOSE OXIDASE

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ABSTRACT

Several 4-deoxy analogs of methyl β-D-galactopyranoside are oxidized by D-galactose oxidase. The rates associated with their various, axially attached 4-substituents follow the sequence $OH > NH_2 > F \gg Cl > H$; these differences are attributed mainly to variations in K_m. Other 4-deoxy analogs, namely, the 4-azido-4-deoxy, 4-bromo-4-deoxy-, 4-deoxy-4-iodo, and 4-thio derivatives were found to be inactive. These observations indicate that the axial 4-hydroxyl group of D-galactopyranose does not play a hydrogen-bonding role primarily, but constitutes a substituent of a size optimal for interaction with the enzyme.

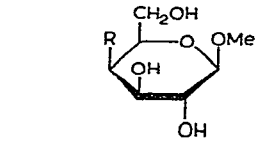
INTRODUCTION

D-Galactose oxidase (O_2-oxido-reductase 1.1.3.9.)[1–3] abstracts the pro-*S* hydrogen atom[4,5] of the primary hydroxymethyl group of D-galactose. In continuation of our studies on the stereochemistry of this reaction, the significance of the 4-hydroxyl group of the sugar as a substrate-specifying group has now been examined. Earlier workers[3,6] established that chemical modification at C-1, C-2, or C-3 of D-galactose has relatively little effect on the enzyme reaction. Thus, neither an anomeric change nor replacement of OH-1 by hydrogen is critical[3], and the reactivities of 2-deoxy-D-*lyxo*-hexose, 2-*O*-methyl-D-galactose, and 3-*O*-methyl-D-galactose show that the hydroxyl groups at C-2 and C-3 are not essential[3,6].

Methylation of OH-4, however, renders the substrate unreactive[6]; this suggests that the methoxyl substituent is too bulky to permit a satisfactory polar interaction of O-4 with the enzyme, or that OH-4 is normally a proton donor in hydrogen-bonding between the substrate and the enzyme. Alternatively, it is possible that this (or some other) modification at C-4 has a disruptive effect on the normal pattern of intermolecular, hydrophobic [7,8] interactions necessary for the ultimate dehydrogenation step.

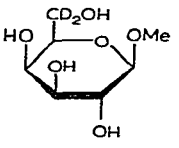
To assess (*a*) the sensitivity of the enzyme towards the size of the 4-substituent and (*b*) the type of affinity involved, several compounds have been synthesized[9] in which the 4-hydroxyl group of methyl β-D-galactopyranoside (**1**)—a particularly highly reactive substrate[3]—is replaced by various protic and aprotic groups. The

protic groups chosen were amino and thio, both of which are larger than a hydroxyl group, and the aprotic groups were hydrogen, the halogens, and the azido group, which were expected to participate in weak, or no, hydrogen bonding. Accordingly, analogs of **1** synthesized as potential substrates were methyl 4-deoxy-β-D-*xylo*-hexopyranoside (**2**), and methyl 4-deoxy-4-fluoro- (**3**) 4-amino-4-deoxy- (**4**), 4-chloro-4-deoxy- (**5**), 4-bromo-4-deoxy- (**6**), 4-deoxy-4-iodo- (**7**), 4-thio- (**8**), and 4-azido-4-deoxy-β-D-galactopyranoside (**9**). With the exception of **8** all are crystalline, and all are readily soluble in water. The reactivities of compounds **1–9** and of the 6,6-dideuterio derivative[4] (**10**) of **1** with D-galactose oxidase are herein compared.



	1	2	3	4	5
R =	OH	H	F	NH_2	Cl

	6	7	8	9
R =	Br	I	SH	N_3



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RESULTS AND DISCUSSION

Several of the D-galactopyranoside analogs, namely, the 4-fluoro (**3**), 4-amino (**4**), and 4-chloro (**5**) derivatives, and methyl 4-deoxy-β-D-*xylo*-hexopyranoside (**2**), were found to be oxidized by D-galactose oxidase, although more slowly than the unmodified glycoside (**1**) (see Table I). Of the deoxy substrates, the amino compound **4** was the most reactive, followed by compounds **3**, **5**, and **2**, in that order (see Table I). Within experimental error, these substrates obeyed the Michaelis–Menten equation, although, possibly because of its basicity, **4** deviated slightly at high concentrations (see Fig. 1); K_m and V_{max} values were not obtained for **2** because of its particularly low reactivity. No reaction was detected for compounds **6**, **7**, **8**, and **9**

TABLE I

ACTION OF D-GALACTOSE OXIDASE ON METHYL β-D-GALACTOPYRANOSIDE AND ANALOGS

Compound[a]	k (×100)[a]	$1/K_m$	V_{max}
Methyl β-D-galactopyranoside (**1**)	96.7	650	6.7
Methyl 4-amino-4-deoxy-β-D-galactopyranoside (**4**)	22.2	553	1.7
Methyl 4-deoxy-4-fluoro-β-D-galactopyranoside (**3**)	17.4	50	10.0
Methyl β-D-galactopyranoside-6,6-d_2 (**10**)	9.8	180	1.6
Methyl 4-chloro-4-deoxy-β-D-galactopyranoside (**5**)	0.4	40	3.3
Methyl 4-deoxy-β-D-*xylo*-hexopyranoside (**2**)	<0.2	—	—

[a]The 4-bromo-4-deoxy (**6**), 4-deoxy-4-iodo (**7**), 4-thio (**8**), and 4-azido-4-deoxy (**9**) analogs gave no detectable reaction.

under the conditions used for rate measurements, as well as in the presence of catalase. It is noteworthy that the unreactive compounds are also not competitive inhibitors of D-galactose oxidase; thus, the rate of reaction of methyl β-D-galactopyranoside was found to be unaffected by the bromo or iodo analog, or by the relatively reactive chloro derivative.

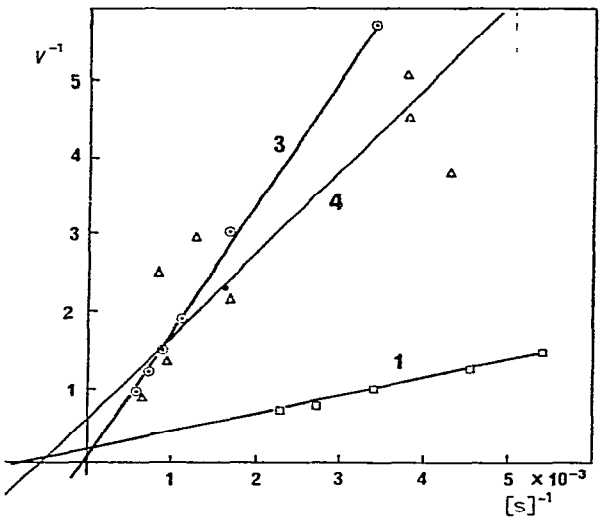


Fig. 1. Lineweaver–Burk plot for initial rate of reaction of D-galactose oxidase with methyl β-D-galactopyranoside (**1**), methyl 4-deoxy-4-fluoro-β-D-galactopyranoside (**3**), and methyl 4-amino-4-deoxy-β-D-galactopyranoside (**4**) (plotted by the least-squares-fit method).

The interaction of the enzyme with these various substrates as expressed by $1/K_m$ is greatest for methyl β-D-galactopyranoside (**1**) and lowest for **5**, although the value for the amino analog is close to that of **1** (see Table I and Fig. 1). However, $1/V_{max}$, is highest for the fluoride and lowest for the 6,6-dideuterio compound (**10**). If $1/K_m$ is regarded as being a measure of the affinity between the enzyme and the glycoside, and V_{max} a measure of the rate of decomposition of the enzyme–substrate complex[11], it is seen that the "natural" substrate (**1**) is not unusually effective in either context. Hence, the affinity of D-galactose oxidase for the alcohol (**1**) is only slightly greater than that for the amine (**4**). Similarly, it appears that, once the enzyme–substrate complex of the fluoro derivative (**3**) has formed, its breakdown to products is even more favorable than that for methyl β-D-galactopyranoside. In general, however, the differences in oxidation rates lie mainly in the K_m term; variations in V_{max} are relatively uniform. The characteristics of **10** (see Table I) are particularly noteworthy, inasmuch as this compound must satisfy the steric requirement of the enzyme in the region of C-4 as fully as does methyl β-D-galactopyranoside itself. It is clear from these data that the impact of the isotope effect on the D-galactose oxidase reaction[4] is manifested equally prominently on both of these descriptors of the reaction kinetics.

Two properties of the 4-hydroxyl group of D-galactose may be considered in relation to its role as a determinant in the dehydrogenation of the sugar by D-galactose oxidase: namely, its ability to form a hydrogen bond, and the size of the group.

The fact that methyl 4-deoxy-*β*-D-*xylo*-hexopyranoside (**2**) reacts at all, even though very slowly, suggests that OH-4 of D-galactose does not have an essential, hydrogen-bonding function, as it is improbable that a hydrogen atom bonded to a carbon atom can participate in hydrogen bonding. Furthermore, as a fluorine atom bonded to a carbon atom cannot act as a hydrogen acceptor[12], the relatively high reactivity of the 4-deoxy-4-fluoro derivative (**3**) also indicates that hydrogen-bonding is not a prerequisite for the oxidation. The other halogens are known to engage in weak, intramolecular hydrogen-bonding, as in the *ortho*-halophenols[13], but only the chloro analog (**5**) shows a detectable reaction with the enzyme, and **5** is far less reactive than **3**.

These various data concur, therefore, in showing that hydrogen bonding is not an important function of the 4-hydroxyl group in the oxidation of D-galactose. It is proposed, rather, that the size of the 4-substituent is a much more critical factor.

A comparison of the reactivities of the D-galactoside and its 4-deoxy analogs relative to the molecular dimensions at C-4 is presented in Fig. 2, using bond lengths and substituent volumes. The former are expressed as the lengths of C–X, where X

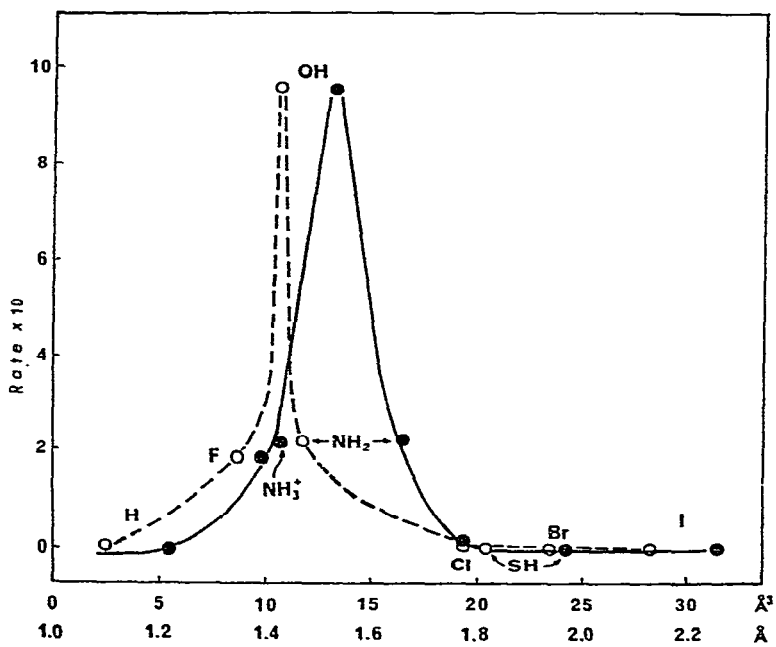
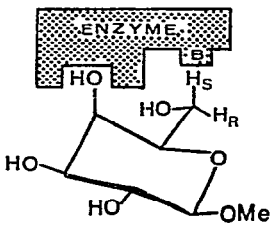


Fig. 2. Effect of size of substituent at C-4 of methyl *β*-D-galactopyranoside (**1**) and its 4-deoxy analogs **2** to **9** on the rate of reaction with D-galactose oxidase. The upper scale of the abscissa gives substituent volumes (Å^3) (dark circles) whereas the lower scale gives the C–X (X = O, H, F, etc.) bond-length[15] (in Å). For compound **4**, the volume for a protonated amino group[14] is used (as the reaction medium was at pH 7.0). These estimates of volume make no allowance for hydration[14].

is the central atom of the group bonded to the carbon atom. Although this does not take account of the hydrogen atom of the hydroxyl, amino, or thiol group (but corresponds closely to the relative radii of the other groups), the discrepancy is probably no larger than the variation found[14] in estimates of the effective volumes of such substituents. In any event, both types of measurement lead to similar conclusions. It is seen that the hydroxyl group is at the apex of each curve: on passing from the hydrogen compound (2) through the fluoro compound (3) to the "ideal" hydroxyl compound, the rate increases and then falls off with increasing size of substituent. That is, the closer in size is the substituent to the hydroxyl group, the more reactive is the substrate.

The effect of having a smaller or larger substituent at C-4 is, presumably, so to alter the geometry of interaction that sites normally juxtaposed in their proper orientation are distorted. As visualized in **11**, when X = OH, there is optimal alignment between the basic group (B:) on the enzyme surface and the reactive hydrogen



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atom (H_s) on C-6. However, when X is larger (*e.g.*, iodine), group B will be ineffectively remote from H_s; when smaller, the substituent itself will experience poor contact with the enzyme surface. Accordingly, those substituents (H, F, NH_2, Cl) which, with various efficiencies, permit the abstraction of H_s provide a measure of the range of induced fit[16] possible for D-galactose oxidase. In terms of the theory[17] of "orbital steering", these rate differences may be ascribed to variations in the efficiency of overlap between the orbitals of reacting groups during the transition state.

In summary, then, it appears that the 4-hydroxyl group of D-galactose and the 4-substituent of certain D-galactopyranose derivatives serves as a filler, or anchor, for a particular cleft on the surface of D-galactose oxidase. The geometric requirements of this association are not extremely rigorous and, hence, the substituent at C-4 of the sugar molecule can be modified substantially without completely disrupting the enzyme–substrate interaction.

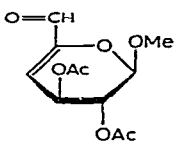
EXPERIMENTAL

General. — The 4-deoxy analogs of methyl β-D-galactopyranoside were synthesized and characterized as described in an accompanying article[9]. T.l.c. plates were

prepared with Silica Gel G, the chromatograms were developed with 3:2:1 (v/v) propyl alcohol–ethyl alcohol–water, and visibilized with (2,4-dinitrophenyl)hydrazine in ethanol containing phosphoric acid[18] or with 5% sulfuric acid in ethanol. Solutions were evaporated *in vacuo* at 45°.

Rates of oxidation by D-*galactose oxidase.* — Kinetic measurements were conducted with "Galactostat" (Worthington Biochemical Corporation). The Galactostat solution consisted of D-galactose oxidase (20 units), peroxidase (2.5 mg), and the chromogen *o*-tolidine (2.5 mg) in 0.01M phosphate buffer (50 ml; pH 7.0). Portions (1.5 ml) of this solution were added to the substrate solution (2 ml) having concentrations ranging from 300μM to 1.3mM for methyl β-D-galactopyranoside (**1**) and its dideuterio analog (**10**), and from 500μM to 10.4mM for the deoxy substrates **2**–**9**. Enzyme-reaction mixtures were incubated at 37°, and the absorbance was monitored at 420 nm with a Beckmann spectrophotometer coupled to a Gilford automatic recording accessory. Plots of absorbance *versus* time were close to straight lines over the interval 2–20 min. Initial rate-constants (k) were estimated from the gradients of these plots, and the values of K_m and V_{max} through use of the Lineweaver–Burk plot[10].

Examination of the reaction products. — *A. Oxidation of methyl* β-D-*galactopyranoside* (**1**). Methyl β-D-galactopyranoside (40 mg) was dissolved in phosphate buffer (4 ml; 0.2M, pH 7.0) and incubated with D-galactose oxidase (5 mg, 125 units) and catalase (Worthington Biochemical Corporation) (5 mg) for 3 h at 37°. A mixture of Amberlite 1R-120 (H^+) and Dowex-1 (HCO_3^-) resins was added to the digest with stirring, the suspension was filtered, and the filtrate concentrated and then lyophilized. The product (36 mg) was treated with pyridine (0.8 ml) and acetic anhydride (0.4 ml) for 18 h at room temperature, the mixture was evaporated, and the syrupy residue was examined by t.l.c.; this showed the presence of two products, each of which has been fully characterized[19], namely, methyl 2,3-di-*O*-acetyl-4-deoxy-6-*aldehydo*-α-L-*threo*-hex-4-enodialdo-1,5-pyranoside (**12**) (R_F 0.62) and an acetylated dimer of methyl β-D-*galacto*-hexodialdo-1,5-pyranoside (R_F 0.27).



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B. Oxidation of methyl 4-deoxy-4-fluoro-β-D-*galactopyranoside* (**3**). The experimental procedure for the oxidation of **1** was followed, 15 mg of **3** being used. After the enzyme reaction, and processing, t.l.c. of the reaction mixture showed the presence of an α,β-unsaturated aldehyde (presumably **12**) (R_F 0.62) as the major fraction, unreacted **3** (R_F 0.46) and an unidentified compound (R_F 0.29).

C. Oxidation of methyl 4-amino-4-deoxy-β-D-*galactopyranoside* (**4**). The experimental procedure in Section *A* was used. T.l.c. examination of the products showed the

presence of a slow-moving compound having aldehydic staining properties; its low mobility suggested that the aldehyde was involved in an intermolecular association. The α,β-unsaturated aldehyde **12** appeared to be absent.

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