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BINDING OF ALKYL 1-THIO-β-D-GALACTOPYRANOSIDES TO β-D-GALACTOSIDASE FROM *E. coli*

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ABSTRACT

The binding of a series of alkyl 1-thio-β-D-galactopyranosides to β-D-galactosidase from *E. coli* has been investigated. The inhibition constants were compared to the partition coefficients for the transfer of these substrate-analogues from water to 1-octanol. The relationships between the observed binding-constants and the partition coefficients indicate that part of the aglycon group binds to a hydrophobic area that is limited in relation to the length of hydrocarbon chain that can be accommodated. Outside this area, the hydrocarbon chain is only partially desolvated. The main driving-force for binding of the aglycon group is the increase in entropy resulting from the return of water molecules from the more-organized layer around the solute molecule to the bulk-water phase.

INTRODUCTION

The enzyme β-D-galactosidase from *E. coli* (EC 3.2.1.23) has been the subject of several recent investigations[1–5] and a recent review[6]. However, relatively little is known about the binding of substrate and inhibitor molecules to the active site of the enzyme. In most cases, the Michaelis–Menten constant K_m is a complex constant which shows no simple relation to the binding constant K_s. Therefore, we decided to study the binding of galactopyranosides by using the corresponding 1-thio-β-D-galactopyranosides, which are fully competitive inhibitors. In the first place, we used a series of alkyl 1-thio-β-D-galactopyranosides to inhibit the β-D-galactosidase-catalyzed hydrolysis of a good substrate, in order to calculate the inhibition constant K_i. Since we suspected that, for these galactosides, hydrophobic interactions would be part of the driving forces, we also studied the influence of the aglycon group on the transfer of these galactopyranosides from an aqueous to a hydrophobic phase (1-octanol). By comparing the results of this model system to the K_i values for the enzymic transfer, we hoped to gain more insight into the binding process.

RESULTS AND DISCUSSION

Alkyl β-D-galactopyranosides. — For a number of alkyl β-D-galactopyranosides, the partition coefficient (P) between aqueous phosphate buffer (pH 7.5; 0.1M; mM

TABLE I

PARTITION COEFFICIENTS OF ALKYL β-D-GALACTOPYRANOSIDES IN WATER–1-OCTANOL (25°)

Aglycon group	*β-D-Galactopyranosides*				*1-Thio-β-D-galactopyranosides*		
	P	*log P*	*ΔG°* (*kcal.mol*$^{-1}$)	*log P* (*alcohols*)[a]	*P*	*log P*	*ΔG°* (*kcal.mol*$^{-1}$)
Methyl	—	−2.88[b]	+3.93	−0.66	—	−2.29[b]	+3.12
Ethyl	0.005	−2.28	+3.11	−0.16	0.031	−1.51	+2.26
Propyl	0.022	−1.66	+2.26	0.34	0.066	−1.18	+1.40
Butyl	0.091	−1.04	+1.42	0.84	0.289	−0.54	+0.54
Pentyl	0.38	−0.42	+0.57	1.34	2.478	+0.39	−0.33
Hexyl	1.41	+0.16	−0.22	1.84	7.030	+0.86	−1.19
Heptyl	5.32	+0.73	−1.00	2.34	31.6	+1.50	−2.05
Octyl	29.10	+1.46	−1.99	2.84	135	+2.13	−2.91
Isopropyl	—	−1.91[b]	+2.61	0.14	0.055	−1.26	+1.71
3-Pentyl	0.16	−0.80	+1.09		0.66	−0.18	+0.25
Benzyl	—	—	—		0.341	−0.47	+0.64
2-Phenethyl	—	—	—		0.790	−0.10	+0.14
3-Phenylpropyl	—	—	—		2.388	+0.38	−0.52

[a]From Ref. 7. [b]Calculated values.

$MgCl_2$) and 1-octanol was determined at 25° (Table I). For the galactosides with n-alkyl aglycon groups, log P, and thus the change of the standard free energy $\Delta G° = -RT\ln P$, is linearly related to the number of C-atoms (N) in the chain, according to the regression function, $\log P = 3.493 + 0.614N$, with standard error of the estimate $s_{y/x} = 0.038$, standard error of the slope $s_b = 0.007$, and correlation coefficient $r = 0.999$.

From this equation, the value of log P for methyl β-D-galactopyranoside can be calculated as −2.88. To a first approximation, ΔG° for the transfer of the β-D-galactopyranose moiety can be evaluated by using N = 0 in the above equation, *i.e.*, log P = 3.49 and ΔG° ∼ +4.7 kcal/mol. The increase in ΔG° per CH_2 group ($\Delta\Delta G°/CH_2$) amounts to 0.84 kcal/CH_2 at 25°.

The log P values for the galactosides are linearly related to those for the corresponding alcohols[7] and to the Hansch π parameter[8],

$$\log P(\text{osides}) = -2.082 + 1.229 \log P(\text{alcohols}),$$

with $s_{y/x} = 0.038$, $s_b = 0.014$, and $r = 0.9996$.
This equation can then be used to calculate log P(osides) for the isopropyl derivative, for example, from the known value of log P for 2-propanol (Table I).

Alkyl 1-thio-β-D-galactopyranosides. — Table I shows values of log P for a number of alkyl and aryl-substituted alkyl 1-thio-β-D-galactopyranosides. For the 1-thio-D-galactopyranosides with an n-alkyl aglycon group (methyl to octyl), log P (and ΔG°) is again linearly related to the number of C-atoms (N) in the chain:

$$\log P = -2.922 + 0.632N, \text{ with } s_{y/x} = 0.134,\ s_b = 0.025, \text{ and } r = 0.996.$$

In this series, $\Delta\Delta G°/CH_2 = 0.87$ kcal/CH_2, and $\Delta G°$ for the transfer of 1-thio-β-D-galactopyranose is +4.0 kcal/mol.

The values of log P for the 1-thiogalactosides are linearly related to the log P values for the corresponding alcohols and to the Hansch π parameter:

$$\log P(\text{osides}) = -1.456 + 1.264 \log P(\text{alcohols}),$$

with $s_{y/x} = 0.17$, $s_b = 0.1$, and $r = 0.99$.

It cannot be proved that the difference between the values of the slope (thio series, 0.632; oxygen series, 0.614) is statistically significant. Thus, it seems more realistic to assume that $\Delta\Delta G°/CH_2$ has approximately the same value in the two series, and to accept a mean difference between the two series of ~0.9 kcal/mol, the galactosides being less hydrophobic than their 1-thio analogues.

The phenyl-substituted alkyl 1-thio-β-D-galactopyranosides belong to a different series. Log P and $\Delta G°$ are linearly related to the number of CH_2 groups, but the increase in $\Delta G°$ per CH_2 group is significantly lower, as can be seen from the equation,

$$\log P = 0.910 + 0.423N, \quad \text{with} \quad s_{y/x} = 0.047,\ s_b = 0.033,\ r = 0.997, \quad \text{and}$$

$\Delta\Delta G°/CH_2 = 0.58$ kcal mol.

Phenyl 1-thio-β-D-galactopyranoside itself does not belong to the above series. The observed hydrophobicity (log P = −0.56; $\Delta G° = +0.77$ kcal/mol) is higher than the theoretical value (log P = −0.91; $\Delta G° = +1.24$ kcal/mol) calculated from the above equation for arylalkyl galactosides.

From the results reported above, some conclusions can be drawn. (*1*) The transfer of the glycon moiety of the substrate to the octanol phase is an endergonic process ($\Delta G°$ ~4 kcal/mol). (*2*) Special effects of the glycon group on the relative hydrophobicity of the whole galactopyranoside molecule are not observed. The hydrophobicity of the galactosides increases in a regular manner with increasing hydrophobicity of the aglycon group. (*3*) For n-alkyl β-D-galactopyranosides (and the 1-thio derivatives), the relative hydrophobicity increases linearly with the number of CH_2 groups in the aglycon chain. The value of $\Delta\Delta G°/CH_2$ (0.8 kcal/CH_2) is in accordance with published values[9,10] for analogous series. 1-Thiogalactosides are more hydrophobic (~0.9 kcal/mol) than the corresponding galactosides.

The β-D-galactopyranosides are molecules containing both hydrophilic and hydrophobic groups. The presence of the latter groups within the aqueous medium is possible because they are bound to the hydrophilic groups. More-extensive water organization is enforced around the hydrophobic groups, thereby decreasing the entropy of the surrounding water layer. The major driving-force for the transfer of the aglycon group of the galactoside to the octanol phase is thus the increase in entropy resulting from the return of the water molecules from the lower entropy state to the less organized bulk-water. Since, in our series, the same hydrophilic group is built into all molecules, the relative hydrophobicity of the galactosides must reflect this increasing positive change of the entropy.

Binding of alkyl 1-thio-β-D-galactopyranosides to β-D-galactosidase. — The 1-thio-β-D-galactopyranosides were used as inhibitors of the β-D-galactosidase-catalysed hydrolysis of *o*-nitrophenyl β-D-galactopyranoside at pH 7.5. As expected, the 1-thio-D-galactosides behaved as fully competitive inhibitors. Consequently, the experimentally determined inhibition constant (K_i) is the true equilibrium constant for the process $E+I \rightleftharpoons EI$, *i.e.*, the transfer of the free inhibitor molecule from the aqueous phase to the active site of the enzyme, resulting in the formation of the enzyme–inhibitor complex. The value of $\Delta G°$, calculated from K_i, is a measure of the difference in free energy between the enzyme–inhibitor complex and the free enzyme plus free inhibitor molecule.

Table II includes the values of K_i (association constant at 25°) and $\Delta G°$ for the alkyl 1-thio-β-D-galactopyranosides, and also the K_m values for the corresponding β-D-galactopyranosides[11]. The close parallelism between the K_i and K_m values corroborates our earlier suggestion[11] that, for the slow-hydrolyzing alkyl β-D-galactopyranosides, K_m is a good approximation of K_s. This earlier suggestion was based on the experimental observations[11] that (*1*) the maximal rate V ($=k_2E_T$) is nearly unaffected by the nature of the alkyl chain, whereas K_m increases by a factor of ~100 (Table II); and (*2*) the value of k_2 itself is small ($\sim 10^2$ sec^{-1} for methyl β-D-galactopyranoside), and slightly decreases with increasing chain-length (in contrast to the K_m value) to $\sim 0.7 \times 10^2$ sec^{-1} for the octyl derivative. These experimental findings suggest that in the equation $K_m = (k_{-1}+k_2)/k_1$, k_2 is small with regard to k_{-1}, so that $K_m \approx k_{-1}/k_1 \approx K_s$.

In contrast to what was found in the water–octanol system, neither log K_i nor log K_m (or $\Delta G°$) is linearly related to the number of C-atoms in the aglycon group

TABLE II

K_i AND K_m VALUES FOR ALKYL β-D-GALACTOPYRANOSIDES AT pH 7.5 AND 25°

No.	*Aglycon group*	*Alkyl 1-thio-β-D-galactopyranosides*		*Alkyl β-D-galactopyranosides*	
		K_i (mM^{-1})	$-\Delta G°$ (*kcal.mol*$^{-1}$)	K_m (mM^{-1})	$-\Delta G°$ (*kcal.mol*$^{-1}$)
1	Methyl	0.59	3.78	0.12	2.84
2	Ethyl	3.72	4.87	0.23	3.22
3	Propyl	10.0	5.46	0.96	4.06
4	Butyl	13.0	5.61	2.57	4.65
5	Pentyl	17.9	5.80	4.22	4.94
6	Hexyl	27.2	6.05	7.35	5.27
7	Heptyl	35.0	6.20	8.55	5.36
8	Octyl	50.2	6.41	12.82	5.60
9	Isopropyl	3.8	4.88		
10	3-Pentyl	44.9	6.35		
11	Benzyl	302	7.48		
12	2-Phenethyl	1,032	8.20		
13	3-Phenylpropyl	204	7.25		
14	4-Nitrobenzyl	115	6.90		

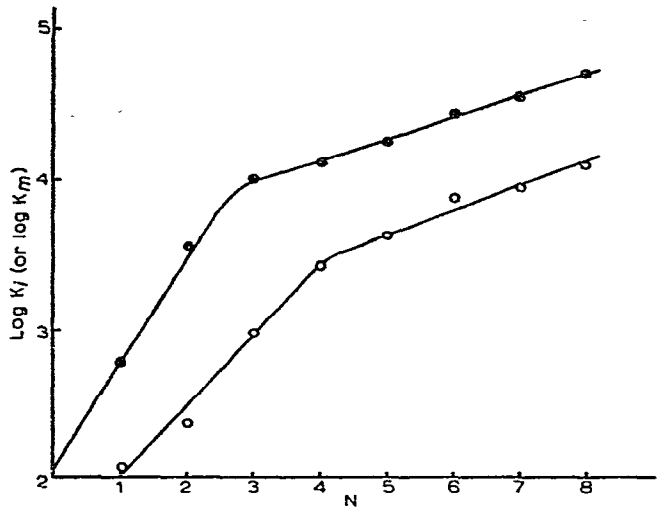

Fig. 1. Log K_i *versus* the number of carbon atoms (N): ●, alkyl 1-thio-β-D-galactopyranosides; ○, alkyl β-D-galactopyranosides.

throughout the whole n-alkyl series. As shown in Fig. 1, log K_i increases strongly and regularly up to three C-atoms. Thereafter, the increase of log K_i is still regular, but far less pronounced.

Graphical extrapolation (N = 0) of the function lines in Fig. 1 yields log K_i = 2.10 or $\Delta G°$ (25°) = −2.86 kcal/mol for 1-thio-β-D-galactopyranose, and log K_m = 1.6 or $\Delta G°$ (25°) = −2.18 kcal/mol for β-D-galactopyranose. A direct determination of K_i for D-galactose yielded $K_i = 45\ M^{-1}$ at 25° or $\Delta G° = -2.25$ kcal/mol. Thus, the transfer of the D-galactose moiety of the substrate to the active site of the enzyme is an exergonic process, in contrast to the endergonic transfer to the hydrophobic octanol phase. It is difficult to see how exergonic transfer would be possible if the glycon site of the active centre were strongly hydrophobic. It seems more logical to assume that this site is built up from hydrophilic groups, which can bind the hydroxyl groups of the glycon ring (*e.g.*, by hydrogen bonds).

However, it is clear from the above results that part of the binding forces depends on the hydrophobic character of the aglycon group. In Fig. 2, the values of $\Delta G°$ calculated from K_i or K_m are compared with the values of $\Delta G°$ from the water–octanol system. The comparison is simple for n-alkyl 1-thio-β-D-galactopyranosides **1–3**: the slope of the function line in Fig. 2 approaches unity and shows that the two processes are very much alike. This similarity can also be seen from a comparison of the calculated regression function for thio derivatives **1–3**:

$$\log K_i = 2.220 + 0.614N, \quad \text{with} \quad s_{y/x} = 0.150,\ s_b = 0.1,\ r = 0.985, \quad \text{and}$$

$$\Delta G°/CH_2 = 0.84 \text{ kcal},$$

with the regression function for the water–octanol system:

$$\log P = -3.493 + 0.614N \text{ and } \Delta\Delta G°/CH_2 = 0.84 \text{ kcal}.$$

It seems logical to assume that the transfer of the first three CH_2-groups of the aglycon chain to the active site parallels the transfer to the octanol phase, in the sense that these groups leave the bulk-water phase and are completely buried in a hydrophobic pocket or micro-region next to the glycon site. The main driving-force would then be the increase in entropy caused by the return of water molecules from the more-ordered layer around the solute molecule to the bulk-water phase.

For thio derivatives **4–9**, the increase in $\Delta G°$ is still regular, but each CH_2-group contributes only 0.2 kcal to the overall $\Delta G°$ of transfer, as can be seen from the equation,

$$\log K_i = 3.573 + 0.140N, \quad \text{with} \quad s_{y/x} = 0.019, \; s_b = 0.006, \; r = 0.997, \quad \text{and}$$
$$\Delta\Delta G°/CH_2 = 0.19 \text{ kcal}.$$

The function line for the oxygen derivatives runs parallel (Fig. 2) to that for the thiogalactosides, and the constant difference ($\Delta\Delta G°$) is ~0.87 kcal/mol. Comparison with the value for the water–octanol system ($\Delta\Delta G°$ ~0.9 kcal/mol) clearly indicates that the better binding of these thio derivatives is caused by their higher intrinsic hydrophobicity, and not by a different manner of binding.

The function lines for the lower homologues of the two series are not parallel (Figs. 1 and 2), and the difference ($\Delta\Delta G°$) between the series decreases with decreasing chain-length. A possible reason seems to be the steric limitation and/or heterogeneity of the active site, in contrast to the homogeneity of the octanol phase. By replacing the glycosidic oxygen atom by a sulfur atom, bond angles and bond lengths are altered. Although this will have an effect on the intrinsic hydrophobicity of the

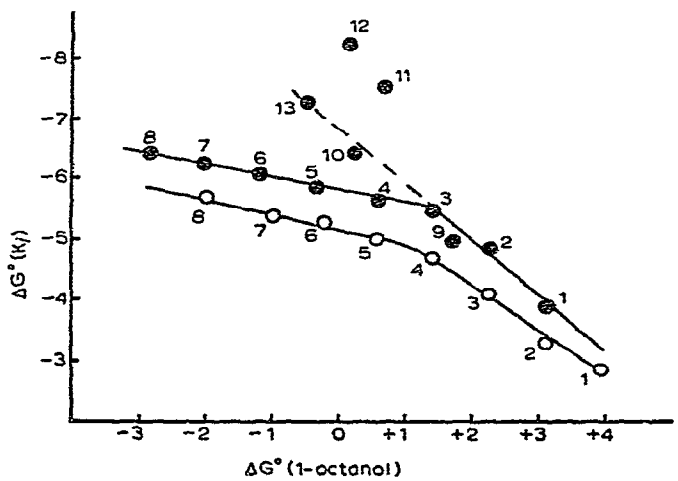

Fig. 2. $\Delta G°$ calculated from K_i *versus* $\Delta G°$ for transfer in the water–octanol system (numbers as in Table II): ●, alkyl 1-thio-β-D-galactopyranosides; ○, alkyl β-D-galactopyranosides.

galactosides, the effect will be constant and independent of the number of C-atoms in the aglycon chain, as long as the hydrophobicity is measured in the octanol–water system. This is the reason why a constant difference of 0.9 kcal/mol between the thio and oxygen series was accepted as being more realistic.

However, when the transfer occurs to a sterically limited, hydrophobic micro-region in the active site, substitution of the glycosidic oxygen atom by a sulfur atom may have more-pronounced effects on K_i than on the transfer of the substrate to the octanol phase. A clear indication of these special effects is the experimental finding that the strong increase in $\Delta G°$ (K_i) ends with propyl 1-thio-β-D-galactopyranoside, but continues up to the butyl derivative in the oxygen series. A first consequence of the heterogeneity of the active site thus seems to be that the terminal CH_3 group of the butyl aglycon-chain in the thio series only partially enters the hydrophobic pocket, whereas the same group in the oxygen series is more completely desolvated. A further consequence will be that, due to the differences in bond angles and lengths, the position of the CH_2 groups next to the glycosidic bond will be different in the two series. It would be highly unreasonable to assume that the positions shift in a regular way, in the sense that, for example, the third C-atom in the aglycon chain of an oxygen derivative positions itself in the same place as the second C-atom in the aglycon of the thio analogue. Only in this (improbable) case would the function lines for the two series run parallel. If, however, due to their different positions, some of the CH_2 groups of members of the oxygen series are less completely transferred from the bulk-water phase to the restricted hydrophobic micro-region, the K_i values will change in a rather unpredictable way, and the strict parallelism between the two series may be destroyed. For the four lower members of the oxygen series, the main increase in $\Delta\Delta G°/CH_2$ amounts to 0.63 ± 0.06 kcal/mol, which is less than the value for the three lower members of the thio series (0.84 kcal/mol) and in agreement with a less-complete transfer. For the higher homologues of both series, $\Delta\Delta G°$ amounts to 0.2 kcal/mol. The simplest explanation seems to be that these CH_2 groups are only very partially desolvated and take up a position outside the hydrophobic pocket in a region in which the water was still highly organized (but less than in the bulk-water phase). In this case, the net increase in entropy (and free energy) will be only a fraction of the value calculated from the water–octanol system. According to Webb[12], the free-energy change for removing two water molecules per CH_2 group is 0.2 kcal. However, it is difficult to prove that the agreement with our value is more than a coincidence.

Isopropyl 1-thio-β-D-galactopyranoside binds as strongly as the ethyl derivative, and thus less strongly than could be expected from its octanol-hydrophobicity. The most probable explanation would be that the third C-atom, because of the branched structure of the aglycon group, remains in contact with the water phase along the surface directed outward from the hydrophobic site. Such a situation cannot occur in the water–octanol system, and thus the octanol-hydrophobicity will be higher.

From Fig. 2, it can be seen that $\Delta G°$ (K_i) for the 3-pentyl 1-thio-β-D-galacto-pyranoside is slightly lower (the point lies lower than the extrapolated line) than

could be expected if the 3-pentyl group were buried completely in the hydrophobic pocket, as was assumed for the aglycon groups of derivatives **1–3**. Again the explanation would be that the hydrophobic region accepts three C-atoms, and that the other two, because of the branched structure, remain in contact with water. In this model, the 3-pentyl derivative can bind more strongly than the 1-pentyl galactoside (as was found experimentally), notwithstanding the fact that its octanol-hydrophobicity is lower than that of the 1-pentyl derivative. When 1-pentyl 1-thio-β-D-galactopyranoside is transferred to a hydrophobic site in such a way that the terminal methyl group and one CH_2-group remain in contact with the water phase, these groups can be completely surrounded by water molecules. However, this is not possible for 3-pentyl 1-thio-β-D-galactopyranoside, as the two CH_2-groups can only be partially surrounded by water molecules because of the branched-chain structure of the aglycon group. Consequently, compared to the complete transfer in the water–octanol system, the 1-pentyl derivative can lose the contribution of two CH_2-groups when binding to the hydrophobic region of the enzyme, whereas such a loss is not possible for the 3-pentyl galactoside. Thus, the higher binding-constant of the 3-pentyl derivative seems to be in agreement with the hypothesis of a limited hydrophobic region in the active site.

For some of the alkyl 1-thio-β-D-galactopyranosides, K_i was determined at five different temperatures, and the enthalpy ($\Delta H°$) and entropy ($\Delta S°$) of binding were calculated (Table III). Because of the large standard errors for K_i and the small influence of temperature on K_i, only large differences in $\Delta H°$ and $\Delta S°$ are meaningful. For n-alkyl galactosides, the value of $\Delta H°$ is negative and nearly constant. Notwithstanding the fact that the process represents the formation of the enzyme–inhibitor complex from free enzyme and free inhibitor, the ΔS values are positive and seem to increase with increasing chain-length. These findings indicate that the main driving-force for the "binding" of the aglycon group is the increase in entropy resulting from the return of water molecules from the highly organized layer around the aglycon group to the bulk-water phase.

As can be seen from the data in Table II and Fig. 2, the phenyl-substituted alkyl 1-thio-β-D-galactopyranosides are very good inhibitors, and their binding constants are higher than can be normally expected from their hydrophobicity in the

TABLE III

INFLUENCE OF THE TEMPERATURE ON K_i

Aglycon group	K_i (mM^{-1})				$\Delta H°$ ($kcal.mol^{-1}$)	$\Delta S°$ (20°) ($cal.degree^{-1}.mol^{-1}$)
	15°	*20°*	*30°*	*35°*		
Methyl	0.73	0.69	0.54	0.49	3.6 ±0.2	+0.6 ±0.8
Propyl	12.0	10.9	9.32	7.64	3.7 ±0.2	+5.7 ±1.7
Pentyl	23.5	18.4	17.6	14.3	3.7 ±0.8	+7.0 ±2.9
Heptyl	40.0	37.2	33.1	29.4	2.6 ±0.3	+12.2 ±0.9
Benzyl	512	380	234	209	8.0 ±0.5	−2.0 ±1.9
2-Phenethyl	1,833	1,314	811	533	10.6 ±0.7	−8.2 ±2.4

water–octanol system. However, log K_i (or $\Delta G°$) does not show the regular increase found in the octanol system. Inspection of the values of $\Delta H°$ and $\Delta S°$ (Table III) suggests the reason for this special behaviour. The absolute value of $\Delta H°$ is significantly higher, but $\Delta S°$ is negative. The simplest explanation would be that, for these derivatives, active binding takes place between the phenyl ring and an amino acid side-chain of the active site. Because such direct interaction requires a stricter orientation of the binding groups, it can explain the unusual influence of the number of CH_2 groups in the aglycon group and the fact that the K_i value of 3-phenylpropyl 1-thio-β-D-galactopyranoside is much lower than that for the phenethyl derivative. The addition of a third CH_2-group probably prevents the correct orientation of the phenyl ring, so that direct interaction is no longer possible (or becomes very weak). Since direct interaction will result in a more-specific orientation of the aglycon group, it is possible that the interaction will prevent some C-atoms of the aglycon from entering the hydrophobic microregion. In that case, it is impossible to compare $\Delta H°$ of binding with the normal hydrophobicity in the water–octanol system, or to deduce to what extent the positive $\Delta S°$, caused by the return of water molecules to the bulk-water, contributes to the overall $\Delta S°$. The nature of the interaction forces between the phenyl ring of the aglycon and the group of the active site is not known, but very probably they are π–π or charge-transfer interactions with an aromatic side-chain of an amino acid.

Influence of pH. — Using *o*-nitrophenyl β-D-galactopyranoside (ONPG) as a substrate, butyl 1-thio-β-D-galactopyranoside as a competitive inhibitor, and butyl β-D-galactopyranoside as a co-substrate, the apparent (pH-dependent) maximal velocity (V'), and Michaelis–Menten (K'_m) and inhibition (K'_i) constants were determined at 25° and various pH values. Since the rate of hydrolysis of butyl β-D-galactopyranoside is very low compared to the rate of hydrolysis of ONPG, the butyl derivative can be considered, to a good approximation, as a competitive inhibitor of the ONPG reaction. The experimental values (K' expressed as an association constant: $K' = 1/K'_m$) are collected in Table IV.

TABLE IV

INFLUENCE OF pH ON THE INHIBITION CONSTANTS AT 25°

pH	10^8 *V'* (*mol.u*$^{-1}$.*min*$^{-1}$) (*1*)[a]	K'_m (*mM*) (*1*)	*K'* (M^{-1}) (*1*)	*log* 10^5 *V'*/K'_m (*1*)	K'_i (M^{-1}) (*2*)	K'_i (M^{-1}) (*3*)	*Δ log* K'_i (*4*)
5.50	67.9	0.245	4,081	2.44	7,143	1,172	0.785
5.83	128.5	0.137	7,300	2.97	13,043	2,580	0.703
6.09	133.1	0.131	7,634	4.01	12,825	2,650	0.684
6.89	134.4	0.089	11,160	4.18	14,880	3,260	0.659
7.78	87.3	0.090	11,110	3.99	9,931	2,390	0.619
8.89	58.6	0.197	5,076	3.47	3,300	760	0.637
9.39	30.2	0.233	4,290	3.11	1,873	416	0.654

[a](1) *o*-nitrophenyl β-D-galactopyranoside; (2) butyl 1-thio-β-D-galactopyranoside; (3) butyl β-D-galactopyranoside; (4) $\Delta \log K'_i = \log K'_i$ (thio) $- \log K'_i$ (oxygen derivative).

Theory shows[5,13,14] that, for the proton-transfer equilibria,

(*a*) free enzyme: $EH_2 \overset{K_b}{\rightleftharpoons} EH \overset{K_a}{\rightleftharpoons} E$, and

(*b*) enzyme inhibitor complex: $EH_2I \overset{K_{b,i}}{\rightleftharpoons} EHI \overset{K_{a,i}}{\rightleftharpoons} EI$,

the apparent kinetic parameters are given by the equations,

$$\log V'/K'_m = \log V/K_m + \log\left[1 + \frac{K_a}{H} + \frac{H}{K_b}\right] = \log V/K_m + \log f, \quad \text{and}$$

$$\log K'_i = \log K_i + \log \frac{1 + \frac{K_a}{H} + \frac{H}{K_b}}{1 + \frac{K_{a,i}}{H} + \frac{H}{K_{b,i}}} = \log K_i + \log \frac{f}{f_i}.$$

Fig. 3 illustrates the correlation between $\log V'/K'_m$ (or $\log K'_i$) and pH. The data are insufficient to calculate the respective pK_a values, but the $\log V'/K'_m$ dependence is in agreement with the findings of Tenu *et al.*[5], who proposed two dissociable groups with pK_a values of ~6 and 8, respectively.

Since $\log K'_i = \log K_i + f/f_i$, the dependence of $\log K'_i$ on pH indicates that $\log f$ differs from $\log f_i$, which is only possible when the pK value of one or both of the dissociable groups is changed by the binding of the inhibitor to the active site. This is illustrated in Fig. 3 by the purely hypothetical case: $K_i = 10^5 M^{-1}$, $K_a = 10^{-8.5}$,

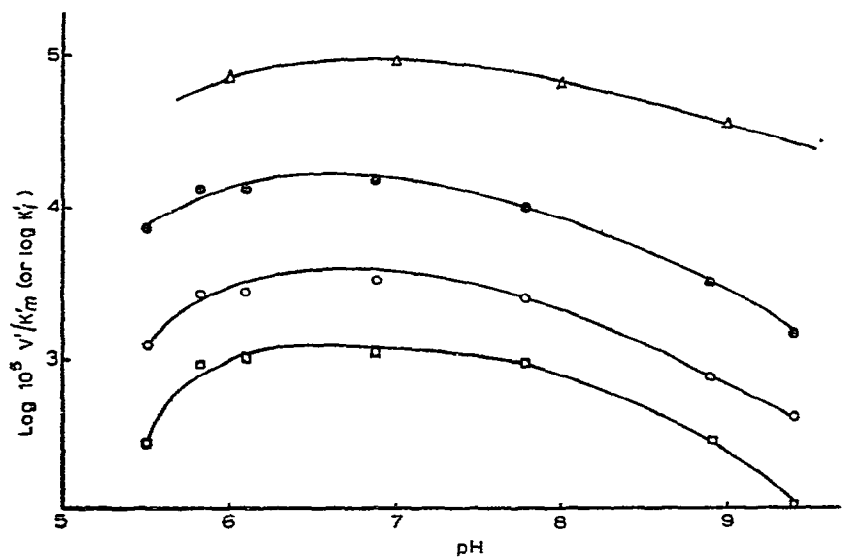

Fig. 3. pH-Dependence of K_i: ●, butyl 1-thio-β-D-galactopyranoside; ○, butyl β-D-galactopyranoside; □, $\log 10^5\ V'/K'_m$; △, calculated hypothetical line.

$K_b = 10^{-5.6}$, $K_{a,i} = 10^{-8}$, and $K_{b,i} = 10^{-6}$. Of course, by changing (more or less) one of the pK values, one can obtain more or less curvature. However, our data are insufficient to allow a quantitative calculation of the shift in pK. Careful inspection of Fig. 3 reveals that the function line for butyl 1-thio-β-D-galactopyranoside is not completely parallel to the line for the oxygen homologue. In Table IV, the difference $\Delta \log K_i' = \log K_i'(\text{thio}) - \log K_i'(\text{oxygen})$ is shown. The regularity (a decrease followed by an increase) and the order of magnitude seem to indicate (although it cannot be proved) that this difference is real, and not due to experimental error. This would mean that the binding of a thiogalactoside has a slightly different influence on pK than the binding of the oxygen analogue. This conclusion seems acceptable, because of the difference between the two structures.

Conclusions. — From the results obtained, it can be concluded that the glycon moiety of β-D-galactopyranosides binds to a rather hydrophilic part of the active site, whereas the aglycon group binds to a more hydrophobic area. This area is circumscribed and limited in the length of hydrocarbon chain that can be accommodated. Outside this area, the hydrocarbon chain is only partially desolvated. The main driving-force for binding of the aglycon group is the increase in entropy arising from the return of water molecules to the bulk-water phase. The better binding of 1-thio-β-D-galactopyranosides results from their higher intrinsic hydrophobicity, but not from a different way of binding. There is no evidence that the binding process is accompanied by a major change in conformation, nor that it is altered by a change of pH.

EXPERIMENTAL

The substrates and inhibitors were synthesized as described[15–20]. The β-D-galactosidase was a crystalline suspension from Boehringer, Lot No 6369505, and was worked-up as described[11]. All other chemicals were analytical grade. Partition coefficients were determined by adding a known volume of 1-octanol–saturated, aqueous phosphate buffer (0.1M; pH 7.5), containing a known amount of galactopyranoside, to a given volume of buffer-saturated 1-octanol. The mixture was then equilibrated with gentle agitation at 25° for 6 h. Aliquots were then removed from the aqueous phase, and the concentration of the remaining galactoside was determined. The concentration of aryl-substituted alkyl 1-thiopyranosides was determined spectrophotometrically by measuring the extinction of the aqueous solutions at 260 nm. The concentration of the other galactopyranosides was determined by measuring the concentration of D-galactose after complete acid-catalyzed hydrolysis[21] (M HCl; 80°) of the galactoside. D-Galactose was measured by the *o*-toluidine method of Winckers[22]. The relative volumes of aqueous buffer and 1-octanol were choosen in such a way that ~50% of the galactoside was transferred to the octanol phase. Standardisation of the enzyme solutions and measurements of the rate of hydrolysis of *o*-nitrophenyl β-D-galactopyranoside were performed as described previously[11]. All enzymic reaction-rates were calculated on the same

enzyme-activity basis (unit). Only initial velocities were measured and each value is the estimated mean of at least three determinations. Since all substrates followed formal Michaelis–Menten kinetics, the classical methods[13,14] for the calculation of the enzymic parameters could be used. All determinations at pH 7.5 were carried out in sodium phosphate buffer (0.1M; mM $MgCl_2$). The influence of pH on K_i was measured with the following buffer systems: pH range 5–6, sodium acetate; 6–8, sodium phosphate; 8.5–9.5, glycine. The concentration of the buffer solutions was 33mM; 145mM NaCl was added to maintain the ionic strength at 0.17 $\pm$0.02 and to have the same sodium-ion concentration of 160 $\pm$20mM, which represents saturating conditions for this activating ion; Mg^{2+} was added to mM.

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