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PURIFICATION AND PROPERTIES OF COFFEE-BEAN α-D-GALACTOSIDASE

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ABSTRACT

A purification method for α-D-galactosidase from *Coffea canephora* is described Two enzymes, α-D-galactosidases I and II, having molecular weights of 28,000 and 36,500, respectively, were found and extensively purified The reaction mechanism of α-D-galactosidase II was studied The enzyme hydrolyzed aryl and alkyl α-D-galactopyranosides and was severely inhibited by excess of these substrates No inhibition occurred with raffinose The influence of para substituents on the reaction rate of phenyl α-D-galactopyranosides, the effect of added alcohols, and the non-competitive inhibition by methyl α-D-galactopyranoside were investigated A two-step mechanism with the formation of an enzyme–galactosyl complex is proposed With aryl galactopyranosides, the reaction of the enzyme–galactosyl complex with water is rate-limiting Influences of the substituents on the inhibition constant were investigated by linear free-energy relationships, and significant correlations between this constant and electronic parameters could be calculated The influence of pH on the reaction is complex

INTRODUCTION

Although α-D-galactosidases (α-D-galactoside galactohydrolases [EC 3 2 1 22]) have been reported to occur widely in animal tissue, micro-organisms, and plants, relatively few kinetic studies have been undertaken Detailed kinetic investigations have been carried out with highly purified preparations from *Vicia faba*[1] and sweet-almond emulsin[2,3] Further details on α-D-galactosidases may be found in a recent review by Dey and Pridham[4] The present paper deals with the purification of α-D-galactosidase from *Coffea canephora* It is also an attempt, by kinetic measurements, to obtain more information about the mechanism of action of this enzyme

RESULTS AND DISCUSSION

Enzyme purification — The purification procedure is an adaptation of the method of Courtois[5] Beans (500 g) of *Coffea canephora* var. "Santos" were ground,

washed with benzene (3 l), and air-dried The coarse powder was then homogenised in 0 9% NaCl (2 5 l) and further stirred for 24 h at 4° The slurry was filtered through glass-wool and washed with cold water This yielded 2,580 ml of extract, containing 920 units of α-D-galactosidase activity Several other glycosidases were present in the extract (milliunits in parentheses) β-D-galactosidase (5,506), α-D-glucosidase (1,207), β-D-glucosidase (6,748), α-D-mannosidase (53,437), β-D-xylosidase (5,562), and *N*-acetyl-β-D-glucosaminidase (426,000) In order to test the efficiency of the purification procedure, the remaining activity of these glycosidases was measured after each step All further operations were performed between 0° and 4°, unless otherwise specified The results of each step of the procedure are summarised in Table I

TABLE I

PURIFICATION OF α-D-GALACTOSIDASE

Fraction	*α-D-Galactosidase activity (units)*	*Specific activity ($u.mg^{-1}$)*	*Total yield (%)*	*Purification (-fold)*
Raw extract	920	0 95	100	—
Step 1 $(NH_4)_2SO_4$ fraction	736	1 9	80	2
Step 2 Acetone fraction	513	22 8	56	24
Step 3 pH fractionation	468	153	51	161
Step 4 $(NH_4)_2SO_4$ fraction	460	229	50	241
Step 5 Al-oxide fraction				
pH 4 5	9	5 9	1	—
pH 5 1 (I)	120	489	13	515
pH 6 2 (II)	238	915	26	963
Step 6 First Sephadex G-75 (I)	101	733	11	771
(II)	201	1,830	22	1,926
Step 7 Second Sephadex G-75 (I)	86	806	9	848
(II)	170	2,200	19	2,311

Step 1 Ammonium sulphate precipitation The extract (2,580 ml) was made 90% saturated by slow addition of 1,708 g of solid $(NH_4)_2SO_4$ The precipitate was isolated by centrifugation (30 min, 8,000*g*), suspended in, and dialyzed against, distilled water for 48 h An insoluble residue was isolated by centrifugation (10 min, 30,000*g*) and discarded

Step 2 Acetone precipitation Acetone (1,038 ml, at −18°) was slowly added to 1,038 ml of the ice-cold extract of step 1, with constant stirring The precipitate was isolated by centrifugation (15 min, 30,000*g*), suspended in, and dialyzed against, water for 24 h Insoluble material was collected by centrifugation (10 min, 30,000*g*) and discarded

Step 3 pH Fractionation To the above enzyme solution (487 ml), McIlvaine buffer (0 1M, pH 3 2, 487 ml) was added slowly, with constant stirring, during 24 h by means of a peristaltic pump Insoluble material was removed by centrifugation (10 min, 30,000*g*) The supernatant was then brought to pH 5.1 by addition of the alkaline buffer component.

Step 4 Second ammonium sulphate precipitation The above extract (1,034 ml) was made 95% saturated by slow addition of 736 g of solid $(NH_4)_2SO_4$. The precipitate was isolated by centrifugation (10 min, 30,000*g*), redissolved in, and dialyzed against, 50mM McIlvaine buffer (pH 4.05) for 24 h.

Step 5 Chromatography on aluminium oxide The extract (100 ml) was applied (10 ml/h) to a column (30 × 3 cm) of alumina equilibrated with 50mM McIlvaine buffer (pH 4.05). The column was first eluted with 50mM McIlvaine buffer (pH 4.5) at 60 ml/h, and fractions of 10 ml were collected. The first 1130 ml contained only a small amount (9 units or 2%) of α-D-galactosidase activity and were discarded. Elution was continued with 50mM McIlvaine buffer (pH 5.1) until no more protein could be detected. The combined fractions contained 120 units of α-D-galactosidase I activity. Elution was then continued with 50mM McIlvaine buffer (pH 6.2). This fraction (1,190 ml) contained 238 units (52%) of α-D-galactosidase II activity.

Step 6 Sephadex G-75 chromatography of α-D-galactosidase I The α-D-galactosidase I solution was concentrated on a Diaflo PM-10 membrane and dialyzed against 50mM McIlvaine buffer (pH 5.1). After this step, 112 units (94%) were recovered and applied to a column (93 × 3 cm) of Sephadex G-75. Equilibration and elution (15 ml/h) were performed with the same buffer. Fractions containing the α-D-galactosidase activity were combined (83 ml, 101 units or 90%), concentrated on a Diaflo PM-10 membrane (4 ml, 95 units), and rechromatographed on the same column. Final yield 86 units (90%) of α-D-galactosidase I. After this step, other carbohydrases could no longer be detected.

Step 7 Sephadex G-75 chromatography of α-D-galactosidase II In the same way, the solution of α-D-galactosidase II was twice chromatographed on Sephadex G-75. Final yield 170 units (89%) of α-D-galactosidase II. Other glycosidases were no longer detectable. In all further experiments, this solution of α-D-galactosidase II was used. The second enzyme having α-D-galactosidase activity (I) was not further investigated, except for an estimation of its molecular weight.

The whole purification-procedure results in a 2,300-fold purification, a specific activity of 2,200, and an overall yield of 19%. The polyacrylamide disc-gel electrophoresis pattern of the purified α-D-galactosidase II showed only one sharp band, corresponding to the enzyme activity, irrespective of whether the staining was performed with Amidoblack 10B, or with the enzyme substrates *p*-nitrophenyl or 4-methylumbelliferyl α-D-galactopyranoside.

Enzyme stability — Whereas raw extracts could be stored at 4° for several months without loss of activity, the enzyme became less stable after purification. Therefore, the relative enzymic activity of purified α-D-galactosidase II was determined after various incubation (25°) periods in buffers pH 2.7 to 8.6. The enzyme was most stable at pH 5.0 to 5.2. Above and below this pH, denaturation occurred rapidly. When stored at 4° in 50mM McIlvaine buffer (pH 5.2), the enzyme showed no significant loss of activity for several months.

Effect of ionic environment — The activity at 25° and pH 6.1 of α-D-galactosidase II was determined in the presence of increasing concentration of buffer salts (10–

100mM McIlvaine buffer) Optimal activity was found at a concentration of 50mM Above and below this concentration, the activity decreased only slightly (maximum, 10%). The use of a Tris-HCl buffer having the same pH did not alter the enzymic activity.

When metal ions were added (10mM) under standard conditions (~10-min assay), it was found that Mg^{2+} and Cu^{2+} had no effect, that Ba^{2+} slightly (~8%) inhibited, and Zn^{2+} slightly (~10%) activated, the enzyme However, Hg^{2+} completely inactivated the enzyme Since several methods for the synthesis of glycosides use mercury salts as catalysts, severe inhibition can occur if Hg^{2+} is not carefully removed

Influence of pH — Using *p*-nitrophenyl α-D-galactopyranoside (mM) as substrate, the activity of the enzyme (initial rate of phenol release) was measured at 25° in 50mM McIlvaine buffers of different pH From the data in Table II, it follows that maximal activity was found at pH 6 04 Therefore, the standardization of the enzyme solutions was always performed at pH 6 04 (see Experimental) Above this pH, the activity decreased sharply and finally became zero. Below pH 6 04, the rate decreased at first, but then increased It was proved experimentally that the increased activity at low pH was not due to an acid-catalysed hydrolysis of the substrate Below pH 3, the irreversible denaturation of the enzyme precluded reliable measurements of the rate Consequently, it remains unknown whether the increase of the rate between pH 4 and 3 would lead to a kinetically significant, second optimum or rather to a constant value

TABLE II

EFFECT OF pH ON α-D-GALACTOSIDASE ACTIVITY (25°)

pH	*Activity* (*μmole min*$^{-1}$ *u*$^{-1}$)	*pH*	*Activity* (*μmole min*$^{-1}$ *u*$^{-1}$)
3 00	0 51	5 35	0 71
3 20	0 53	5 60	0 85
3 35	0 51	5 75	0 91
3 55	0 47	5 93	0 95
3 75	0 43	6 04	1 00
4 10	0 41	6 13	0 99
4 55	0 43	6 20	0 97
4 85	0 49	6 50	0 92
5 00	0 57	7 05	0 38
5 20	0 62	7 30	0 11

Since the enzyme is severely inhibited by excess substrate (see below) and this inhibition is also a function[6] of pH, the experimental pH-optimum depends on the initial concentration of the substrate The influence of pH on the activity of the enzyme is thus complex, and further explanation in the present state of knowledge must be speculative and awaits further experimental facts These investigations are

now in progress The pH-dependence described in this work can only be used to choose suitable working conditions

Molecular weight — The molecular weights of α-D-galactosidases I and II were determined by gel chromatography[7,8] on Biogel P-150 The elution volume of the two α-D-galactosidases was compared with the elution volumes of standard proteins (see Experimental) on the same column. The data are presented in Fig 1 The molecular weight of α-D-galactosidase I was 28,000 (±900) and that of α-D-galactosidase II was 36,500 (±600)

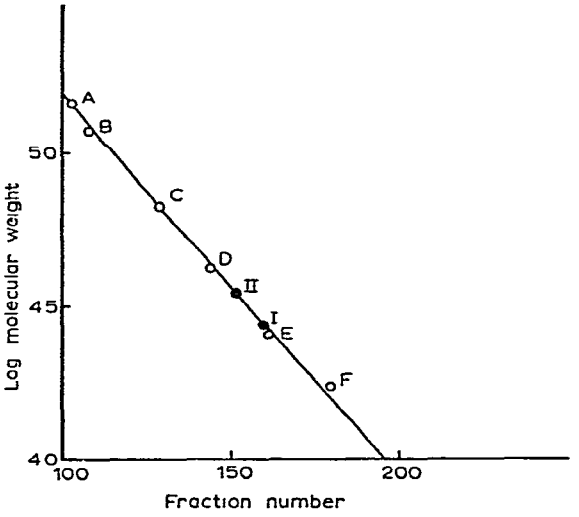


Fig 1 Determination of molecular weight of α-D-galactosidases I and II by gel filtration (see Experimental)

Hydrolysis of para-substituted phenyl α-D-galactopyranosides — The enzymic hydrolysis of eight para-substituted phenyl α-D-galactopyranosides was followed at 25° in 50mM McIlvaine buffer (pH 6 04) by determination of the liberated phenol Since, in each case, severe substrate inhibition was observed, K_m and V could not be calculated by the usual linear transformations of the formal Michaelis–Menten equation Rough, graphical estimations of these parameters could be made by using only the data at low concentrations of substrate It appeared, however, that this type of inhibition could be explained by presuming that, at higher concentrations of substrate, some sort of inactive or dead-end complex of the type ESS was formed[9] The initial rate (v_i) could then be correlated with the substrate concentration by means of the formal equation

$$v_i = \frac{VK[S]}{1+K[S]+K.K_i[S]^2}$$

In a simple model, V, K, and K_i would have an exact meaning, namely, theoretical

maximum rate (without inhibition), equilibrium or steady-state constant of the Michaelis–Menten complex, and equilibrium (association) constant for the reaction $ES + S \rightleftharpoons ESS$, respectively

However, since the proposed reaction scheme will not be simple (see further), these parameters will be complex functions of several rate and equilibrium constants The calculations of the parameters V, K, and K_i were then performed with the aid of an iterative computer programme, designed to compute the most probable values of these parameters, in such a manner that the sum of squares $(v_{exp} - v_{recalc})^2$ was minimized V, K, and K_i were then used to recalculate the rate (v_i) at any given concentration (S) Table III shows two examples of experimental and recalculated values of v_i, together with the percentage error As can be seen, the agreement is excellent and no systematic deviations are observed The same agreement was found for the other galactosides The fact that, even at the highest concentrations (50mM) of substrate, the experimental rate still decreased and did not deviate from the calculated rate seems to corroborate the assumption that ESS is a dead-end complex In no case did the rate become constant at a finite value, independent of the substrate concentration

TABLE III

EFFECT OF SUBSTRATE CONCENTRATION ON v_i (25°, pH 6 04)

p-*Chlorophenyl* α-D-*galactopyranoside*				p-*Methylphenyl* α-D-*galactopyranoside*			
S (mM)	v_{exp} (μmole	v_{recalc} min^{-1} u^{-1})	*Deviation* (%)	*S* (mM)	v_{exp} (μmole	v_{recalc} min^{-1} u^{-1})	*Deviation* (%)
0 1	0 1873	0 1765	6 1	0 3	0 2683	0 2672	0 4
0 2	0 2124	0 2115	0 4	0 5	0 4519	0 4318	−3 7
0 3	0 3694	0 3493	5 8	0 7	0 5400	0 5411	−0 2
0 4	0 4309	0 4436	−2 9	1 0	0 6234	0 6174	1 0
0 5	0 4846	0 5105	−5 1	1 2	0 6711	0 6723	−0 2
0 6	0 5783	0 5590	3 4	1 5	0 7143	0 7128	0 2
0 7	0 5935	0 5946	−0 2	1 8	0 7557	0 7430	1 7
0 9	0 6140	0 6209	−1 1	2 0	0 7869	0 7658	2 8
1 0	0 6299	0 6404	−1 6	2 5	0 7970	0 7958	0 2
1 2	0 6662	0 6648	0 2	3 0	0 8006	0 8120	−1 4
1 5	0 6812	0 6765	0 7	3 5	0 8179	0 8179	−0 2
1 8	0 6945	0 6802	2 1	4 0	0 8071	0 8217	−1 8
2 0	0 6731	0 6788	−0 8	4 5	0 8095	0 8198	−1 3
2 5	0 6694	0 6668	0 4	5 0	0 8006	0 8153	−1 8
3 0	0 6511	0 6486	0 4	7 5	0 7949	0 7739	2 7
3 5	0 6236	0 6278	−0 7	10 0	0 7273	0 7236	0 5
4 0	0 6094	0 6062	−0 3	12 5	0 6731	0 6752	−0 3
4 5	0 5895	0 5848	0 8	15 0	0 6392	0 6308	1 3
5 0	0 5586	0 5640	−1 0	20 0	0 5592	0 5554	0 7
5 5	0 5592	0 5441	2 8	25 0	0 4912	0 4949	−0 8
6 0	0 5203	0 5251	−0 9	30 0	0 4469	0 4458	0 2
7 0	0 4894	0 4902	−0 2	35 0	0 3963	0 4054	−2 2
8 0	0 4547	0 4590	−0 9				
9 0	0 4263	0 4312	−1 1				

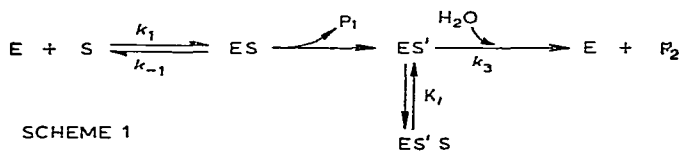
The calculated values of K, K_i, and V for eight para-substituted phenyl α-D-galactopyranosides are collected in Table IV. For a given amount of enzyme, saturated with substrate, V (expressed per unit of enzyme activity) represents the maximum, attainable rate of phenol release, assuming, of course, that no inhibition took place From the data, it follows that the substituent group, with the possible exception of the nitro group, has no effect on V The same, small influence of the aglycon group was found for the α-D-galactosidases from sweet almonds[2,3] and *Vicia faba*[1], although, in the first case, a correlation between log V and the Hammett substituent constant σ for electron-releasing groups, but not for electron-withdrawing groups, was suggested No such relationship exists between our log V values and σ.

TABLE IV

SUBSTITUTED PHENYL α-D-GALACTOPYRANOSIDES
ENZYME PARAMETERS AT 25° AND pH 6 04

Substituent	$10^6 V$ (*moles* min^{-1} u^{-1})	$\overline{K}$ (M^{-1})	$\overline{K_i}$ (M^{-1})	$10^6 V\,\overline{K}$ ($l\ min^{-1}\ u^{-1}$)	$10^{-6}\overline{K_i}/V$ (l *min u moles*$^{-2}$)
None	0 805	929	60	747	74 4
p-Chloro	1 059	2,006	156	2,124	147 3
p-Bromo	1 126	2,433	143	2,740	127 0
p-Iodo	1 113	2,152	154	2,396	138 3
p-Methyl	1 169	1,190	53	1,391	45 3
p-Ethyl	1 022	1,234	67	1,262	65 5
p-Ethoxy	1 094	2,093	85	2,291	77 7
p-Nitro	1 694	4,031	430	6,827	253 9

Since V is independent of the substituent within this series of para-substituted phenyl galactopyranosides, it seems improbable that the release of the phenyl aglycon group constitutes the rate-limiting step of the enzymic reaction mechanism Therefore, we propose that the hydrolysis proceeds by a pathway involving at least two intermediates, according to the minimal scheme·



SCHEME 1

After the formation of the Michaelis–Menten complex (ES), the aglycon group (P_1) is released with the simultaneous formation (glycosylation step) of an enzyme–galactosyl complex (ES'), which then reacts with water, yielding galactose (P_2) and free enzyme (deglycosylation) The k_3-step may eventually involve the binding of the acceptor molecule (water or another nucleophile) prior to the reaction itself At higher concentrations of substrate, a second molecule adds to ES' with the formation of the inhibitory complex ES'S Theoretically, it is conceivable that an inhibitory

complex ESS would be formed from the Michaelis–Menten complex ES However, the experimental evidence (see below, methyl α-D-galactopyranoside) suggests that the second substrate-molecule binds to ES′ In the above scheme, all retro-reactions (k_{-2}, k_{-3}) are omitted, as only initial velocities were measured

Assuming steady-state conditions for ES and ES′, and equilibrium for ES′S, equation *I* may be calculated

$$v_i = \frac{E_t k_{cat} \bar{K}[S]}{1+\bar{K}[S]+\bar{K}.\bar{K}_i[S]^2} \qquad (I)$$

Formally, this is the same equation as was used for the calculations of V, K, and K_i, but now the calculated parameters are, in fact, complex functions of rate and equilibrium constants

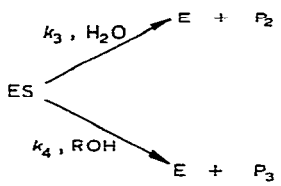
$$k_{cat} = \frac{k_2 k_3'}{k_2+k_3'}, \qquad \text{with} \qquad k_3' = k_3[H_2O],$$

$$\bar{K} = K\frac{k_2+k_3'}{k_3'}, \qquad \text{with} \qquad K = k_1/(k_2+k_{-1}),$$

$$\bar{K}_i = K_i\frac{k_2}{k_2+k_3'}, \qquad \text{with} \qquad K_i = \text{true association constant}$$

If it is assumed that, for phenyl α-D-galactopyranosides or substrates with a good-leaving aglycon group, the deglycosylation reaction is rate-limiting ($k_3' \ll k_2$ and thus $k_3 \ll k_2$), k_{cat} simplifies to $k_{cat} \sim k_3'$ and $V \sim E_t k_3'$. Consequently, the experimental value of V will be independent of the aglycon group, and no information about the k_2-step will be available Secondly, in our model, the inhibition by the substrate will be experimentally detectable, as it then occurs before the rate-limiting step If $k_2 \ll k_3'$, $\bar{K}_i \sim K_i k_2/k_3'$ and $\bar{K}\bar{K}_i[S]^2$ will be negligible compared to $\bar{K}[S]$, whereas, if $k_2 \gg k_3'$, then $\bar{K}_i \sim K_i$

Influence of added nucleophiles — If alcohols are added to an enzymic reaction mixture in which an enzyme–glycosyl complex is formed, these alcohols can react competitively[10,11] with water for the ES′ complex, with the formation of alkyl galactosides (P_3), according to the scheme



Experimentally, it was found by t l c. that the addition of methanol or 1-propanol to an enzymic reaction mixture containing *p*-nitrophenyl α-D-galacto-

pyranoside as substrate resulted in the formation of the corresponding alkyl galactopyranosides No alkyl galactoside was formed when the enzyme was omitted, or when the enzyme was incubated with a mixture of D-galactose and the alcohol. This indicated that the alkyl galactoside was formed by nucleophilic competition, during the hydrolysis of a substrate molecule

Table V shows the relative, initial reaction rates (release of *p*-nitrophenol) at 25° [McIlvaine buffer (pH 6 04), *p*-nitrophenyl α-D-galactopyranoside (1mM) as substrate], as a function of increasing concentrations of the alcohols In both cases, the rate was a linear function of the alcohol concentration The values of the slope and of the correlation coefficient, calculated by the method of least-squares, are, respectively methanol, $0\,253\times10^{-6}$ l min^{-1} u^{-1}, r = 0 995, 1-propanol, $0\,376\times10^{-6}$ l min^{-1} u^{-1}, r = 0 999 The increase of the rate by added alcohols indicates that the galactosyl–enzyme intermediate reacts faster with alcohols than with water At the same time, it proves that the aglycon-releasing step (k_2) cannot be rate-limiting If this were the case, the effect of the alcohols on dP_1/dt would not be observable

TABLE V

INFLUENCE OF ALCOHOLS AND METHYL α-D-GALACTOPYRANOSIDE ON THE HYDROLYSIS OF *p*-NITROPHENYL α-D-GALACTOPYRANOSIDE (25°, pH 6 04)

Methanol (*m*M)	v_i[a]	*1-Propanol* (*m*M)	v_i[a]	*Methyl α-D-galactopyranoside* (*m*M)	v_i[a]
0	1	0	1	0	1
9 91	1 006	50	1 017	0 1	0 997
49 5	1 0163	100	1 038	1	0 866
99 1	1 0237	150	1 056	2	0 756
148 6	1 0389	200	1 075	4	0 566
				6	0 483
				8	0 407
				10	0 346

[a]Initial rate of phenol release (μmoles min^{-1} u^{-1}) Mean value from five determinations (standard deviation <1%)

Hydrolysis of raffinose and methyl α-D-galactopyranoside — No substrate inhibition could be detected with these two substrates, and thus V and K_m (25°, pH 6 04) were calculated by the method of Wilkinson[12] (Table VI) For raffinose, $K = 1/K_m = 135M^{-1}$ and V = 0 305 μmole min^{-1} u^{-1}, for methyl α-D-galactopyranoside, $K = 218M^{-1}$ and V = 0 0116 μmole min^{-1} u^{-1} Both substrates, especially methyl galactoside, were hydrolysed at a lower rate than phenyl galactosides Our results can be compared with those for other α-D-galactosidases The enzyme from sweet-almond[2,3] showed substrate inhibition with phenyl and alkyl α-D-galactopyranosides, but not with raffinose Raffinose and alkyl galactosides were hydrolysed at a lower rate than the phenyl galactosides The α-D-galactosidases I and II from *Vicia faba*[1] are inhibited by alkyl and aryl α-D-galactopyranosides

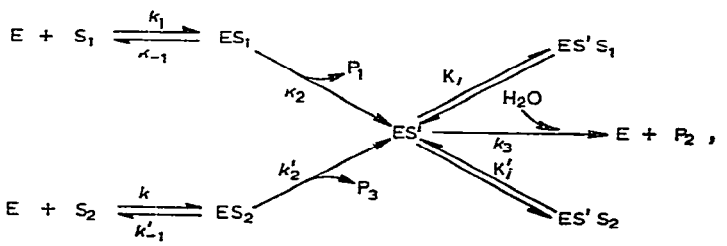
TABLE VI

ENZYMIC HYDROLYSIS OF RAFFINOSE AND METHYL α-D-GALACTOPYRANOSIDE (25°, pH 6 04)

Raffinose		*Methyl α-D-galactopyranoside*	
S (mM)	v_{exp} (μmole min^{-1} u^{-1})	S (mM)	v_{exp} (μmole min^{-1} u^{-1})
0 002	0 0645	0 005	0 0051
0 004	0 1049	0 010	0 0078
0 006	0 1368	0 030	0 0101
0 008	0 1575	0 050	0 0108
0 010	0 1772	0 070	0 0109
0 015	0 2027	0 090	0 0110
0 020	0 2219		
0 025	0 2345		

The reason why raffinose does not show substrate inhibition is unknown, but probably lies in the large structural differences between aryl galactosides and oligosaccharides For methyl α-D-galactopyranoside, however, it remained possible that the absence of substrate inhibition was only apparent, due to the fact that it occurred after the rate-limiting step For this substrate, the deglycosylation step cannot be rate-limiting (*cf* V) unless the reaction mechanism is different, which is highly improbable On the contrary, it seems more plausible that the glycosylation step becomes rate-limiting ($k_2 \ll k_3'$) due to the poor-leaving alkyl group If a second substrate molecule binds to ES′ with the formation of ES′S, this type of inhibition will not be detectable when the release of P_1 is followed, as the inhibition then occurs after the rate-limiting step If the second substrate molecule binds to ES with the formation of ESS, then the inhibition should be experimentally detectable by following P_1. No inhibition could be detected even at a concentration of 0 1M methyl galactoside whereas for *p*-nitrophenyl α-D-galactopyranoside, a marked inhibition was noticeable at concentrations as low as mM

If methyl α-D-galactopyranoside binds to ES′, it must do so regardless of the origin of ES′ Consequently, when the methyl galactoside (S_2) is added to a reaction mixture containing *p*-nitrophenyl α-D-galactopyranoside (S_1) as substrate, two types of inhibition must be detectable full-competitive with the free enzyme (ES_2), and non-competitive with ES′, according to the scheme.



where P_1 = nitrophenol, $ES'S_1$ = inhibitory complex with S_1, P_2 = galactose, $ES'S_2$ = inhibitory complex with S_2, P_3 = methanol, ES' = galactosyl–enzyme complex, and K_i and K_i' are association constants

Under the experimental conditions used, the hydrolysis of the methyl galactoside is negligible compared to that of *p*-nitrophenyl α-D-galactopyranoside (0 8% at the highest concentration of S_2 used) Assuming steady-state conditions for ES_1, ES_2, and ES', and equilibrium conditions for $ES'S_1$ and $ES'S_2$, the initial velocity $v_i = dP_1/dt$ is given by Equation *2*

$$v_i = \frac{E_t k_{cat} \bar{K}[S_1]}{1+\bar{K}[S_1]+\bar{K}_i\bar{K}[S_1]^2+K'[S_2]+\bar{K}_i'\bar{K}[S_1][S_2]} \qquad (2)$$

Equation *2* is of the same general form as equation *1*, but with the addition of two correction terms $K'[S_2]$ for the full-competitive, and $\bar{K}_i'\,\bar{K}[S_1][S_2]$ for the non-competitive inhibition From the hydrolysis of S_1 (without S_2), k_{cat}, $\bar{K}$, and $\bar{K}_i$ can be calculated (Table IV) $K' = k_1'/(k_{-1}'+k_2')$ and refers to ES_2, $\bar{K}_i' = K_i'k_2/(k_2+k_3')$ and refers to $ES'S_2$

When the release of P_1 is measured in a reaction series with constant S_1 and various concentrations of S_2, $1/v_i$ must be a linear function of $[S_2]$, according to the equation

$$\frac{1}{v_i} = \frac{1+\bar{K}[S_1]+\bar{K}_i\bar{K}[S_1]^2}{E_t k_{cat}\bar{K}[S_1]} + \frac{K'+\bar{K}_i'\bar{K}[S_1]}{E_t k_{cat}\bar{K}[S_1]} \times [S_2]$$

For $[S_1]$ = mM, the value of the intercept must be $9\,9\times10^5$ moles^{-1} min u If no inhibitory complex $ES'S$ is formed, $\bar{K}_i' = 0$ and the slope equals $K'/E_t k_{cat}\bar{K}[S_1]$ From the hydrolysis of methyl α-D-galactopyranoside, it is known that $\bar{K} = K'(k_2'+k_3')/k_3' = 218\text{M}^{-1}$ and, since $k_2 \ll k_3'$ (alkyl galactosides), $K' \sim \bar{K}'$ If it is assumed that for phenyl galactosides $k_{cat} \sim k_3'$, and for alkyl galactosides $k_{cat} \sim k_2$, then $k_2/k_3' = V(\text{alkyl})/V(\text{phenyl}) = 0\,0116/1\,69 = 0\,00686$, and $K' = 216\text{M}^{-1}$ With $\bar{K}_i' = 0$ and $K' = 216\text{M}^{-1}$, the slope must be $31\,6\times10^6$ min u l moles^{-2}

In Table V, the values of v_i and $1/v_i$ (pH 6 04, 25°, S_1 = mM) as a function of increasing concentrations of S_2 are collected Graphical analysis showed that $1/v_i$ was a linear function of $[S_2]$ Calculations by the method of least squares yielded intercept, $9\,7\times10^5$ moles^{-1} min u, slope, $(189 \pm 3)\times10^6$ min u l.moles^{-2} The calculated and experimental values of the intercept do agree, whereas the experimental value of the slope is six times the expected one Consequently, $\bar{K}_i'$ must differ from zero, which means that the complex $ES'S$ is formed From the value of the slope, $\bar{K}_i'$ can be calculated as 266M^{-1}, and from $\bar{K}_i' = K_i'k_2/(k_2+k_3')$, with $k_2 \gg k_3'$, it follows that K_i' must be of the same order of magnitude as $\bar{K}_i'$ The association constant of the $ES'S$ complex (K_i') is thus very similar to the constant (K') of the ES complex (216M^{-1}) This similarity was also found for the α-D-galactosidase from *Vicia faba*[1] Since the constant K_i' for methyl galactoside is even higher than K_i for the phenyl galactosides, still stronger substrate-inhibition should occur with the

former substrate The reason why it is not found experimentally must then reside in the fact that it occurs after the rate-limiting step If, for hydrolysis of methyl α-D-galactopyranoside, $k_2 \ll k'_3$, $\bar{K}_i$ will be small even if K_i itself is relatively large In equation *1*, the term $\bar{K}\ \bar{K}_i[S]^2$ will be small compared to $\bar{K}[S]$ and the equation will approach the simple Michaelis–Menten form Only when [S] is very large, will the term in $[S]^2$ become important For phenyl galactosides, $k_2 \gg k'_3$, $\bar{K}_i \sim K_i$, and $\bar{K}_i$ will not be small Consequently, the term $\bar{K}\ \bar{K}_i[S]^2$ may become important at much lower concentrations of S

Influence of the substituents on $\bar{K}_i$ — According to Scheme 1, $\bar{K}_i = K_i k_2/(k_2+k'_3)$ and $V = E_t k_2 k'_3/(k_2+k'_3)$, hence $\bar{K}_i/V = K_i/k'_3 E_t$ and $\log \bar{K}_i/V = \log K_i - \log k'_3 E_t$ Since $\log k'_3 E_t$ is independent of the aglycon group, the experimentally available value of $\log \bar{K}_i/V$ can be used to calculate linear free-energy relations (LFER) between K_i and substituent parameters Of the many possible combinations, the highest correlation was found with σ_I[13] and σ_R[14] ($\sigma_R = \sigma_P - \sigma_I$) Using the values of K_i in Table IV, regression analysis yielded the equation

$$\log 10^{-6}\ \bar{K}_i/V = 1\ 836 + 0\ 784\sigma_I + 0\ 301\sigma_R,$$ with a standard error of the estimate $s_{y/x} = 0\ 057$, multiple correlation coefficient $R = 0\ 977$, partial correlation product of σ_I, $PCP(\sigma_I) = 0\ 91$, $PCP(\sigma_R) = 0\ 04$, confidence level $CL > 99\ 5$.

Although the specific binding of the substrate to ES′ will be through the glycon part of the molecule, the above equation shows that the substituent on the aglycon group also affects the binding through electrical factors Electron-withdrawing substituents increase the affinity, through their inductive, as well as through their resonance, effect, although the latter is not very significant When $\log \bar{K}_i$ itself is correlated with σ_I and σ_R, a similar equation can be calculated

$$\log \bar{K}_i = 1\ 834 + 0\ 998\sigma_I + 0\ 376\sigma_R,$$ with $s_{y/x} = 0\ 044$, $R = 0\ 978$, $PCP(\sigma_I) = 0\ 940$, $PCP(\sigma_R) = 0\ 04$, $CL > 99\ 5$

According to our scheme, $\log \bar{K}_i = \log K_i + \log k_2 - \log (k_2+k'_3)$ The similarity between the two equations suggests that $\bar{K}_i$ approaches the values of the association constant (K_s), which again indicates that $k'_3 \ll k_2$

One of the possible reasons for the electronic effect of the substituent on K_i could be an interaction between a charged group on the enzyme and a permanent dipole of the aglycon group By analogy with Tute[15], we tried to correlate $\log 10^{-6}\ \bar{K}_i/V$ with some parameters characteristic for dipole moments The highest correlation was found with μ_v, the vertical component (through the glycosidic oxygen atom and the para-substituent) As calculated by Tute, μ_v is treated as of negative sign in the direction 0 to the substituent Using the μ_v values of Tute[15], regression analysis yielded the equations

$$\log 10^{-6}\ \bar{K}_i/V = 1\ 858 - 0\ 147\mu_v,$$ with $s_{y/x} = 0\ 08$, $r = 0\ 95$, and $CL > 99\ 9$, and

$$\log \bar{K}_i = 1.865 - 0\ 191\mu_v,$$ with $s_{y/x} = 0\ 08$, $r = 0\ 97$, and $CL > 99\ 9$

Including a term in σ_H (horizontal component at 90° to the μ_v axis) or in α (electronic polarizability) did not improve the correlation The negative sign of the slope indicates

that a partial, negative charge on the substituent increases the relative affinity of the aglycon group for the enzyme, suggesting an interaction with a positively charged enzyme-group However, the exact nature of this interaction is not known

Influence of the substituent on $\bar{K}$ and $V\bar{K}$ — The most significant correlation between log $\bar{K}$ or log $V\bar{K}$ and substituent parameters was found with σ_I Regression analysis yielded the equations

$\log \bar{K} = 3.074 + 0.644\sigma_I$, with $s_{y/x} = 0.067$, $r = 0.975$, and CL >99.5, and

$\log 10^6\, V\bar{K} = 3.061 + 0.877\sigma_I$, with $s_{y/x} = 0.126$, $r = 0.912$, and CL >99.5

Including a term in σ_R did not improve the correlation, and no significant LFER could be calculated with μ_v

Although both correlations are significant, their performance as a summary of the data is less so, especially for the equation with $V\bar{K}$ (*cf* $s_{y/x}$), probably because $\bar{K}$ and $V\bar{K}$ still remain complex parameters According to Scheme 1, $\bar{K}V = E_t\, k_1\, k_2/(k_2 + k_{-1})$, only if $k_{-1} \ll k_2$, $\bar{K}V \sim E_t k_1$ and thus becomes a measure of the single rate-constant k_1 for the formation of the ES complex However, no experimental evidence is available to substantiate the assumption that $k_{-1} \ll k_2$ On the other hand if $k_2 \ll k_{-1}$ and K approaches the true association constant of ES, $V\bar{K} \sim E_t k_2 K_a$ and thus remains complex In this case, however, the LFER with log $V\bar{K}$ will be a measure of the relative free-energy difference between the free enzyme/substrate and the transition state of the k_2-step According to Scheme 1, the experimental parameter $\bar{K}$ itself is given by $\bar{K} = K(k_2 + k_3')/k_3'$, and if $k_3' \ll k_2$, it reduces to $\bar{K} = Kk_2/k_3'$ If $k_{-1} \ll k_2$, it simplifies to $\bar{K} \sim k_1/k_3'$, and if $k_2 \ll k_{-1}$, it reduces to $\bar{K} = K_a k_2/k_3'$ Except for the substituent-independent constant k_3', both $V\bar{K}$ and $\bar{K}$ must yield the same LFER (regardless of the ratio k_2/k_{-1}), if $k_3' \ll k_2$ Experimentally, it was found that both LFER are indeed very similar However, the mechanistic interpretation of the LFER does depend on the ratio k_2/k_{-1}, and since this ratio is unknown, the interpretation remains speculative From the LFER, it follows that an electron-withdrawing substituent increases $\bar{K}$ and $\bar{K}V$ If $\bar{K}$ and $\bar{K}V$ approach k_1 (*i e*, $k_{-1} \ll k_2$), the LFER indicates that an electron-withdrawing substituent lowers the energy of the transition complex leading to ES If, during the formation of ES, a hydrogen bridge is formed between an enzyme group and the glycosidic oxygen atom the reaction constant ρ in the LFER should have a negative sign, as an electron-withdrawing group decreases the electron density around this oxygen atom and thus lowers the extent of proton transfer On the other hand, if the effect of the substituent resides in an interaction between a charged group and a dipole moment, a correlation with μ (or μ_v) might be expected Since neither the correlation nor the negative reaction constant ρ were found experimentally, we believe that the assumption $k_{-1} \ll k_2$ is not true. Consequently, both $\bar{K}V$ and $\bar{K}$ remain complex parameters, composed of the equilibrium constant K_a and the rate constant k_2 Since the substituent may have an influence on both constants, further analysis of the LFER seems too speculative

EXPERIMENTAL

The synthesis of the α-D-galactopyranosides used was described previously[16]. One unit (u) of enzyme activity is defined as the hydrolysis of 1 μmole of substrate per min under the following standard conditions *p*-nitrophenyl α-D-galactopyranoside (mM) as substrate, 50mM McIlvaine buffer (pH 6 04), temperature 25° For the other glycosidases, the same standard conditions were used, except that the corresponding *p*-nitrophenyl glycopyranoside was used and that the measurements were performed at the experimentally determined pH optimum of the particular glycosidase. β-D-galactosidase, β-D-glucosidase, β-D-xylosidase, and *N*-acetyl-β-D-glucosaminidase (5 15), α-D-glucosidase (5.55), α-D-mannosidase (4 50) Routine enzymic assays were performed under standard conditions Release of *p*-nitrophenol was followed by adding 1-ml aliquots of the reaction mixture to 3 ml of 10% (w/v) aqueous Na_2CO_3 and measuring the absorbance at 400 nm Hydrolysis of other phenyl galactosides was followed by measuring the liberated phenol by the method of Folin–Ciocalteu[17], and hydrolysis of methyl α-D-galactopyranoside and raffinose by measuring reducing sugar by the method of Somogyi–Nelson[18] Protein concentration was routinely measured according to Warburg and Christian[19] Specific activity was expressed as enzyme units per mg of protein Polyacrylamide gel-electrophoresis was performed according to the method of Hjertén[20] Electrophoresis was carried out in 7.5% gel, 10mM Tris-HCl buffer (pH 8 8) at room temperature, for 1 h at 3 mA per tube By incubating the unstained gels in a solution of *p*-nitrophenyl or 4-methylumbelliferyl α-D-galactopyranoside, the enzyme was localized by the appearance of a yellow or fluorescent band Proteins were stained with Amidoblack 10B (1% in 7% acetic acid) For the determination of the molecular weights by gel filtration, 4 mg of α-D-galactosidase I, 2 mg of α-D-galactosidase II, and 2 mg of the following proteins (molecular weight in parentheses), (A) alcohol dehydrogenase (150,000), (B) glyceraldehyde 3-phosphate dehydrogenase (120,000), (C) bovine serum albumin (68,000), (D) ovalbumin (43,000), (E) chymotrypsinogen (25,700), (F) myoglobin (17,300), were applied to a column (1 × 100 cm) of Biogel P-150 equilibrated with 50mM phosphate buffer (pH 7 2) Elution was performed with the same buffer at a flow rate of 10 ml/h Fractions of 1 ml were collected

The Diaflo–Amicon ultrafiltration system was used to concentrate solutions of protein. The best results were obtained with the filter PM-10 (~95% recovery)

Enzyme solutions were standardized each day, and all initial reaction rates (mean value from three to five determinations) were expressed per unit of enzyme activity At least 20 substrate concentrations were used to determine V, $\bar{K}$, and $\bar{K}_i$ with the aid of the iterative computer programme

REFERENCES

1 P M Dey and J B Pridham, *Biochem J*, 115 (1969) 47
2 O P Malhotra and P M Dey, *Biochem J*, 103 (1967) 739
3 P M Dey, *Biochim Biophys Acta*, 191 (1969) 644

4 P M DEY AND J B PRIDHAM, *Advan Enzymol*, 36 (1972) 91
5 J E COURTOIS AND F PETEK, *Methods Enzymol*, 8 (1966) 565
6 H. CARCHON AND C K DE BRUYNE, unpublished results
7 P ANDREWS, *Biochem J*, 96 (1965) 595
8 H DETERMANN, *Gelchromatography*, Springer, Berlin, 1967.
9 K J LAIDLER, *The Chemical Kinetics of Enzyme Action*, Clarendon Press, Oxford, 1958, p 117
10 I HINBERG AND K. J LAIDLER, *Can J. Biochem*, 50 (1972) 1334
11 J P TENU, O. VIRATELLE, AND J. YON, *Eur J Biochem*, 20 (1971) 363
12 G N WILKINSON, *Biochem J*, 80 (1961) 324
13 M CHARTON, *J. Org Chem*, 29 (1964) 1222
14 O EXNER, IN N B CHAPMAN AND J SHORTER (Eds), *Advances in Linear Free Energy Relationships*, Plenum Press, London, 1972, p 35
15 M S TUTE, *J Med Chem*, 13 (1970) 48
16 C K. DE BRUYNE AND H CARCHON, *Carbohyd Res*, 33 (1974) 117
17 O FOLIN AND V CIOCALTEU, *J Biol Chem*, 73 (1927) 627
18 H U BERGMEYER, *Methoden der Enzymatischen Analyse*, Verlag Chemie, Weinheim, 1962, p 75
19 O WARBURG AND W CHRISTIAN, *Biochem Z*, 310 (1941) 384
20 S HJERTÉN, S JERSTEDT, AND A TISELIUS, *Anal Biochem*, 11 (1965) 219