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α-D-MANNOSIDASE-CATALYZED HYDROLYSIS OF SUBSTITUTED PHENYL α-D-MANNOPYRANOSIDES

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ABSTRACT

The influence of substituents on the hydrolysis of substituted phenyl α-D-mannopyranosides by α-D-mannosidase from *Medicago sativa L.* has been investigated. As indicated by structure–activity relations, the electronic effect of the substituent has an influence on the rate of formation of the intermediate mannosyl–enzyme complex. This effect depends not only on the nature of the substituent, but also on its position (meta or para) and on the temperature of the experiment. Hammett-type linear free energy relationships show that the reaction constant ρ changes its sign at ~27°. Substrates with strong electron-withdrawing groups show values of log V that are linearly related to 1/T, whereas the Arrhenius plots for other substrates are severely curved. This complex behaviour is tentatively explained by assuming that some meta-substituents have an unusual, temperature- and substituent-dependent influence on the formation of the Michaelis–Menten complex.

INTRODUCTION

In previous[1,2] papers, we described the purification of the enzyme α-D-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) from *Medicago sativa L.*, and proposed a mechanism[2] involving the rate-limiting formation of a mannosyl–enzyme complex. In order to obtain more information about the reaction step in which the aglycon is split off, we have studied the hydrolysis by α-D-mannosidase of a series of substituted phenyl α-D-mannopyranosides, and have tried to correlate the enzymic reaction coefficients with parameters characteristic for the electronic and hydrophobic effect of the substituent. We now report on some results of this study.

RESULTS AND DISCUSSION

Influence of added nucleophiles

From a study of the influence of added nucleophiles on the rates of formation of the different reaction products, we proposed[2] a two-step mechanism with formation of a mannosyl–enzyme complex (ES′), according to Scheme 1.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} ES' + P_1 \begin{cases} \xrightarrow{k_3W} E + P_2 \\ \xrightarrow{k_4N} E + P_3 \end{cases}$$

(Scheme 1)

We also proposed that the reaction of ES' with water (k_3W), or with an added nucleophile (k_4N), is fast compared to the rate of aglycon release (k_2), so that the k_2-step represents the rate-limiting step. However, these conclusions were based on experiments in which *p*-nitrophenyl (PNPM) and *p*-chlorophenyl α-D-mannopyranoside (PCPM) only were used as substrates. In view of the complex influence of the substituent on the reaction rate of substituted phenyl α-D-mannopyranosides (see further), it seemed important to prove that our propositions (especially that the k_2-step is rate-limiting) were still correct when other substrates were used. Therefore, we determined the influence of an added nucleophile (methanol) on v_1 (rate of aglycon release) and v_2 (rate of mannose formation) for a number of para- and meta-substituted phenyl α-D-mannopyranosides (Table I).

From the data, it follows that (*a*) the rate of phenol release is independent of the concentration of the alcohol, but depends on the substituent; and (*b*) for all of the substrates, v_2 decreases with increasing concentration of methanol. Both findings strongly suggest that the reaction proceeds by a two-step mechanism, according to Scheme 1. As shown previously[2], the steady-state treatment of this mechanism, according to Hinberg and Laidler[3], yields formal Michaelis–Menten kinetics. Moreover, it requires the ratio $v_3/v_2 = k_4(N)/k_3(W)$ to be independent of

TABLE I

INFLUENCE OF METHANOL ON v_1 AND v_2 (pH 4.0, 25°) FOR THE HYDROLYSIS OF SUBSTITUTED PHENYL α-D-MANNOPYRANOSIDES[a]

Substituent	v_1 (*μmol.min*$^{-1}$*.U*$^{-1}$)				v_2 (*μmol.min*$^{-1}$*.U*$^{-1}$)		
	0M MeOH	*0.5M MeOH*	*M MeOH*	*1.5M MeOH*	*0.5 MeOH*	*M MeOH*	*1.5M MeOH*
None	0.291	0.289	0.281	0.283	0.274	0.259	0.249
p-Methyl	0.438	0.437	—	0.436	—	0.388	0.364
p-Ethyl	0.526	—	0.523	0.529	—	0.458	0.435
p-Nitro-*o*-chloro	0.689	—	0.686	0.692	0.645	0.595	—
p-*tert*-Butyl	0.610	0.617	0.614	0.621	—	0.533	—
p-Nitro	1.000	0.983	1.006	1.004	0.938	0.895	0.834
p-Chloro	0.592	—	0.595	0.597	0.558	0.525	0.500
m-Bromo	0.548	0.546	0.550	—	0.508	—	0.454
m-Chloro	0.383	0.380	—	0.382	—	0.343	0.324
m-Methyl	0.241	—	0.244	0.240	—	0.221	0.202

[a]Concentration of substrate, 5mM, except for *p*-nitro-*o*-chloro (2.5mM).

TABLE II

INFLUENCE OF THE SUBSTITUENTS ON THE RATIO v_1/v_2

Substituent	v_2/v_1		
	0.5M MeOH	*M MeOH*	*1.5M MeOH*
None	0.958 ± 0.051	0.905 ± 0.014	0.870 ± 0.050
p-Methyl	—	0.888 ± 0.030	0.833 ± 0.011
p-Ethyl	0.958 ± 0.022	0.871 ± 0.021	0.826 ± 0.010
p-Nitro-*o*-chloro	0.936 ± 0.020	0.864 ± 0.020	—
p-*tert*-Butyl	0.958 ± 0.012	0.865 ± 0.020	0.810 ± 0.011
p-Nitro	0.938 ± 0.016	0.889 ± 0.002	0.828 ± 0.010
p-Chloro	0.938 ± 0.010	0.882 ± 0.021	0.840 ± 0.011
m-Bromo	0.927 ± 0.016	—	0.833 ± 0.036
m-Chloro	—	0.896 ± 0.081	0.846 ± 0.012
m-Methyl	—	0.909 ± 0.015	0.838 ± 0.010

the nature of the aglycon group. If a common, intermediate mannosyl–enzyme complex is formed in a step which is rate-limiting irrespective of the nature of the aglycon group, neither k_3 nor k_4 will be dependent on the substituent. As can be seen from the data in Table II, the ratio v_2/v_1 (or v_3/v_1, as $v_3 = v_1 - v_2$), for a given concentration of methanol, is independent of the nature of the substituent. These findings indicate that all of the substrates studied are hydrolyzed *via* the mechanism depicted in Scheme 1.

The results of our previous[2] study were explained by an ambident mechanism, in which the aglycon group is split off by the concerted action of a proton donor and a nucleophile, with the formation of a glycosyl-carbonium ion, which then reacts with water (or the nucleophile). These assumptions were based on the experimental finding that the relative reactivity of the alcohols [expressed as $R = k_4/k_3 = v_3(W)/v_2(N)$] towards the mannosyl–enzyme complex was >1 ($R \sim 8$), suggesting a nucleophilic attack on a glycosyl cation (C-1 with sp^2 character).

As a model system, we investigated the acid-catalyzed hydrolysis of PNPM. The generally accepted mechanism[5] involves a fast, equilibrium-controlled protonation of the exocyclic oxygen atom, followed by the unimolecular, rate-limiting heterolysis of the conjugate acid to a glycosyl oxonium–carbonium ion, which then reacts rapidly with water. If an alcohol is added, competition between alcohol and water for the glycosyl cation will occur in a manner similar to the enzymic reaction, with the formation of mannose and methyl D-mannoside. However, in this case, it is certain that the relative reactivity, R, reflects the reactivity towards a glycosyl cation. In 1.6M aqueous hydrochloric acid, the initial rate of phenol release (v_1) at 60° was determined for PNPM (10mM) as substrate, with and without the addition of methanol (M). The same value $v_1 = 55.4$ μmol.min^{-1} was found in both cases. Under identical conditions, the initial rate of mannose formation (M MeOH) was $v_2 = 48.3$ μmol. min^{-1}.

According to Scheme 2 for the acid-catalyzed reaction, and with $k_2 \ll k_3(W)$ and $k_2 \ll k_4(N)$, $v_3/v_2 = k_4(N)/k_3(W)$;

$$\begin{array}{l} \qquad\qquad\qquad\quad k_3W \\ \qquad\quad k_2 \qquad\quad \rightarrow \quad P_2 \\ S + H^+ \rightleftarrows SH^+ \rightarrow S' \\ \qquad\quad \downarrow \qquad\quad k_4N \\ \qquad\quad P_1 \qquad\quad \rightarrow \quad P_3 \end{array} \qquad \text{(Scheme 2)}$$

since $v_1 = v_2 + v_3$, $R = k_4/k_3 = v_3(W)/v_2(N) = (v_1 - v_2)(W)/v_2(N)$. With $v_1 = 55.3$ μmol.min^{-1}, $v_2 = 48.3$ μmol.min^{-1}, and (W)/(N) = 53.15, calculation yields R = 7.7.

As the methyl D-mannoside formed is also hydrolyzed (but ~10 times slower than PNPM), the experimentally determined value of v_2 is probably too high, so that the real value of R must be slightly larger than 7.7. The striking similarity between $R = k_4/k_3$ for the enzymic hydrolysis (R ~ 8) and the acid-catalyzed hydrolysis (R ~ 7.7) strongly suggests that, in the enzymic reaction, the nucleophile reacts with a glycosyl cation, and that a specific acceptor site for the nucleophile does not exist.

Influence of the substituent on the maximal reaction velocity

Because the rate-limiting step of the enzymic reaction is the formation of the enzyme–mannosyl complex, the experimental rate constant for the release of the aglycon, $k_{cat} = k_2k_3(W)/[k_2 + k_3(W)]$, can be simplified to $k_{cat} \approx k_2$. When the maximal rate (V) is always expressed on the same enzyme activity basis (per unit), $V = k_2(E_t)$ is a relative measure of k_2. For a number of meta-substituted (and one para-substituted) phenyl α-D-mannopyranosides, V was determined at different temperatures (pH 4.0) in order to obtain more information about the reaction step in which the aglycon group is split off. The data in Table III show that the effect of the substituent is small, but complex and temperature-dependent. This is illustrated in Fig. 1, where log V is plotted against the Hammett substituent constant σ. At 40°, log V is linearly related with σ, and the reaction constant ρ is negative. At the other temperatures, the pattern gradually changes. At a temperature of ~25° to 30°, the substituent has no effect on the rate ($\rho \sim 0$). At still lower temperatures, the Hammett plots becomes biphasic, and, depending on the substrates, ρ can be taken as positive or negative. Moreover, the biphasic character becomes more pronounced with decreasing temperature. Using the σ values of McDaniel and Brown[6], together with the data of Table III, the following regression functions were calculated:

at 40°: $\log 10^7V = 1.602 - 0.518\ \sigma$, with the standard error on the estimate $s_{y/x} = 0.014$, the standard error on the slope $s_b = 0.023$, the correlation coefficient r = 0.997, and the number of points n = 5.

at 30°: $\log 10^7V = 1.090 - 0.106\ \sigma$, with $s_{y/x} = 0.015$, $s_b = 0.023$, r = 0.94, and n = 5.

at 25°: no correlation was found: r = 0.4.

TABLE III

INFLUENCE OF THE SUBSTITUENT ON THE MAXIMAL REACTION VELOCITY (V) AND ON K_{spp}

No.	*Substituent*	10^6 V (*mol.min*$^{-1}$.*u*$^{-1}$)						10^4 K_{app} (M)			
		15°	*20°*	*25°*	*30°*	*35°*	*40°*	*15°*	*25°*	*30°*	*40°*
1	None	0.47	0.63	0.79	1.28	1.57	3.84	56	90	178	333
2	*m*-Methyl	0.43	—	0.78	1.20	—	4.52	63	107	225	450
3	*m*-Chloro	0.78	—	0.90	1.14	—	2.57	53	66	70	102
4	*m*-Bromo	0.73	—	0.85	1.12	—	2.50	48	50	55	83
5	*m*-Nitro	0.45	0.60	0.78	1.02	1.32	1.72	16	17	26	31
6	*p*-Nitro	0.66	0.91	1.25	1.74	2.40	3.26	9	12	17	26

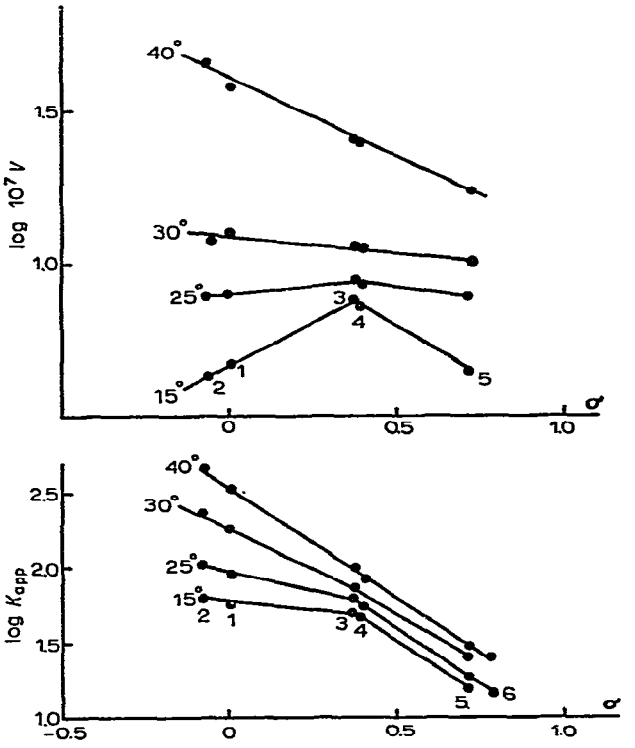

Fig. 1. Log V and log K_{spp} *versus* σ; numbers as in Table III.

at 15°: log $10^7V = 0.671 + 0.539\ \sigma$, with $s_{y/x} = 0.019$, $s_b = 0.046$, $r = 0.993$, and $n = 4$.

(The *m*-nitro derivative was not used in the calculation.)

The t- and F-tests on the significance of ρ reveal that, at 15° and 40°, the ρ value is significantly different from zero at better than the 99.9% level of significance. The deviation of the point for the meta-nitro derivative (15°) from the function line

calculated for derivatives **1** to **4** is highly significant (>99.9% level). Of course, the same is true for the deviation of points 1 and 2 from the function line for **3**, **4**, and **5**. Values of log V for the *p*-nitro derivative do not fit the lines for the meta derivatives.

Isokinetic relationship

The change of the sign of the reaction constant ρ can be explained by the Leffler isokinetic relationship. As pointed out by Leffler[9], it is possible that, within a reaction series, a mutual compensation between the activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ may exist, according to the empirical equation $\Delta H^{\ddagger} = \text{constant} + \beta\Delta S^{\ddagger}$, in which β represents the so-called isokinetic temperature. Since $\Delta H^{\ddagger} = \Delta G^{\ddagger} + T\Delta S^{\ddagger}$, the first equation can be rewritten as $\Delta G^{\ddagger} = \text{constant} + (\beta - \mathrm{T})\Delta S^{\ddagger}$. Hence, when the temperature of the experiment (T) equals β, $\Delta G^{\ddagger} = \text{constant}$, and all compounds of the series will react at the same rate. Since the reaction constant k_2 [or $V = k_2(\mathrm{E_t})$] is proportional to $\Delta G^{\ddagger}$, the ρ value in the Hammett linear free energy relationship with log V can be expressed as $\rho = \text{constant}\ (1 - \beta/\mathrm{T})$. Hence, ρ will be negative when $\beta > \mathrm{T}$, positive when $\beta < \mathrm{T}$, and zero when $\beta = \mathrm{T}$.

As pointed out by Exner[10,11], β cannot be determined by a linear regression of $\Delta H^{\ddagger}$ on $\Delta S^{\ddagger}$, because both quantities are *a priori* mutually dependent, both being calculated from the same original kinetic constants. According to Exner, this difficulty can be overcome by plotting values of log K [or log $V = \log k_2\ (\mathrm{E_t})$] obtained at two different temperatures ($\mathrm{T_1} > \mathrm{T_2}$) against each other. From the slope of this line, the value of β can be calculated by the Exner equation[10].

Using the data of Table III, regression analysis yields the equation (for derivatives **1** and **4**):

$$\log 10^7 V(40°) = 2.297 - 1.014 \log 10^7 V(15°),$$
$$\text{with } s_{y/x} = 0.017,\ s_b = 0.12,\ r = 0.99,\ n = 4, \text{ and } \beta = 300\ \mathrm{K} = 27°\mathrm{C}.$$

From the equation $\rho = \text{constant}\ (1 - \beta/\mathrm{T})$, β can be calculated by plotting the ρ values (at different temperatures) *versus* 1/T. Using, at 15° and 25°, only derivatives **1** to **4**, the following ρ values can be calculated: +0.539 (15°) and +0.11 (25°). Regression analysis then yields the equation

$$\rho = -12.69 + (3.81 \times 10^3)/\mathrm{T}, \text{ with } s_{y/x} = 0.009,$$
$$s_b = 0.046,\ r = 0.9998, \text{ and } n = 4. \text{ Hence,}$$
$$\beta = 3.81 \times 10^3/12.69 = 300\ \mathrm{K} = 27°\mathrm{C}.$$

However, the above equations do not prove that the β-value has a real mechanistic meaning, because the temperature interval is not large, the differences between the V-values are small, and the standard deviation (s_b) on b is relatively large. The strongest evidence that the value of β is not an artefact stems from the experimental finding that ρ does indeed become zero at ~27°, and changes its sign between 15° and 40°. The existence of the isokinetic relationship indicates that all derivatives (except, perhaps, the *m*-nitrophenyl derivative) react by the same basic mechanism.

TABLE IV

INFLUENCE OF THE TEMPERATURE ON k_2 (pH 4.0); ACTIVATION PARAMETERS

Substituent	$k_2(sec^{-1})$						E_a *kcal.mol*$^{-1}$		$\Delta H^{\ddagger}$ *kcal.mol*$^{-1}$		$\Delta S^{\ddagger}$ *cal.degree*$^{-1}$. *mol*$^{-1}$	
	15°	*20°*	*25°*	*30°*	*35°*	*40°*	*a*	*b*	*a*	*b*	*a*	*b*
None	1.57	2.10	2.63	4.27	5.23	12.83	9.0	22.2	8.5	21.5	−28	+15
m-Methyl	1.43	—	2.60	4.00	—	15.07	10.2	23.5	9.6	22.8	−24	+19
m-Chloro	2.60	—	3.00	3.80	—	8.57	2.8	15.4	2.2	14.8	−49	− 7
m-Bromo	2.43	—	2.83	3.73	—	8.33	2.4	15.2	1.8	14.6	−51	− 8
m-Nitro	1.50	2.00	2.60	3.40	4.40	5.73	9.7	9.7	9.1	9.0	−26	−26
p-Nitro	2.20	3.03	4.17	5.80	8.00	10.87	11.5	11.5	10.9	10.9	−19	−19

[a]Calculated in the range 15° to 25°; [b]Calculated in the range 25° to 40°.

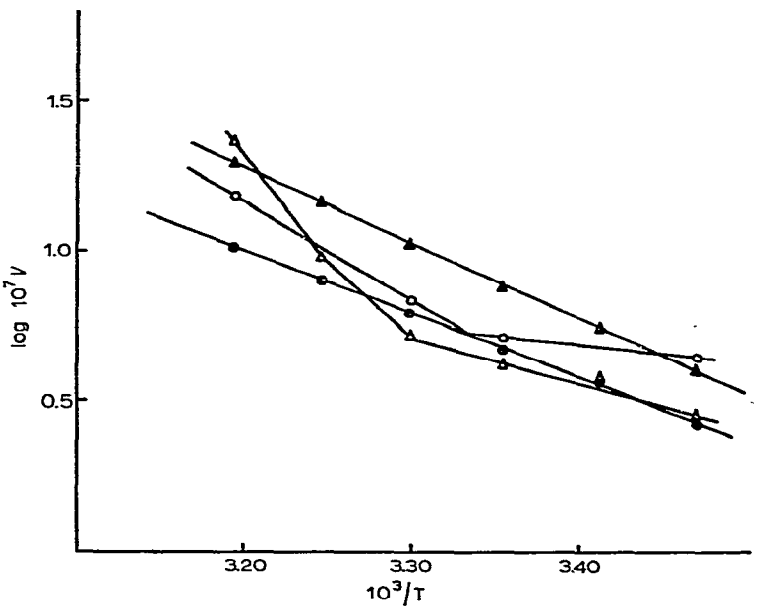

Fig. 2. Log *V versus* 1/T: ▲, *p*-nitrophenyl α-D-mannopyranoside; ●, *m*-nitrophenyl α-D-mannopyranoside; △, phenyl α-D-mannopyranoside; ○, *m*-bromophenyl α-D-mannopyranoside.

Influence of the temperature on V

As expected from the Hammett and isokinetic relationships, the influence of the temperature on the maximal rate *V* proved to be complex. For several substituted phenyl α-D-mannopyranosides, *V* was determined at five or six temperatures. As indicated in our previous paper[2], one unit of enzyme activity (pH = 4.0) equals $\sim 5 \times 10^{-9}$ "mol" of active site (on the basis of a molecular weight[2] of 220,000). This allows the calculation of the reaction constant k_2 and of $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ from the

theory[12] of absolute reaction rates. However, due to possible errors in the determination of the number of "mol" of active site per unit (*e.g.*, because of the presence of small amounts of inactive protein molecules), only the order of magnitude and the relative differences of the $\Delta S^{\ddagger}$ values are meaningful. Each value of k_2 in Table IV is the arithmetic mean of at least three determinations. From these values and from Fig. 2, it follows that log V ($\sim$log k_2) is linearly related to 1/T for the *p*- and *m*-nitrophenyl derivatives only, whereas the plots for the other derivatives are severely curved.

As can be seen from Table IV, the *p*-nitro derivative hydrolyses faster than its meta homologue, notwithstanding the higher $\Delta H^{\ddagger}$ of the former, because the change of entropy is less negative. The difference in rate increases with increasing temperature.

It is impossible to calculate the real activation parameters for the other derivatives, as the Arrhenius plots are curved. Therefore, we calculated two series of apparent activation parameters, the first in the range 15° to 25°, and the second in the range 25° to 40°. These calculations yield only crude approximations, and the physical meaning of the figures is not quite clear. However, they illustrate the large and complex influence of the temperature on the reaction. In general, an increase in the temperature results in a higher apparent $\Delta H^{\ddagger}$, which is, however, more than compensated by a less-negative $\Delta S^{\ddagger}$. For the meta series, the apparent activation parameters $\Delta H^{\ddagger}$ and $T\Delta S^{\ddagger}$ (calculated for the range 25° to 40°) are linearly dependent on σ, according to the equations:

$$\Delta H^{\ddagger} = 21.52 - 17.731\ \sigma, \text{ with } s_{y/x} = 0.121,\ s_b = 0.190,\ r = 0.9998, \text{ and } n = 5.$$
$$T\Delta S^{\ddagger} = 4.752 - 18.46\ \sigma, \text{ with } s_{y/x} = 0.125,\ s_b = 0.197,\ r = 0.9998, \text{ and } n = 5.$$

For the activation parameters calculated in the range 15° to 25°, an analogous dependence can be calculated for the methyl, bromo, chloro, and non-substituted derivative. However, the *m*-nitro compound does not fit the equation (it would yield a negative $\Delta H^{\ddagger}$-value):

$$\Delta H^{\ddagger} = 8.465 - 17.05\ \sigma, \text{ with } s_{y/x} = 0.027,\ s_b = 0.066,\ r = 0.9999, \text{ and } n = 4.$$
$$T\Delta S^{\ddagger} = -8.148 - 16.34\ \sigma, \text{ with } s_{y/x} = 0.026,\ s_b = 0.062,\ r = 0.9999, \text{ and } n = 4.$$

The actual values of the coefficients of these equations probably have no real mechanistic meaning. However, the equations indicate that there exists some kind of compensation between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ (*cf.* the isokinetic relationship), in the sense that an electron-withdrawing substituent lowers both $\Delta H^{\ddagger}$ and $T\Delta S^{\ddagger}$.

At the lower temperatures, the effect on $\Delta H^{\ddagger}$ is predominant; hence, the rate increases with increasing electron-withdrawing power of the substituent. By contrast, at the higher temperatures, electron-withdrawing substituents will decrease the rate, because their effect on $T\Delta S^{\ddagger}$ becomes predominant. Consequently, for derivatives **1** to **4**, $\Delta G^{\ddagger}$ is linearly dependent on σ, not because the derivatives belong to an isoentropic or isoenthalpic series, but because a compensation of the $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ parameters exists.

However, the large differences between the apparent activation parameters and their temperature dependence clearly indicate that these parameters do not represent merely the bond-breaking step, but a far more complex process. Consequently, also, the compensating effect does not operate on a single step, but on a more complex process.

Influence of the substituent on K_{app}

The values of K_{app} at various temperatures are given in Table III. The analysis of the dependence of log K_{app} on σ (Fig. 1) is difficult, because the exact meaning of K_{app} is not known with certainty. Application of the steady-state treatment of our proposed two-step mechanism shows that $K_{app} = (k_{-1} + k_2)k_3W/(k_2 + k_3W)k_1$. If it is assumed that $k_2 \ll k_3$ (formation of the enzyme–mannosyl complex ES' is rate-limiting), K_{app} can be simplified to $K_{app} = (k_{-1} + k_2)/k_1 = K_m$. Two further simplifications are possible: (*1*) either $k_2 \gg k_{-1}$, hence $K_{app} = k_2/k_1$; (*2*) or $k_2 \ll k_{-1}$, so that $K_{app} = k_{-1}/k_1 = K_s$, with K_s the dissociation constant of the enzyme–substrate complex ES. The first assumption is not very plausible, because the k_2-values themselves are small (Table IV). With $k_2 \sim 10\ sec^{-1}$ (*p*-nitro derivative; 40°) and $k_2 \gg k_{-1}$, the maximal value of k_{-1} would be 1 sec^{-1}. In this case, $K_{app} \approx k_2/k_1$, and k_1 would become 10/0.0026 = 3800 $l.mol^{-1}.sec^{-1}$. For the other derivatives, still lower values for k_1 would be calculated, and such values seem far too low to represent the rate constant for the formation of the enzyme–substrate complex. Consequently, we assume, as a working-hypothesis, that $k_2 \ll k_{-1}$ and thus that $K_{app} \approx K_s$.

However, if $K_{app} \approx K_s$, then log $1/K_{app}$ is a measure of the standard free energy of binding (formation of the ES complex). In this case, the plots of log K_{app} *versus* σ refer to the influence of the substituent (and the temperature) on the relative stability of the enzyme–substrate complex (Fig. 3).

When the data in Table III are used to calculate the ratio V/K_{app}, it is found that, at each temperature and for all the meta derivatives, log K_{app} is linearly related

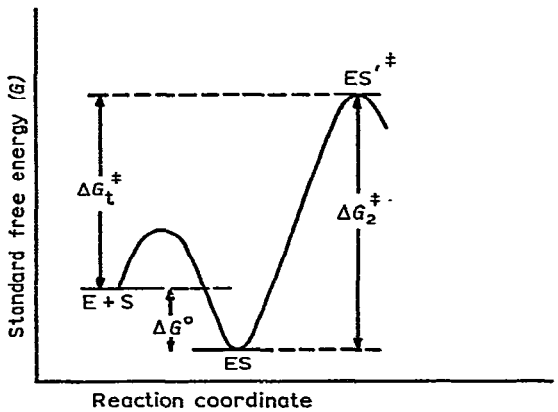

Fig. 3. Free-energy diagram; for details see text.

with σ. The *p*-nitro compound, however, does not fit the equations. This again indicates the peculiar influence of the location of the substituent. At each temperature, the reaction constant ρ is positive, as can be seen from the following equations:

$$\log 10^5 V/K_{app}\ (15°) = 0.898 + 0.758\ \sigma\ (r = 0.997),$$
$$\log 10^5 V/K_{app}\ (25°) = 0.945 + 0.950\ \sigma\ (r = 0.973),$$
$$\log 10^5 V/K_{app}\ (30°) = 0.832 + 1.095\ \sigma\ (r = 0.994),$$
$$\log 10^5 V/K_{app}\ (40°) = 1.066 + 0.965\ \sigma\ (r = 0.997).$$

Since $K_{app} = (k_{-1} + k_2)k_3/(k_2 + k_3)k_1$ and $V = k_{cat}(E_t) = (E_t)k_2k_3/(k_2 + k_3)$, the ratio $V/K_{app} = (E_t)k_1k_2/(k_2 + k_{-1})$.

With $k_2 \ll k_{-1}$, the ratio becomes $V/K_{app} = E_tk_1k_2/k_{-1} = E_tk_2/K_s = (E_t)k_2K_a$, with K_a $(= 1/K_s)$ the association equilibrium constant for the formation of the enzyme–substrate complex ES. In this case, $V/K_{app}(E_t)$ has the dimensions of an overall second-order constant for the reaction: $E + S \rightleftharpoons (ES')^{\ddagger}$, *i.e.*, the reaction step leading to the transition complex $(ES')^{\ddagger}$ of the bond-breaking sequence. When one plots log V/K_{app} *versus* σ, this is equivalent to plotting log V/K_{app} = log (E_t) + log k_2 + log K_a, and since (E_t) is constant throughout the whole series, log V/K_{app} is a relative measure of $\Delta G_t^{\ddagger} = \Delta G^{\circ} + \Delta G_2^{\ddagger}$, *i.e.*, the overall change in free energy between the transition state of the k_2-step and the free enzyme plus free substrate molecule (Fig. 3). Consequently, V/K_{app} values will give no information about the step leading to and from the ES complex itself.

From Fig. 1, it follows that, at 40°, the dissociation constant of the enzyme–substrate complex ($K_{app} \approx K_s$) linearly decreases with increasing electron-withdrawing power of the substituent, whereas, at the lower temperatures, the Hammett plot becomes biphasic. From Fig. 1, it also follows that the temperature has a more pronounced effect on the K_{app} values of derivatives **1** and **2** than on the values of the other derivatives. Such behaviour suggests that the influence of the aglycon group on the mechanism by which the binding-complex ES is formed (and on the relative stability of the complex itself) depends on the temperature, but in the sense that the extent to which the temperature can influence the effect of the substituent depends on the electronic character of the substituent itself.

Since it is improbable that such a small temperature interval would have a large influence on the conformation of the substrates themselves, it seems more logical to assume a temperature- and substituent-dependent change of the enzyme and/or of the surroundings. In this way, the peculiar influence of the position of the substituent on the phenyl ring and the existence of an isokinetic relationship with the isokinetic temperature ~27° becomes more acceptable, as they then refer to the binding of the substrate to a flexible protein molecule and not to a chemical process of bond-breaking. The analogy between the log V and log K_{app} plots suggests that the reason for the complex temperature-dependent influence of the substituent on k_2 (or V) could be found (at least partially) in the influence of the substituent on the ground-state energy of ES in the reaction step ES → $(ES')^{\ddagger}$. Consequently, the biphasic log V *versus* σ plot, and the nonlinear Arrhenius plots, would be caused, not

by a change of the rate-limiting step, but by a different initial binding-complex ES. It is noteworthy that derivatives with strong electron-withdrawing substituents (nitro), whose K_{app} does not show an abnormal temperature-dependence (Fig. 1), also show a linear log V *versus* 1/T plot.

Since the combined effects of the substituent and of the temperature on ΔG° ($\sim$log K_{app}) are also detectable in the $\Delta G^\ddagger$ ($\sim$log V) values (*cf.* the analogy between the Hammett plots in Fig. 1), they must change the free energy of the transition complex of the k_2-step in a different way than they change the free energy of the initial binding complex ES. Consequently, it is possible that these combined temperature–substituent effects will cancel out (at least partially) in $\Delta G_t^\ddagger = \Delta G^\circ + \Delta G_2^\ddagger$, so that log V/K_{app} *versus* σ will yield linear Hammett plots at each temperature.

CONCLUSIONS

All of the evidence obtained suggests that the α-D-mannosidase-catalyzed hydrolysis of substituted phenyl α-D-mannosides proceeds by a two-step mechanism with formation of a mannosyl–enzyme intermediate (Scheme 1). The fast reaction of the mannosyl–enzyme intermediate with water probably occurs through a mannopyranosyl cation. However, since only the α anomer of methyl D-mannopyranoside is formed[13] when methanol is added to the reaction mixture, the glycosyl oxonium–carbonium ion must be shielded in some way.

For the substrates used, the formation of the intermediate complex (glycosylation step) is the rate-determining step, and V is a relative measure of the rate at which this complex is formed. However, the experimental findings indicate that the glycosylation step is rather complex. The small overall influence of the substituent on the reaction rate is in agreement with (although it does not prove) a mechanism in which an enzyme group protonates the exocyclic oxygen atom, thus making the aglycon a good leaving group. An electron-withdrawing group will facilitate heterolysis of the glycosidic bond, but at the same time it will lower the extent of proton transfer by lowering the electron density around the glycosidic oxygen atom. Consequently, the small overall effect of the substituent may be due to cancellation of opposite effects. However, the influence of the temperature on both K_{app} and V, the isokinetic relationship, and the nonlinear Arrhenius plots clearly indicate that the effect of the substituent comprises more than its electronic influence on the bond-breaking step. The evidence available suggests that some substituent groups in the meta position have an unusual effect on the ground-state energy of the initial Michaelis–Menten complex, rather than on the bond-breaking step itself. The fundamental reason for this effect remains unknown, but it must reside in the protein nature of the catalyst. An altered conformation of the enzyme may have quite a large effect on, for example, the extent of directed proton-transfer to the glycosyl oxygen atom.

At this time, a further analysis of these substituent effects would be too hypothetical, because of the complex nature of the process and because insufficient data

are available. A more thorough study of the effect of the temperature, and of the substituent, on the activation parameters of the other members of the series is in progress.

EXPERIMENTAL

The substrates used were synthesized as described previously[14]. The purification, standardisation, and some characteristics of α-D-mannosidase from *Medicago sativa* L have been described[1,2]. Hydrolysis of non-chromogenic phenyl α-D-mannopyranosides was followed by measuring the liberated phenol by the 4-aminoantipyrine method[15] or by the method of Folin and Ciocalteu[16], and the liberated mannose with the *o*-toluidine reagent[17]. Release of nitrophenols was followed by adding aliquots of the reaction mixture to 10% (w/v) aqueous Na_2CO_3, and measuring the absorbance of the phenolate ion. Since all substrates used followed formal Michaelis–Menten kinetics, $[v = E_t k_{cat} K_{app} S/(1 + K_{app} S)]$, V and K_{app} were calculated from Hanes[18] plots, and/or by the method of Wilkinson[19]. All reaction rates were calculated on the same enzyme-activity basis (1 unit). Protein concentrations were determined by the method of Warburg and Christian[20] and were used, together with a molecular weight[1] of 220,000 for the α-D-mannosidase, to calculate the "mol" active site per unit. All determinations were carried out in 50mM McIllvaine buffer (pH 4.0).

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