Carbohydrate Research, 43 (1975) 173–182

EFFECT OF ADDED NUCLEOPHILES AND pH ON α-D-MANNOSIDASE-CATALYZED REACTIONS

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(Received December 2nd, 1974; accepted for publication, February 3rd, 1975)

ABSTRACT

The effect of (*1*) added nucleophiles, (*2*) the concentration of water, and (*3*) pH on the α-D-mannosidase-catalyzed hydrolysis of substituted phenyl α-D-mannopyranosides has been investigated. A two-step mechanism, with formation of a mannosyl–enzyme complex, is proposed. With the phenyl α-D-mannopyranosides, the formation of this intermediate complex is the rate-limiting step. The activity of the enzyme is controlled by two dissociable groups having pK ~3 and pK ~6, respectively. It is proposed that one of the groups functions as a proton-donor, whereas the other group stabilizes the mannosyl–enzyme complex by ion-pair formation or covalent binding.

INTRODUCTION

The enzyme α-D-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) is widely distributed in animal tissue, micro-organisms, and plant seeds. In a recent review, Snaith and Levvy[1] have summarized the purification procedure, characteristics, and reaction mechanism of α-D-mannosidase from various sources. In a previous[2] paper, we reported on the purification and some properties of α-D-mannosidase from the seeds of *Medicago sativa* L. The present paper deals with the mechanism of action of this enzyme, and especially with the influence of pH and added nucleophiles (alcohols) on the reaction rate.

RESULTS AND DISCUSSION

Enzyme stability

In order to obtain sufficient information about the influence of pH, the activity of α-D-mannosidase had to be measured over a broad pH range. However, as described previously[2], the spontaneous and irreversible denaturation of the enzyme precluded reliable measurements at and below pH 3. Since it was also found that the addition of Zn^{2+} resulted in a stabilization of the enzyme, it appeared worthwhile to investigate whether stabilization by this ion would be sufficient to allow rate measurements at pH 3.

A purified enzyme extract was dialyzed for 48 h at 4° against 50mM McIlvaine buffer (pH 4.0) containing mM $ZnSO_4$. Using *p*-nitrophenyl α-D-mannopyranoside (PNPM) (5mM) as substrate, the activity of the stabilized enzyme was then assayed at 25° and pH 3.0, 3.7, and 4.0, respectively. The rate of release of *p*-nitrophenol was followed by transferring, at regular time intervals, samples of the reaction mixture into 10% aqueous sodium carbonate and measuring the absorbance at 400 nm. Since, in each case, only initial velocities (maximum, 1% hydrolysis) were measured, a linear increase in absorbance would have been expected, provided no denaturation of the enzyme occurred. The results, however, clearly indicated that, notwithstanding the addition of Zn^{2+}, the enzyme was still strongly denatured at pH ~3.

In a second series of measurements, the rate of release of *p*-nitrophenol at pH 3 and 25° was followed during the first few minutes after the addition of the enzyme. The initial concentration of the substrate (PNPM) was varied from 0.5 to 6mM. In each case, the absorbance increased linearly with time up to ~5 min; thereafter, denaturation of the enzyme caused deviation from linearity.

Below pH 3, even with a stabilized enzyme, no initial linear part of the curve could be observed. In the absence of stabilization, immediate denaturation took place even at pH values between 3 and 3.5.

Consequently, all rate measurements between pH 3 to 3.5 were confined to the first 5 min after the addition of the enzyme, and performed with an enzyme extract stabilized with Zn^{2+}. In this way, reproducible measurements of the initial rate became feasible up to pH 3.

Influence of added nucleophiles

Valuable information about the mechanism of action of hydrolytic enzymes can be gained from the study of the influence of an added nucleophile (alcohol) on the rates of formation of different reaction products.

The reaction can proceed through a single-step mechanism according to Scheme 1.

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \begin{cases} \xrightarrow{k_3W} E+P_1+P_2 \\ \xrightarrow[k_4N]{} E+P_1+P_3 \end{cases}$$

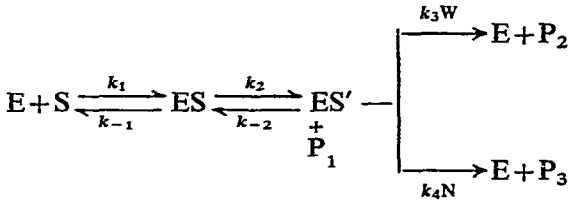
(Scheme 1), where

W and N are, respectively, water and nucleophile concentrations, P_1 is the aglycon group, P_2 is mannose, and P_3 the product (*e.g.*, methyl α/β-D-mannopyranoside) formed by the nucleophile N. By application of the steady-state treatment of this mechanism, Hinberg and Laidler[3] have shown that the reaction velocities $v_i = dP_i/dt$ and $i = 1, 2, 3$, were given by an equation of the form,

$$v_i = \frac{k_{cat,i} E_t [S]}{K_{app} + [S]}$$

with $k_{cat,1} = k_3W + k_4N$, $k_{cat,2} = k_3W$, $k_{cat,3} = k_4N$, and $K_{app} = \dfrac{k_{-1} + k_3W + k_4N}{k_1}$.

Another possibility is that the reaction proceeds by a two-step mechanism, with the formation of an intermediate enzyme–mannosyl complex ES', according to Scheme 2.



(Scheme 2)

According to Hinberg and Laidler[3], application of the steady-state treatment leads again to an equation of the form,

$$v_i = \frac{k_{cat,i} E_t [S]}{K_m + [S]} \qquad i = 1, 2, 3,$$

with $k_{cat,1} = \dfrac{k_2(k_3W + k_4N)}{k_2 + k_3W + k_4N}$, $k_{cat,2} = \dfrac{k_2 k_3 W}{k_2 + k_3W + k_4N}$, $k_{cat,3} = \dfrac{k_2 k_4 N}{k_2 + k_3W + k_4N}$,

$K_{app} = K_s \left[\dfrac{k_3W + k_4N}{k_2 + k_3W + k_4N}\right]$, and $K_s = \dfrac{k_{-1} + k_2}{k_1}$.

This mechanism predicts[3] that the ratio v_3/v_2 must be independent of the nature of the leaving group P_1.

Using *p*-nitrophenyl α-D-mannopyranoside (PNPM) and *p*-chlorophenyl α-D-mannopyranoside (PCPM) as substrates (5mM), v_1 (rate of phenol release) and v_2 (rate of mannose formation) were determined in the presence of various concentrations of methanol (Table I). From the data, it follows that (*1*) the rate of phenol release v_1 depends on the nature of the aglycon group, but not on the concentration of the alcohol; (*2*) since, for one and the same substrate, v_1 remains constant up to at least 2M methanol, the addition of the alcohol causes neither denaturation of the enzyme nor inhibition of the reaction; (*3*) for both substrates, v_2 decreases with increasing concentration of the alcohol; (*4*) the ratio v_1/v_2 (or v_3/v_2) is independent of the nature of the aglycon group. Especially, this last finding strongly suggests that the reaction proceeds by a two-step mechanism according to Scheme 2.

With PNPM as substrate, V_1 and V_2 (maximum rate of phenol and mannose release, respectively) were determined together with K_{app}, in the presence of various concentrations of methanol (Table II). The single-step mechanism requires that $k_{cat,2}$ ($\sim V_2$) is independent of the concentration of the nucleophile, hence $k_{cat,2}/W$ must remain constant. The data in Table II show that V_2/W decreases with increasing concentrations of the alcohol. Such an effect of methanol on V_2 makes the one-step mechanism highly improbable. However, if the hydrolysis proceeds by the two-step

TABLE I

INFLUENCE OF METHANOL ON THE HYDROLYSES[a]

Substrate[b]	*MeOH* (M)	H_2O (M)	v_1[c]	v_2	v_3	v_3/v_2	v_1/v_2
PNPM	0	55.28	1.000	1.000	—	—	1.000
(5mM)	0.5	54.20	0.983 ±0.015	0.938 ±0.016	0.0620	0.066	1.048
	1.0	53.11	1.036 ±0.014	0.895 ±0.032	0.105	0.117	1.158
	1.5	52.03	1.004 ±0.025	0.834 ±0.003	0.166	0.199	1.204
	2.0	50.94	1.018 ±0.013	0.750 ±0.066	0.250	0.333	1.357
PCPM	0	55.28	0.5923 ±0.012	0.5923 ±0.012	—	—	1.000
(5mM)	0.5	54.20	0.5911 ±0.015	0.5580 ±0.059	0.0343	0.062	1.061
	1.0	53.11	0.5916 ±0.014	0.5245 ±0.013	0.0678	0.129	1.129
	1.5	52.03	0.5900 ±0.011	0.5004 ±0.001	0.0919	0.184	1.184
	2.0	50.94	0.5967 ±0.011	0.4408 ±0.015	0.1515	0.343	1.354

[a]At 25° and pH 4.15; v is expressed as μmol $min^{-1}.u^{-1}$. [b]PNPM = *p*-nitrophenyl α-D-mannopyranoside; PCPM = *p*-chlorophenyl α-D-mannopyranoside. [c]Mean values: PNPM, 1.010 ±0.014; PCPM, 0.592 ±0.006.

TABLE II

RATE PARAMETERS FOR THE HYDROLYSIS OF *p*-NITROPHENYL α-D-MANNOPYRANOSIDE[a]

Methanol (M)	*Water* (M)	V_1 ($\mu mol.min^{-1}.u^{-1}$)	V_2 ($\mu mol.min^{-1}.u^{-1}$)	K_{app} (mM)		V_2/W
				a	*b*	
0	55.28	1.16 ±0.04	1.18 ±0.04	1.27 ±0.16		0.0213
1	53.11	1.22 ±0.06	1.08 ±0.03	1.42 ±0.05	1.37 ±0.11	0.0203
2	50.92	1.19 ±0.02	0.90 ±0.03	1.45 ±0.16	1.30 ±0.22	0.0177

[a]At pH 4.4 and 25° in the presence of methanol. [b]Determined by measuring *p*-nitrophenol (v_1). [c]Determined by measuring mannose (v_2).

mechanism and the release of the aglycon group is rate-limiting[3], then $k_{cat,1} \sim k_2$ and $K_{app} \sim K_s$. Thus, V_1 and K_{app}, although functions of the aglycon group, are independent of the concentration of the nucleophile, whereas the rate parameter $k_{cat,2}(V_2)$ still remains a function of N according to the equation $k_{cat,2} = k_2 k_3 W/(k_3 W + k_4 N)$.

According to Scheme 2, and with the assumption that $k_2 \ll k_3 W$ and $k_2 \ll k_4 N$, the quantity $1/v_2$ must be a linear function of N/W (or of N, since W remains approximately constant) if S is kept constant,

$$\frac{1}{v_2} = \frac{K_s + S}{k_2 E_t S} + \frac{K_s + S}{k_2 E_t S} \cdot \frac{k_4 N}{k_3 W} = a + b.N.$$

Using the values of Table I, the following equations can be calculated: $1/v_2(PNPM) = 0.983 + 0.160[N]$, with standard error of the estimate $s_{y/x} = 0.028$, standard error of the

slope $s_b = 0.018$, and correlation coefficient $r = 0.982$; and $1/v_2(\text{PCPM}) = 1.657 + 0.273[\text{N}]$, $s_{y/x} = 0.059$, $s_b = 0.037$, and $r = 0.973$. Since for both equations, the ratio b/a (slope/intercept) equals $k_4/k_3 W$, the same ratio must be found regardless of the substrate used. The experimental ratio is: for PNPM, $0.160/0.983 = 0.163$, and for PCPM, $0.273/1.657 = 0.165$. Consequently, the ratio k_4/k_3 is ~ 9.

The influence of other alcohols, such as ethanol and 1-propanol (both M), on v_1 and v_2 was investigated (pH 4.15, 25°), using PNPM (5mM) as substrate (Table III). Both alcohols show transfer activity, but 1-propanol also inhibits the reaction. However, the lowering of v_2 is larger than can be explained by this inhibition. From Scheme 2, with k_2 rate-limiting, it follows that, for each alcohol,

$$\frac{v_3}{v_2} = \frac{k_{cat,3}}{k_{cat,2}} = \frac{k_4 N}{k_3 W} = R \times \frac{N}{W}.$$

The factor R is a measure of the relative reactivity of each alcohol and can be calculated from the data of Tables I and III: methanol, 7.18 (mean value); ethanol, 7.66; 1-propanol, 7.08. These R-values are in good agreement with that (~ 9) calculated from the rate equations. They also indicate that the three alcohols used are equally effective as nucleophiles.

TABLE III

INFLUENCE OF ETHANOL AND 1-PROPANOL ON THE HYDROLYSIS OF *p*-NITROPHENYL α-D-MANNOPYRANOSIDE[a]

Nucleophile (1M)	H_2O (M)	v_1 ($\mu mol.min^{-1}.u^{-1}$)	v_2	v_3
None	55.28	1.00	1.00	0
Ethanol	52.06	1.003 ±0.005	0.874 ±0.010	0.129
1-Propanol	51.13	0.934 ±0.007	0.824 ±0.006	0.110

[a]5mM; pH 4.15; 25°.

Influence of 1,4-dioxane

A decrease in the water concentration, resulting from the addition of dioxane to the enzymic reaction mixture, may have an effect on the reaction rate. However, although transfer reactions are impossible, there are many other ways (inhibition of the reaction by the formation of an enzyme–dioxane complex, change of the dielectric constant of the medium, etc.) in which the added dioxane may affect the rate. In such cases, the mechanistic interpretation of the experimental results will be very difficult or even impossible. If, however, relatively high concentrations of dioxane have no effect at all, the analysis of the results will be much easier.

Using PNPM (5mM) as substrate, the rate of phenol release (25°; 50mM McIlvaine buffer, pH 4.15) was determined in the presence of increasing concentrations of 1,4-dioxane. Calculations of the molarity of water in the mixtures were

based on a weight of 996.03 g per litre of water at 25°. Each value of v in Table IV is the mean of at least six determinations, and the estimated standard deviation did not exceed 1.5%. From the data in Table IV, it follows that up to 3.5% dioxane the rate remained constant, and then decreased slowly at the higher concentrations.

TABLE IV

INFLUENCE OF 1,4-DIOXANE ON THE HYDROLYSIS OF *p*-NITROPHENYL α-D-MANNOPYRANOSIDE[a]

Dioxane (%, *v/v*)	H_2O (M)	v_1 ($\mu mol.min^{-1}.u^{-1}$)
0	55.283	1.00
0.5	55.006	1.004 ±0.012
1.0	54.730	1.011 ±0.010
1.5	54.454	1.016 ±0.009
2.0	54.177	1.008 ±0.008
2.5	53.901	0.997 ±0.012
3.0	53.624	0.994 ±0.009
3.5	53.348	0.993 ±0.014
4.0	53.071	0.986 ±0.005
5.0	52.519	0.979 ±0.014
10.0	49.755	0.919 ±0.014
15.0	46.990	0.869 ±0.006
20.0	44.226	0.796 ±0.012

[a]At pH 4.15 and 25°.

In the one-step mechanism, the rate of phenol release must depend on the concentration of water since $v = k_1 k_3 WE_t[S]/(k_{-1} + k_3 W)$. If the reaction proceeds *via* the two-step mechanism, two possibilities exist. When $k_2 \ll k_3 W$, the rate equation becomes $v = k_2 E_t[S]/(K_s + S)$, since $k_{cat,1} \sim k_2$ and $K_{app} \sim K_s$, and v will be independent of the water concentration. When $k_2 \gg k_3 W$, the rate will be given by $v = k_3 WE_t[S]/(K_{app} + S)$, with $K_{app} = K_s k_3 W/k_2$, and v will be function of W. The experimental observation that v remains constant up to 3.5% dioxane is thus a strong indication that the reaction proceeds through the two-step mechanism, with $k_2 \ll k_3 W$.

Influence of pH

Using PNPM as substrate, the maximal rate V and the apparent Michaelis–Menten constant K_{app} over the pH range 2.98–6.81 were determined. Below pH 3, the enzyme was too unstable, and above pH 7, the rate was too low, to allow reliable measurements. The calculated kinetic constants are summarized in Table V, and the usual plots of log V and log V/K_{app} are given in Fig. 1. With the possible exception of the value at pH 6.1, none of the K_{app} values differs significantly from the estimated mean ($K_{app} = 1.21 \pm 0.024$; confidence limits at the 95% level of significance = 1.21 ±0.053). The only possible conclusion is thus that K_{app} is independent of pH in the pH range 3–6.8. The pH dependence of V/K_{app}, which gives a measure of the pro-

totropic equilibria of the free enzyme, shows that V/K_{app} is dependent on two ionizable groups with pK ~6 and ~3, respectively. From a previous[2], less-extensive study on the influence of pH on the activity of α-D-mannosidase, it was concluded that enzyme groups with the above pK values were involved. However, due to the instability of our previous enzyme preparation at pH 3, the presence of the group with pK ~3 could not be firmly established at that time. The results of the present study seem to

TABLE V

INFLUENCE OF pH ON THE HYDROLYSIS OF *p*-NITROPHENYL α-D-MANNOPYRANOSIDE[a]

pH	V ($\mu mol.min^{-1}.u^{-1}$)	K_{app} (mM)	$10^4 V/K_{app}$ ($l.min^{-1}.u^{-1}$)
2.98	0.83 ±0.03	1.32 ±0.12	6.28
3.28	0.98 ±0.03	1.21 ±0.09	8.12
3.53	1.07 ±0.02	1.06 ±0.06	10.11
3.78	1.17 ±0.02	1.09 ±0.05	10.76
3.98	1.25 ±0.01	1.28 ±0.09	9.76
4.16	1.26 ±0.02	1.28 ±0.06	9.78
4.44	1.18 ±0.04	1.27 ±0.17	9.26
4.78	1.14 ±0.01	1.18 ±0.03	9.66
5.53	0.94 ±0.02	1.19 ±0.09	7.89
6.09	0.62 ±0.01	1.24 ±0.05	5.02
6.48	0.32 ±0.01	1.19 ±0.07	2.69
6.81	0.23 ±0.01	1.59 ±0.12	1.44

[a]5mM; at 25°.

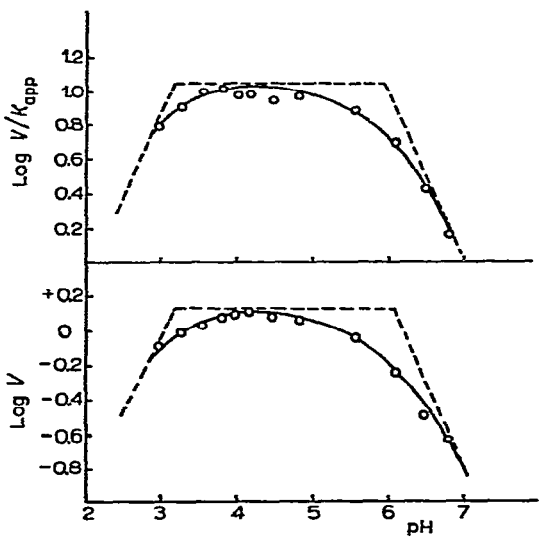


Fig. 1. Log V and log V/K_{app} *versus* pH (5mM *p*-nitrophenyl α-D-mannopyranoside; 25°).

confirm our previous conclusion as to the existence of a dissociable group with pK ~3. Also the log V–pH curve, which gives a measure of the prototropic equilibria of the enzyme–substrate complex, shows that there are two ionizable groups with pK ~3 and ~6, respectively. Only a rough estimate of the pK values can be given, but a comparison of the above estimates with the values listed by Dixon and Webb[4] indicates the possible participation of a carboxyl group (pK ~3) and a histidine imidazolium group (pK ~6). The rigorous identification of these groups needs further investigations.

The influence of pH may be accounted for in terms of a scheme, based on a two-step mechanism, and developed by Tenu *et al.*[5] for β-D-galactosidase.

The initial rate of phenol release will be given by the equation,

$$v_1 = dP_1/dt = \frac{E_t k_{cat}[S]}{K_{app}+[S]}.$$

The rate coefficients k_{cat} and K_{app} will be functions, not only of k_2 and k_3' ($k_3' = k_3 W$), but also of the Michaelis pH-equations (f, f', f''):

$$k_{cat} = k_2 k_3'/(k_2 f'' + k_3' f')$$

$$K_{app} = K_m f k_3'/(k_2 f'' + k_3' f').$$

However, if it is assumed again that the k_2-step remains rate-limiting in the pH range 3 to 6.8, so that $k_2 f'' < k_3 f'$, the above equations can be simplified to

$$k_{cat} = k_2/f'; \; K_{app} = K_m f/f'; \; V/K_{app} = E_t k_2/Kf.$$

With this assumption, both log V and log V/K_{app}, when plotted against pH, must yield the classical curve. K_{app} will be independent of pH when $K_a = K_a'$ and $K_b = K_b'$.

The results of the study on the pH-dependency and on the influence of added nucleophiles can be explained on a molecular basis by a mechanism analogous to the one proposed for lysozyme[6], α-D-galactosidase[7], and β-D-galactosidase[8]. It can be described as an ambident (or push-pull) mechanism, in which the aglycon is split off by the concerted action of a proton donor (*e.g.*, an imidazolium ion) and nucleophile (*e.g.*, a carboxylate group), with the formation of a mannosyl–enzyme intermediate. In going to the transition state of the k_2-step (Scheme 2), C-1 of the glycon group (sp^3) gains more sp^2 character, and the transition state resembles more nearly a glycosyl-carbonium ion[8]. Such a transition-state structure would explain the very strong ($K_i = 53 \mu M$) inhibition[2] of the α-D-mannosidase activity by D-mannono-1,5-lactone, since the lactone, being isosteric with the glycosyl cation, functions as a transition-state analogue. The transition state can be further stabilized through proton transfer from the imidazolium ion and through nucleophilic attack by the carboxylate group. The mannosyl residue in the ES' complex can be depicted as being covalently bound to the nucleophilic group, or as being stabilized by ion-pair formation. As demonstrated by Sinnott *et al.*[8] for β-D-galactosidase, it is possible that an equilibrium exists between the covalently bound mannosyl–enzyme complex and a

more-planar mannosyl cation, the latter being the reactive species in the subsequent attack of a water molecule.

As pointed out by Sinnott and Viratelle[9], methanol, being less nucleophilic than water to saturated carbon in both S_N1 and S_N2 reactions, should be less effective than water if the reaction of the alcohol with the mannosyl–enzyme complex proceeds by nucleophilic attack on a covalently bound sp^3 carbon atom. Actually, it was found that alcohols were more reactive (~8 times) than water towards the mannosyl–enzyme. Consequently, if the nucleophilic attack occurs on sp^3 carbon, preferential binding of alcohols to the active site of the enzyme must be invoked. However, if binding of the alcohols does occur, the factor R (~8) is no longer equal to k_4/k_3, but becomes a complex function of the rate and equilibrium constants. In such a case, it seems difficult to explain why different alcohols still have the same R-value.

According to the same authors, the alcohol should be more reactive than water towards a glycosyl cation, since C-1 in the half-chair conformation resembles a carbonyl-type carbon. Reaction *via* a mannosyl cation can thus explain the higher reactivity, without invoking preferential binding of the alcohol molecule. Consequently, the mechanism proposed by Sinnott[8] for β-D-galactosidase seems also to be the most plausible explanation in the case of α-D-mannosidase, both for the pH dependency and for the influence of added alcohols.

EXPERIMENTAL

The substrates used were synthesized as described previously[10]. The purification, standardisation, and some characteristics of α-D-mannosidase from *Medicago sativa* L have been described[2]. Release of *p*-nitrophenol was followed by adding aliquots of the reaction mixture to 10% (w/v) aqueous Na_2CO_3 and measuring the absorbance at 400 nm. Hydrolysis of non-chromogenic phenyl α-D-mannopyranosides was followed by measuring the liberated phenol using the 4-aminoantipyrine method[11], or the liberated mannose with the *o*-toluidine reagent[12]. Only initial velocities were measured, and each value is the estimated mean of at least three determinations. All substrates used followed Michaelis–Menten kinetics; neither substrate nor product inhibition was observed. In order to detect significant, experimental aberrations, V and K_{app} were first estimated from Hanes[13] plots, and then calculated by the method of Wilkinson[14]. The enzyme solution was standardized prior to use, and all reaction rates were calculated on the same enzyme–activity[2] basis (1 unit). All determinations were carried out in 50mM McIllvaine buffers.

ACKNOWLEDGMENT

One author (J.D.P.) is indebted to the Instituut voor Wetenschappelijk Onderzoek in Landbouw en Nijverheid (I.W.O.N.L.) for financial support.

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