A PRELIMINARY STUDY OF THE NUCLEOPHILIC COMPETITION IN β-GALACTOSIDASE CATALYZED REACTIONS.

O. Viratelle, J.P. Tenu, J. Garnier*, J. Yon.
Laboratoire d'Enzymologie Physico-chimique et Moléculaire
(G.R. CNRS) Faculté des Sciences - 91 ORSAY - France.

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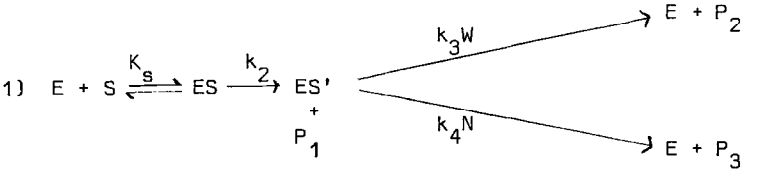
Summary Evidence is given for the formation of an intermediary complex following the Michaelis complex in galactoside hydrolysis catalyzed by β-galactosidase. A two step scheme is proposed. The formation of the intermediary complex is the limiting step in phenylgalactoside hydrolysis, while each of the two steps is partially rate controlling in o-nitrophenylgalactoside hydrolysis.

The mechanism of galactoside hydrolysis catalyzed by β-galactosidase from E. Coli is still unknown. A tentative scheme has been proposed by Wallenfels and Malhotra in 1961 (1) involving histidyl and cysteinyl residues as catalytic groups essentially on the basis of the pH rate profile. Later, data were presented (2,3) suggesting that the cysteinyl group does not play a role.

This enzyme is known to be able to promote transfer reactions in the presence of some nucleophilic compounds (1,4). Therefore, it seemed possible to apply a nucleophilic competition method as for other hydrolytic enzymes (5,6). This method can provide some informations about the pathway of the reaction. The present work is an attempt in this direction.

Two substrates have been studied, o-nitrophenyl β-D-galactoside (ONPG) and phenyl β-D-galactoside (PG). For the interpretation of the data, a scheme involving two intermediates is proposed :



*Laboratoire de Biochimie, Faculté des Sciences - 33 Bordeaux - France.

where E stands for the enzyme, S for the substrate, P_1 for the aglycon part of the substrate, P_2 and P_3 for, respectively, the galactose and the alkylgalactoside resulting from the transfer reaction, and N for the nucleophilic compound competing with water (W).

Under steady-state conditions, the kinetic parameters are :

$$\frac{1}{E_t}\frac{dP_1}{dt} = k_{cat_1} = \frac{k_2 (k'_3 + k_4 N)}{k_2 + k'_3 + k_4 N}$$

$$\frac{1}{E_t}\frac{dP_2}{dt} = k_{cat_2} = \frac{k_2 k'_3}{k_2 + k'_3 + k_4 N}$$

$$\frac{1}{E_t}\frac{dP_3}{dt} = k_{cat_3} = \frac{k_2 k_4 N}{k_2 + k'_3 + k_4 N}$$

$$K_m = K_s \frac{k'_3 + k_4 N}{k_2 + k'_3 + k_4 N}$$

where E_t is the total enzyme concentration, and $k'_3 = k_3 W$.

MATERIALS AND METHODS

β-galactosidase (EC 3.2.1.23) has been prepared from E. Coli 2E01 according to Perrin's method (7,8).

ONPG hydrolysis has been followed by the optical density change at 373 nm with a Cary Model 14 spectrophotometer. PG hydrolysis has been followed either by the optical density change at 280 nm in a Cary Model 16 spectrophotometer or by measuring simultaneously the phenol (9) and the galactose (10) on the same aliquot after addition of Na_2CO_3 (0.1 N final concentration). K_m and k_{cat_1} have been obtained from the integrated Michaelis equation in the spectrophotometric methods, and k_{cat_2} with a saturating concentration of substrate for the galactose in the chemical method.

The enzymatic reactions have been carried out in the following conditions :

T = 25 ± 0.2°C, [NaCl] = 0.145 M, [Mg^{2+}] = 10^{-3}M in a pH 6.9 ± 0.1 TES buffer (N-Tris (hydroxymethyl) methyl-2-amino-ethane sulfonic acid). It was checked that, in our experimental conditions, the reversibility of the reaction can be neglected.

The data have been submitted to a statistical treatment according to the iterative method of Cleland (11) programmed for a Wang computer system.

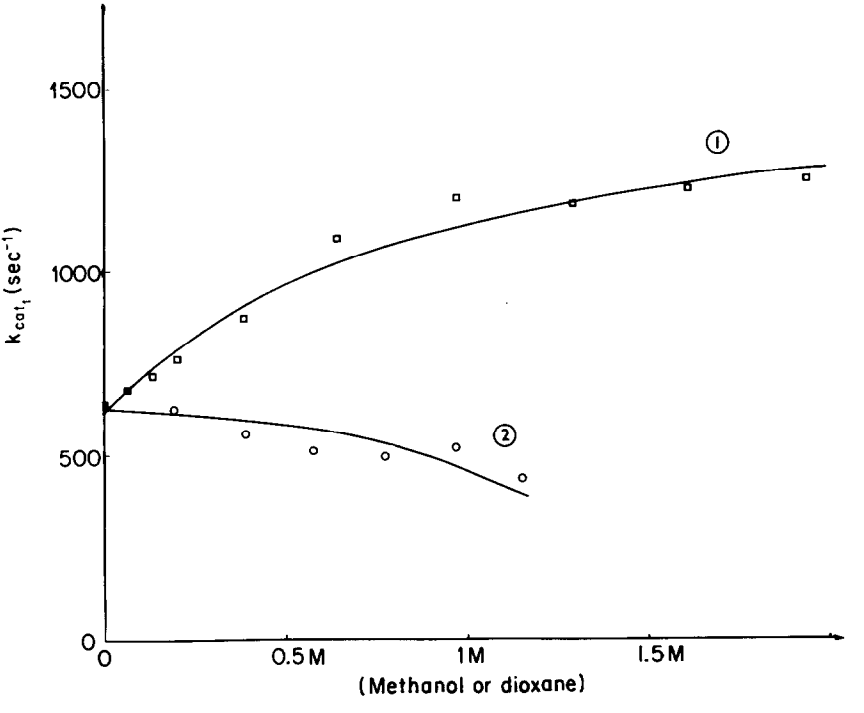


Figure 1 : Variation of k_{cat_1} in ONPG hydrolysis. Experimental conditions are described in "Materials and Methods".
Curve 1 (□) : Effect of methanol concentration on k_{cat_1}. The points are experimental, and the curve is fitted according to the theoretical equation derived from scheme 1.
Curve 2 (○) : Effect of dioxane concentration on k_{cat_1}.

RESULTS AND DISCUSSION

Both K_m and k_{cat_1} obtained from ONPG hydrolysis increase with the methanol concentration and they tend to level off (see figures 1 and 2) as can be expected if the alcohol competes with water according to scheme 1. Shifrin and Hunn (4) have also observed that methanol stimulates enzyme activity. In

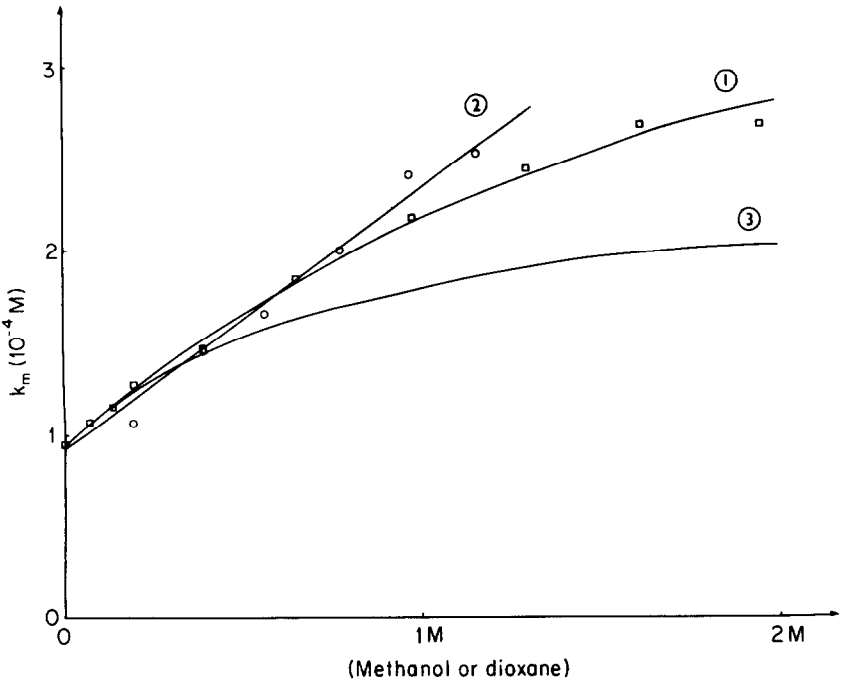


Figure 2 : Variation of K_m in ONPG hydrolysis. Experimental conditions are described in "Materials and Methods".
Curve 1 (□) : Effect of methanol concentration on K_m. The points are experimental, and the curve is fitted according to the theoretical equation derived for scheme 1.
Curve 2 (○) : Effect of dioxane concentration on K_m.
Curve 3 : Theoretical curve of the effect of methanol concentration on K_m calculated from the variation of k_{cat_1}. (see text).

addition, they have shown that alcohols do not affect significantly either the tertiary or the quaternary structure of the enzyme.

On the other hand, if dioxane, instead of methanol, is added to the reaction mixture, only K_m is significantly increased but not k_{cat_1} (figures 1 and 2). The fact that curves 1 of figures 1 and 2 approache a limiting value is clearly consistant with an intermediary complex which does form after the Michaelis complex and is susceptible to nucleophilic attack by water or alcohol (6). The various kinetic parameters obtained from the analysis of the experimental data are reported in Table I. As it was difficult to take account of the solvent effect on K_m, K_s has been calculated with the values of k_2 and k'_3 obtained from the variation of k_{cat_1}. The formation of the intermediary complex is only a partially rate controlling step for ONPG hydrolysis, contrary to what has been pre-

TABLE I

Kinetic parameters for the hydrolysis of PG and ONPG by β-galactosidase [a].

Substrate	$10^{-3}k_2$ sec^{-1}	$10^{-3}k'_3$ sec^{-1}	$10^{-3}k_4$ M^{-1} sec^{-1}	k_4/k'_3 M^{-1}	K_s $10^{-4}M$	K_m $10^{-4}M$	$10^{-3}k_{cat}$ [d] sec^{-1}
PG	0.035	-	-	1.96 [b]	0.9±0.2	0.9± 0.2	0.035
ONPG	1.6±0.3	1.0±0.15	2.6 ± 1.5	2.56 [c]	2.6±0.6	1± 0.2	0.6

a - calculated for one monomer of a M.W. = 135,000
b - standard error 0.19
c - standard error 0.61
d - K_m and k_{cat} have been determined by Eadie's plot without methanol

N.B. The variation ranges indicated correspond to a confidence limit of 0.9

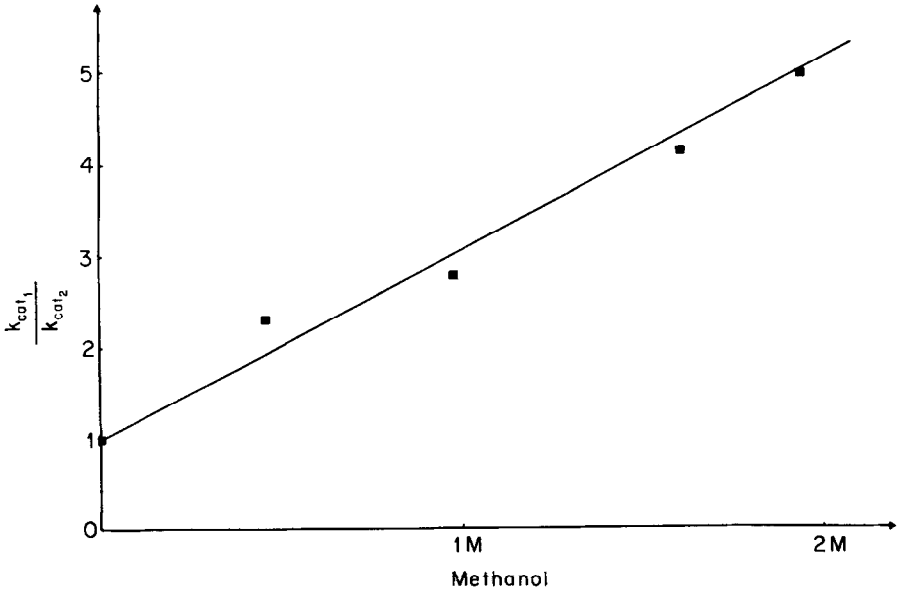


Figure 3 : Effect of methanol concentration on the ratio $\frac{k_{cat_1}}{k_{cat_2}}$ in PG hydrolysis (see text). Experimental conditions are described in "Materials and Methods".

viously reported (1). But, with such rate constants, neither the presteady - state nor the burst can be expected to be observed with a stopped-flow apparatus.

Similar experiments have been carried out with PG where k_{cat_1} is slightly decreased (20%) as in dioxane and K_m does not vary significantly. In this case, such results indicate that the rate limiting step of the reaction is the formation of the intermediary complex ES' ($k_2 << k'_3$). The ratio k_{cat_1}/k_{cat_2} increases linearily (figure 3) and allows one to determine k_4/k'_3 (table I).

Although no direct experimental proof has yet been given for a sequential liberation of products, the similar values of the ratio k_4/k'_3 for the two substrates favour a common intermediate step as proposed above.

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