

# Measurement and Study of Substrate Specificity of Exoglucosidase Activity in Eutrophic Water

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**The  $\alpha$ - and  $\beta$ -glucosidase activity in natural samples can be readily measured during short incubation times (20 min) by using the artificial substrates 4-methylumbelliferyl- $\alpha$ -D-glucoside and 4-methylumbelliferyl- $\beta$ -D-glucoside. The apparent  $K_m$  of both  $\alpha$ - and  $\beta$ -glucosidase for these respective substrates is 0.01  $\mu$ M. The homologous disaccharides maltose and cellobiose competitively inhibit  $\alpha$ - and  $\beta$ -glucosidase, respectively. Absolute substrate specificity of the  $\alpha$ - and  $\beta$ -glucosidase is observed with respect to the configuration of carbon atoms 1 and 4. Enrichment cultures on either  $\alpha$ - and  $\beta$ -glucoside result in increasing activity of the corresponding glucosidase, both in absolute terms and with respect to the other glucosidase.**

Plant biomass is the major source of organic carbon for bacterial activity. This material is mostly macromolecular and can only be taken up by microorganisms after exoenzymatic hydrolysis (13). As pointed out by Dowson and Liebezeit (2), combined sugars constitute a large fraction of this polymeric organic matter. However, little information is available on the exoenzymatic hydrolysis of polysaccharides in natural environments, owing to the lack of a method with adequate sensitivity for determining the activity of the hydrolytic enzymes.

Duddridge and Wainwright (3) evaluated the cellulase activity of river sediments by observing the rate of glucose release. Meyer-Reil (10), by the method of Morrison et al. (11), developed a technique for marine sediments which is based on the addition of a chromophoric analog of a disaccharide compound (*p*-nitrophenyl- $\beta$ -D-glucoside) and spectrophotometrically measuring its hydrolysis by the appearance of *p*-nitrophenol. Neither of these methods are very sensitive, however, and they require long incubation times (24 and 6 h, respectively).

Fluorimetric essays appear to be suitable for measurement of enzymatic activity in natural waters (14).

Hoppe (8) used a sensitive method derived from the fluorimetric essay of Robinson (12) for brackish water environments. A 4-methylumbelliferylglucopyranoside added to the medium was hydrolyzed by glucosidase, liberating 4-methylumbelliferone (4MU) a highly fluorescent compound. Hoppe, however, used incubation times of 3 to 9 h. Long incubation times are not recommended for the measurement of natural processes, owing to the difficulty of maintaining the *in situ* conditions throughout the incubation.

I report here a modification of this fluorimetric method which allows accurate measurements of glucosidase activity in natural water during short-term incubations. I also demonstrate the high specificity of the enzymatic hydrolysis of glucosides and the induction of the glucosidase.

## MATERIALS AND METHODS

**Collection of samples.** The samples were collected at the surface in (i) the Belgian coastal area (Southern bight of the North Sea), (ii) the Western Scheldt estuary, and (iii) a pond (Bois de la Cambre, Brussels), i.e., in three different eutrophic environments.

**Development of the test procedure.** A quartz cell (1 cm optical path) is filled with 2 ml of the sample. A 50- $\mu$ l volume

of a sterile aqueous solution of a 4-methylumbelliferylglucoside, for instance, 4-methylumbelliferyl- $\beta$ -D-glucopyranoside (4MU $\beta$ G; Sigma Chemical Co.) was added to the sample, yielding a final concentration of 1.5  $\mu$ M (Fig. 1). The fluorescence of the 4-methylumbelliferone (4MU) produced by the hydrolysis of the substrate (Fig. 1) was measured as a function of time over 15 to 20 min with a Perkin Elmer 2000 fluorimeter.

For convenience, the excitation wavelength ( $\lambda_{ex}$ , 340 nm) has been chosen equal to that used for the measurement of exoproteolytic activity (14). Therefore, it was possible to determine both enzymatic activities of a natural sample without readjustment of the fluorimeter. The wavelength of maximum absorption of 4MU is 365 nm. The use of a  $\lambda_{ex}$  of 340 nm decreases the emission signal of 4MU by 16%.

Previous studies (7, 12) have recommended assaying the fluorescence of 4MU in a 0.15 M glycine buffer (pH 10.3), to optimize the fluorescence of 4MU and ensure its stability (9). I eliminated this supplementary step by the simultaneous assay of a 2-ml reference sample to which 50  $\mu$ l of a sterile aqueous solution of 4MU was added to a final concentration of  $0.6 \times 10^{-8}$  M. The use of a reference sample allowed the verification of the stability of the fluorescence signal of 4MU during the incubation.

In 90% of the cases, the reference solution had a stable fluorescence. In the remaining cases, the increase of fluorescence in the experimental sample was corrected for the increase or decrease of fluorescence in the reference sample. The variations in fluorescence of the reference sample were always <20% of the experimental results and were independent of the 4MU concentration. In addition, the rise of fluorescence which was due to the addition of 4MU at the pH of the natural sample was used to calibrate the signal produced by the hydrolysis of 4MU $\beta$ G in terms of 4MU liberated per liter  $\cdot$  min. The pH of the samples studied varied from 7 to 7.9. Their adjustment to pH 10.3 by the glycine buffer would have raised the fluorescence of 4MU from 53 to 30%, respectively.

One must note that the substrate concentrations described here (1.5  $\mu$ M) were considerably lower than those used by Morrison et al. (5 mM [11]) and Hågerdal et al. (10 mM [7]), and they were in the range of 0.5 to 40  $\mu$ M, as recommended by Hoppe (8). In view of the native fluorescence of the substrate, the low concentration of substrate used here renders the method more sensitive.

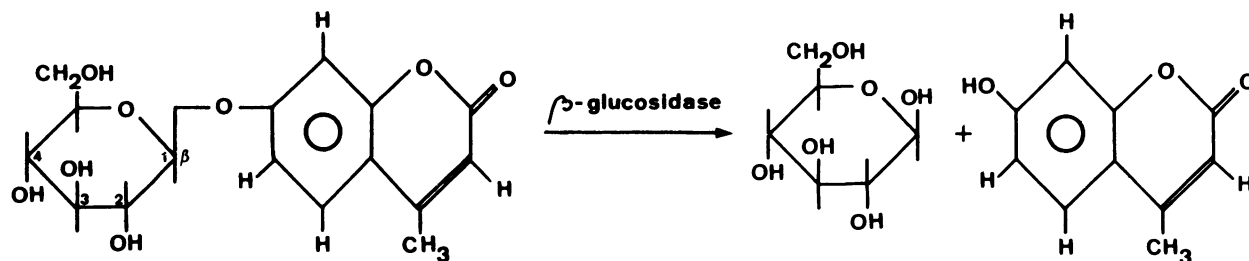


FIG. 1. Hydrolysis of 4MUβG into glucose and 4MU.

The same methods were also used for the determination of  $\alpha$ -glucosidase activity, with 4-methylumbelliferyl- $\alpha$ -D-glucopyranoside (4MU $\alpha$ G; Sigma) as a substrate. The experimental procedure adopted was exactly the same as that described above. The substrate (4MU $\alpha$ G) concentration chosen was identical, 1.5  $\mu$ M.

**Bacteriological activity in aquatic environments.** Parallel determinations of  $\beta$ -glucosidase activity, glucose incorporation, and bacterial production were performed on samples from the North Sea, the Scheldt estuary, and a pond (Bois de la Cambre, Brussels) at in situ temperature in the spring of 1983.

High-specific-activity [ $U$ - $^{14}C$ ]glucose (230 mCi mmol $^{-1}$ ) incorporations were determined by the method of Billen et al. (1). The amount of glucose added can be considered negligible with respect to natural concentrations. Incubations (10 ml of sample plus 1  $\mu$ Ci of [ $U$ - $^{14}C$ ]glucose) were performed in the dark in 2-h periods. The sample was then filtered through a membrane filter (pore size, 0.2  $\mu$ m). The radioactivity retained on the filter was measured by liquid scintillation.

Bacterial production by [*methyl*- $^3H$ ]thymidine (50 Ci mmol $^{-1}$ ) incorporation was determined by the method of Fuhrman and Azam (5, 6). Incubations were performed at 20 nM of thymidine, because experiments in the Scheldt estuary showed that this concentration saturates the bacterial incorporation. After a 1-h incubation, the 5% trichloroacetic acid-insoluble fraction of the sample was collected on a membrane filter (pore size, 0.2  $\mu$ m), and its radioactivity was measured by liquid scintillation.

**Substrate specificity of exoglucosidase.** The substrate specificity of the glucosidase activity was determined by assaying the effect on the hydrolysis of 4MUβG of increasing concentrations (0.15 to 1.5  $\mu$ M) of various disaccharides, including cellobiose ( $\beta$ -D-glucopyranosyl-1-4- $\alpha$ -D-glucopyranose),

saccharose ( $\alpha$ -D-glucopyranosyl-1-2- $\beta$ -D-fructofuranose), maltose ( $\alpha$ -D-glucopyranosyl-1-4- $\beta$ -D-glucopyranose), and lactose ( $\beta$ -D-galactopyranosyl-1-4- $\beta$ -D-glucopyranose). The natural water used in these essays was collected in August 1983, at the surface of an eutrophic pond (Bois de la Cambre, Brussels). The sample was left overnight at room temperature to stabilize the bacterial activity so that all inhibition experiments could be carried out within the same day, without any important modification of the reference activities.

Similar experiments were conducted with 4MU $\alpha$ G as a substrate, again with cellobiose, saccharose, maltose, and lactose concentrations of 0.15 to 1.5  $\mu$ M.

**Enrichment of natural waters by polysaccharides.** To test the reaction of natural bacterial assemblages to addition of various polysaccharides, enrichment cultures were developed with  $\alpha$ - or  $\beta$ -glucopyranosides. Samples (50 ml) of pond water (Bois de la Cambre, Brussels, collected November 1983) were filtered on GF/C fiberglass filters, inoculated with 1% unfiltered water, and then enriched with 20, 50, 100, 150, 200, and 400 mg of C per liter of glucose, cellobiose, maltose, and starch. A (NH $_4$ ) $_2$ SO $_4$  and K $_2$ PO $_4$  solution (47 and 4.4 g/liter, respectively) was added to achieve a C/N/P ratio of 100:10:1. In the exponential-growth phase, 20 ml of the cultures were centrifuged for 10 min at 10,000 rpm. Any remaining glucosidase inhibitors were removed by washing the centrifugate twice with 5 ml of sterile, filtered pond water. The centrifugate (cells) was finally suspended in 20 ml of sterile, filtered pond water. Both  $\alpha$ - and  $\beta$ -glucosidase activities were then measured as previously described. Optical densities were measured simultaneously at 410 nm with a spectrophotometer (Spectronic 710; Bausch & Lomb).

## RESULTS

**Fluorogenic substrate hydrolysis.** Figure 2 shows an example of the variations of fluorescence observed in a hydrolysis experiment with 4MUβG and in the corresponding reference solution. Autoclaved samples showed no activity, indicating that the activity was entirely enzymatic. Samples filtered through membranes (pore size, 0.2  $\mu$ m) showed no glucosidase activity, indicating that the  $\beta$ -glucosidase activity is associated with particles. Chemical hydrolysis of 4MU $\alpha$ G or 4MUβG is unlikely since glucosides were very stable compounds except in strong acidic conditions.

The 4MUβG concentration used (1.5  $\mu$ M) has been chosen on the basis of hydrolysis experiments at different concentrations in substrate. The hydrolysis of 4-MUβG, at a zero concentration of cellobiose, obeys Michaelis-Menton kinetics, with an apparent  $K_m$  of 0.01  $\mu$ M (Fig. 3A).

The 4MU $\alpha$ G concentration used to test  $\alpha$ -glucosidase activities was the same as that for 4MUβG (1.5  $\mu$ M). Indeed, the apparent  $K_m$  of the enzymes for 4MU $\alpha$ G is also 0.01  $\mu$ M.

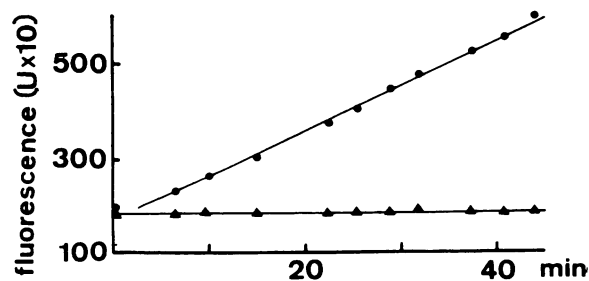


FIG. 2. Evolution with time of the fluorescence of a sample of pond water (Bois de la Cambre, Brussels) with 1.5  $\mu$ M of 4MUβG (●) and with 0.6  $10^{-8}$  M of 4MU (▲). The fluorescence is expressed in the units directly read on the fluorimeter. Addition of 0.6  $\times 10^{-8}$  M of 4MU rises the fluorescence of the sample by 30 U ( $\times 10$ ).

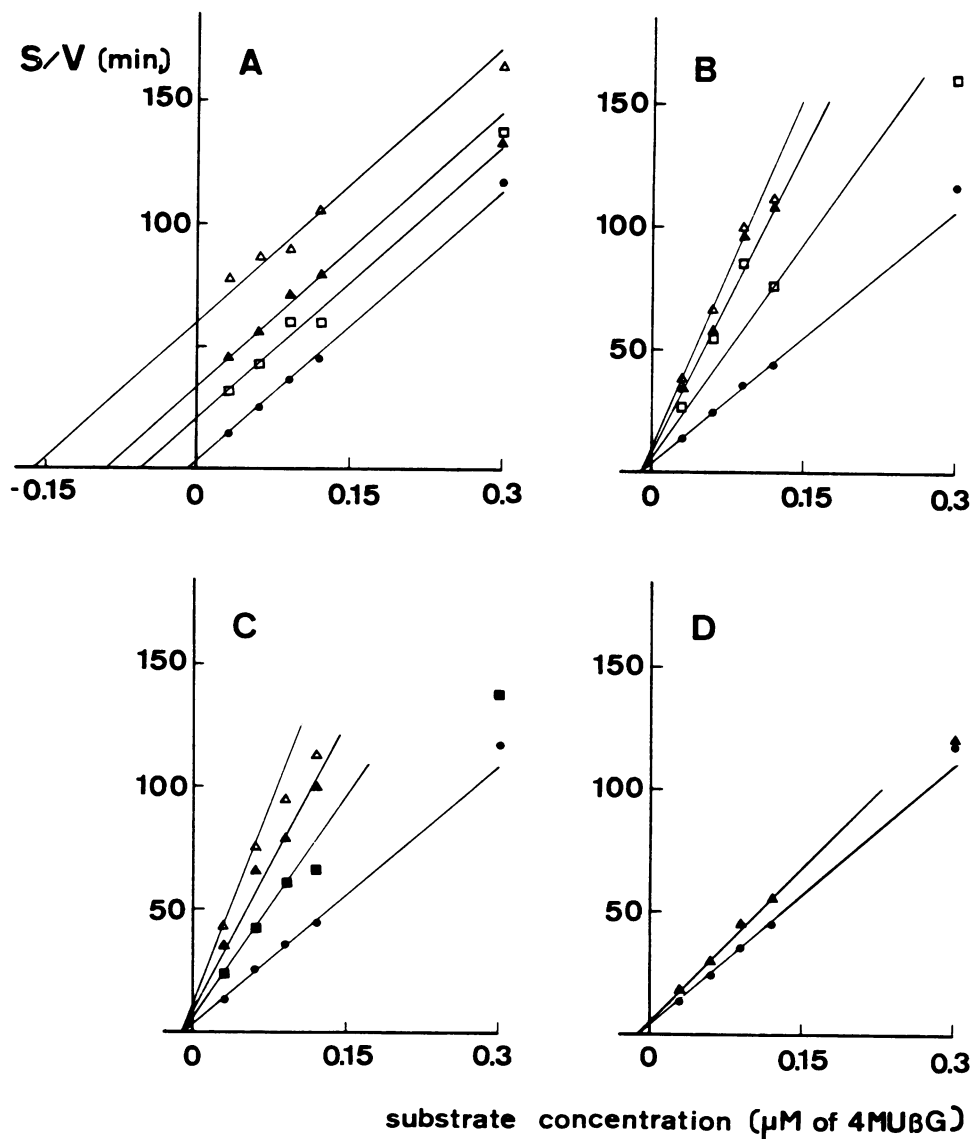


FIG. 3. Inhibitory effect of different concentrations of cellobiose (A), saccharose (B), maltose (C), and lactose (D) on the  $\beta$ -glucosidase activity in pond water (Bois de la Cambre, Brussels). Concentrations:  $\Delta$ , 1.5  $\mu\text{M}$ ;  $\blacktriangle$ , 0.74  $\mu\text{M}$ ;  $\square$ , 0.30  $\mu\text{M}$ ;  $\blacksquare$ , 0.15  $\mu\text{M}$ ;  $\bullet$ , 0  $\mu\text{M}$ .

**Glucosidase and bacterial activities in aquatic environments.** Figure 4 represents the potential  $\beta$ -glucosidase activity as a function of glucose incorporation (Fig. 4A) on the one hand and as a function of the thymidine incorporation (Fig. 4B) on the other hand. In each case, the in situ temperature at which incubations have been carried out is indicated.

Although the glucose incorporations are termed in percent hour<sup>-1</sup>, they are a good approximation of the absolute rate of utilization owing to the constance of glucose concentration in the North Sea and the Scheldt estuary, 0.02 to 0.1  $\mu\text{M}$ . They also are a good evaluation of the general metabolism of polysaccharides by bacteria (1).

The regression lines obtained between  $\beta$ -glucosidase activity and glucose incorporation ( $r = 0.972$ , Fig. 4A) and between  $\beta$ -glucosidase activity and thymidine incorporation ( $r = 0.944$ , Fig. 4B) indicate an excellent agreement between the enzymatic activity measurements and the two independent evaluations of bacterial activity. Their respective slopes

(0.926 and 1.102) are very close to 1, indicating a first-order relationship between the variables.

**Substrate specificity of exoglucosidases.** Inhibition experiments of 4MU $\beta$ G hydrolysis by various disaccharides were carried out.

In these experiments, a competitive inhibition of the hydrolysis rate of the artificial substrate by a disaccharide indicates that the enzyme assayed is specific for the type of polysaccharide-bond present in the disaccharide. In a semi-reciprocal plot (substrate/velocity versus substrate), a competitive inhibition results in parallel straight lines for the different concentrations of the inhibitor.

Only cellobiose, which is structurally homologous to 4MU $\beta$ G ( $\beta$ -D-glucopyranoside), competitively inhibits the hydrolysis of the artificial substrate (Fig. 3). The  $\alpha$ -D-glucopyranosides tested (saccharose and maltose) are non-competitive inhibitors for the hydrolysis of 4MU $\beta$ G, as indicated by the convergence of the straight lines of Fig. 3B

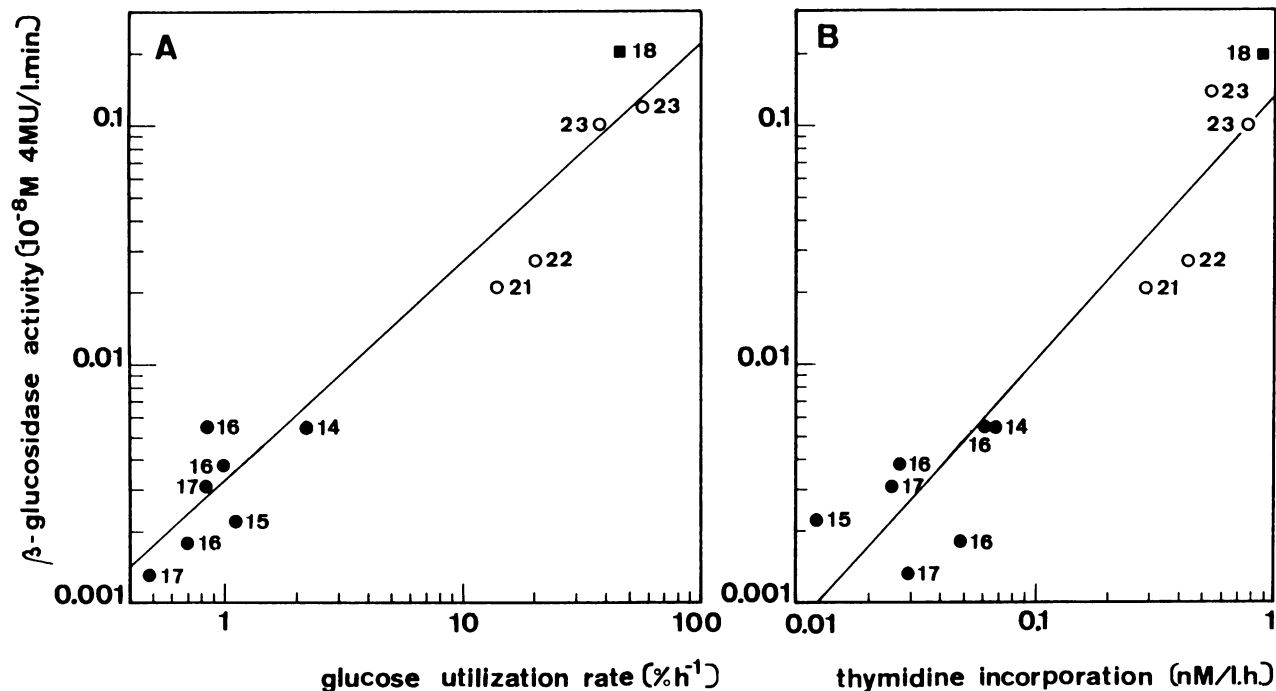


FIG. 4.  $\beta$ -glucosidase activity as a function of glucose utilization rate (A) and of thymidine incorporation (B). Symbols: ●, coastal sea; ○, Scheldt estuary; and ■, eutrophic pond water (Bois de la Cambre, Brussels). For each sample, the in situ temperature at which incubations were carried out is indicated.

and 3C on the  $x$ -axis. Lactose ( $\beta$ -D-galactopyranoside) as shown in Fig. 3D has a very weak effect on the hydrolysis of the substrate. Its inhibition is likely noncompetitive. Table 1 reports the type of inhibition and the range of  $K_i$  observed for the four disaccharides tested. The measurement of the hydrolysis of 4MU $\beta$ G appears thence to be a specific determination of  $\beta$ -D-glucosidases.

For 4MU $\alpha$ G as the substrate, the types of inhibition and the inhibition constants observed are reported in Table 2. Maltose is a competitive inhibitor of the hydrolysis of 4MU $\alpha$ G. Cellobiose and saccharose inhibit 4MU $\alpha$ G hydrolysis noncompetitively, and lactose has a very slight inhibitory effect, likely noncompetitive. Among the two  $\alpha$ -glucopyranosides tested, only the maltose ( $\alpha$ -D-glucopyranosyl-1-4- $\beta$ -D-glucopyranose) competitively inhibited the hydrolysis of 4MU $\alpha$ G. Saccharose ( $\alpha$ -D-glucopyranosyl-1-2- $\beta$ -D-fructofuranose), on the other hand, was a noncompetitive inhibitor.

**Response of bacterial communities to addition of polysaccharides.** Enrichment cultures of natural water with either  $\alpha$ - or  $\beta$ -glucopyranosides show that for comparable optical densities, the  $\alpha$ -glucosidase activity was higher for growth on starch than on maltose than on glucose. Similarly,  $\beta$ -

glucosidase activity was more important for growth on cellobiose than on glucose.

Figure 5 shows the  $\alpha$ -glucosidase activity as a function of  $\beta$ -glucosidase activity for different enrichment cultures. The  $\alpha$ - and  $\beta$ -glucosidase activities of the biomass that developed during growth on glucose, i.e., without any induction by an  $\alpha$ - or  $\beta$ -glucopyranoside, represented the background levels of these enzymes in our water samples. During growth on glucose, the ratio of  $\alpha$ - to  $\beta$ -glucosidase was constant at about 0.73. This ratio was the same as that observed on the natural sample before enrichment. During growth on glucosides, the  $\alpha$ -/ $\beta$ -glucosidase ratio was altered. As noted in Fig. 5, growth on  $\alpha$ -glucopyranosides (maltose and starch) increased the proportion of  $\alpha$ -glucosidase ( $\alpha$ -/ $\beta$ -glucosidase, 1.03 and 1.11, respectively), whereas growth on cellobiose resulted in a lower  $\alpha$ -/ $\beta$ -glucosidase ratio (0.54).

## DISCUSSION

Both  $\alpha$ - and  $\beta$ -glucosidase activity in natural water can be readily measured during short incubation times (20 min) by use of artificial substrates such as 4MU $\alpha$ G or 4MU $\beta$ G. These activities are primarily associated with particles  $>0.2$   $\mu$ m.

TABLE 1. Type of inhibition and  $K_i$  of different disaccharides on the hydrolysis of 4MU $\beta$ G<sup>a</sup>

Disaccharide	Inhibition	$K_i$ ( $\mu$ M of disaccharide)
Cellobiose ( $\beta$ -glucopyranoside)	Competitive	0.22–0.24
Saccharose ( $\alpha$ -glucopyranoside)	Noncompetitive	2.90–5.56
Maltose ( $\alpha$ -glucopyranoside)	Noncompetitive	2.13–5.26
Lactose ( $\beta$ -galactopyranoside)	Noncompetitive (?)	4.9–8.1

<sup>a</sup> The apparent  $K_m$  for 4MU $\beta$ G was 0.01  $\mu$ M.

TABLE 2. Type of inhibition and  $K_i$  of different disaccharides on the hydrolysis of 4MU $\alpha$ G<sup>a</sup>

Disaccharide	Inhibition	$K_i$ ( $\mu$ M of disaccharide)
Cellobiose ( $\beta$ -glucopyranoside)	Noncompetitive	0.34–0.89
Saccharose ( $\alpha$ -glucopyranoside)	Noncompetitive	1.1–1.7
Maltose ( $\alpha$ -glucopyranoside)	Competitive	0.04–0.2
Lactose ( $\beta$ -galactopyranoside)	Noncompetitive (?)	3.8–7.5

<sup>a</sup> The average apparent  $K_m$  for 4MU $\alpha$ G was 0.01  $\mu$ M.

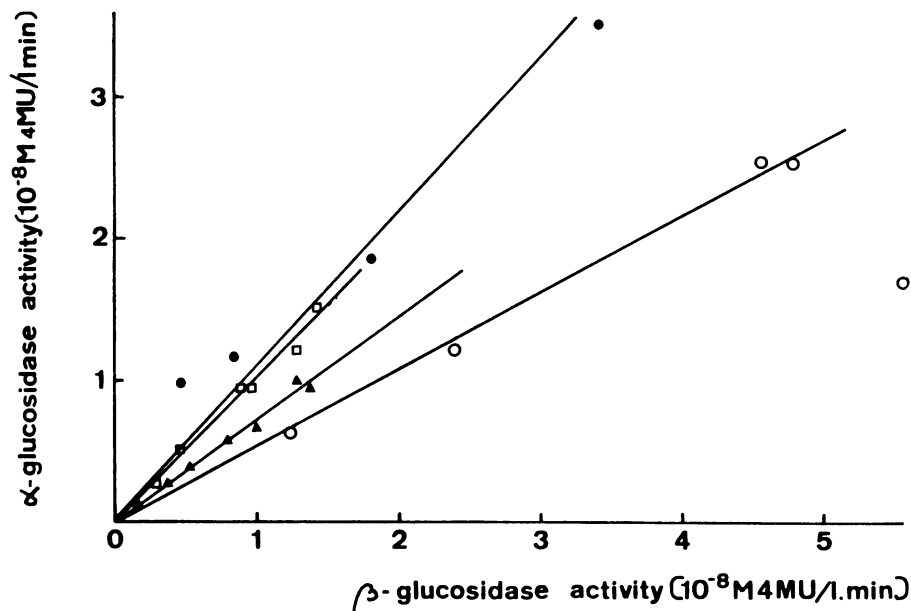


FIG. 5. The  $\alpha$ - versus  $\beta$ -glucosidase activities for enrichment cultures on starch (●), maltose (□), glucose (▲), and cellobiose (○) of pond water (Bois de la Cambre, Brussels).

$\beta$ -glucosidase activity is linked to bacterial activity determined by thymidine incorporation and to bacterial uptake of glucose, likely by a first-order relationship.

Hydrolysis of 4MU $\alpha$ G and 4MU $\beta$ G are competitively inhibited by the homologous disaccharides maltose and cellobiose, respectively. The specificity of the glucosidases with regard to carbon atom 1 appears to be absolute. Absolute specificity of  $\beta$ -glucosidase is also observed for the configuration of the C4 since a  $\beta$ -galactopyranoside (lactose) does not significantly inhibit the hydrolysis of 4MU $\beta$ G. These two observations are in perfect agreement with the observations of Duerksen and Halvorson (4) who observed an absolute specificity at carbon atoms 1 and 4 of a  $\beta$ -D-glucosidase for the affinity of an inducible  $\beta$ -glucosidase of yeast.

A disaccharide containing a fructofuranose ring (the saccharose) exhibits only noncompetitive inhibition of 4MU $\alpha$ G hydrolysis, indicating the importance of the shape of the whole molecule in the specificity of  $\alpha$ -glucosidase.

Growth on either  $\alpha$ - or  $\beta$ -glucopyranoside results in increasing the activity of the corresponding glucosidase both in absolute and with respect to the other glucosidase. In the absence of glucosides, the  $\alpha$ -/ $\beta$ -glucosidase ratio is about 0.73.

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