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# PREPARATION AND USE OF α-MALTOSYL FLUORIDE AS A SUBSTRATE BY BETA AMYLASE*

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ABSTRACT

The preparation of pure (amorphous) α-maltosyl fluoride is described. A modification of the procedure of Brauns was used to obtain analytically pure, crystalline hepta-*O*-acetyl-α-maltosyl fluoride, the structure of which was assigned by $^{19}F$- and $^{1}H$-n.m.r. spectroscopy. α-Maltosyl fluoride was obtained by deacetylating the heptaacetate. It behaved as a single compound on thin-layer and paper chromatography, and was essentially completely hydrolyzed to maltose and hydrogen fluoride by 0.01M sulfuric acid in 10 min at 100°. Crystalline beta amylase, likewise, catalyzed essentially complete hydrolysis of α-maltosyl fluoride to give maltose and hydrogen fluoride. The rates of hydrolysis catalyzed by beta amylase preparations from sweet potatoes and soybeans acting on a range of concentrations of the substrate produced linear curves for the relationship, $1/v$ *vs* $1/S$; reaction constants for crystalline, sweet-potato enzyme were $K_m$ 3.6 mM and $V_{max}$ ~2 μmol/min/mg. The finding that α-maltosyl fluoride is hydrolyzed 30–60 times faster than maltotriose demonstrates for the first time that beta amylase is capable of effecting hydrolysis at an appreciable rate of a substrate having only two D-glucose residues.

## INTRODUCTION

In an earlier study[1], α-maltosyl fluoride was found to serve as a substrate for sweet-potato beta amylase and for alpha amylases from several sources. At the time, only very crude preparations of this previously unreported compound were available for study, and these allowed only a limited amount of information to be generated on the enzymically catalyzed reactions. Nevertheless, evidence was obtained showing that the capacity of beta amylase to catalyze hydrolytic reactions is not restricted to the cleavage of maltosidic linkages, but extends to the glycosylic C–F bond of

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α-maltosyl fluoride. In addition, alpha amylases were found to bring about extensive formation of maltosaccharides from α-maltosyl fluoride at much lower concentrations of substrate than observed with α-D-glucosyl fluoride[1,2]. These observations signalled an unusual opportunity to gain new basic information on the catalytic capacities of these important enzymes through the use of purified α-maltosyl fluoride as a substrate. The investigation of enzymic glycosylation reactions that take place without glycosidic bond-cleavage appears, from recent studies with amylases[1-3], D-glucosyltransferases[4-6], α- and β-D-glucosidases[7,8] and β-D-galactosidase[7,9], to hold considerable promise as a general approach to gaining a more complete understanding of the mechanism of carbohydrase action.

The present paper describes both the preparation of purified α-maltosyl fluoride and the results obtained from experiments relating the action of sweet potato and soybean beta amylase preparations on this compound to their action on other substrates. A separate study of the utilization of the present α-maltosyl fluoride preparations by alpha amylases has been made, and a preliminary account of the findings reported[10].

## RESULTS AND DISCUSSION

*Preparation of α-maltosyl fluoride.* — A reliable procedure has been developed for obtaining α-maltosyl fluoride of high purity for use in enzymic experiments. The initial steps involve the synthesis of hepta-*O*-acetyl-α-maltosyl fluoride (**1**) by treating maltose octaacetate with cold anhydrous hydrogen fluoride, followed by extraction of the product into chloroform, concentration, and precipitation with petroleum ether. Brauns[11] had found that these steps provide crystals of **1** that could be purified by repeated recrystallization from 95% ethanol on cooling. However, in repeated trials, we found the material recovered following fluorination to be a mostly amorphous mixture containing the desired product as the smaller of two components evident on t.l.c. Crystals of **1** were not obtained from ethanolic solutions of the crude product until it was discovered that adjustment of the solutions to pH 6 allowed very rapid formation of crystals. Final purification was achieved by using a column of silica gel 60 with ether as eluant. Recrystallized **1** had m.p. 172–174° and $[\alpha]_D^{23}$ +110° (*c* 0.8, chloroform) [*lit*[11]: m.p. 174–175°, $[\alpha]_D^{20}$ +111.1° (chloroform)]. Unambiguous assignment of the α-anomeric configuration and D-glucopyranosyl structure of the F-bearing ring was afforded by the $^{19}F$ chemical shift (+147.6) and coupling constants ($J_{1,F}$ 53.8 Hz, $J_{2,F}$ 24.5 Hz) found by[19] F-n.m.r. analysis. These values agree closely with those reported by Hall *et al.*[12] for tetra-*O*-acetyl-α-D-glucopyranosyl fluoride.

α-Maltosyl fluoride was generated by deacetylating the heptaacetate at 0° with sodium methoxide, and freed from accompanying impurities by chromatography on silica gel 60. Though amorphous, the product ($[\alpha]_D^{22}$ + 137.4° (*c* 0.6, methanol)] behaved as a pure compound on thin-layer and paper chromatography. Tested by the latter technique, 100 μg produced a single spot ($R_{Glc}$ 1.15) that reduced silver

nitrate extremely slowly; 0.1% of glucose or 0.2% of maltose as impurities would have been detected if present. Treatment of the product with 0.01M sulfuric acid for 10 min at 100° caused essentially complete hydrolysis of the glycosylic C–F bond. No sign of residual material of $R_{Glc}$ 1.15 was found in chromatograms of such hydrolyzates; maltose and a trace of glucose were the only products seen. Analyses (8 different preparations) showed an average of 0.95 mol of fluoride anion released per 2 mol of D-glucose contained in the sample.

The free fluoride content in freshly prepared α-maltosyl fluoride was ~0.1%, as measured with a fluoride ion probe, and this value was found to remain unchanged even after months of storage *in vacuo* in a desiccator at −20°. Samples kept for several days in a desiccator at room temperature were occasionally found to have undergone some deterioration. When freshly dissolved in acetate buffers of pH 4.8–5.6 and kept for 1 h at 30°, α-maltosyl fluoride was observed to be hydrolyzed to the extent of ~0.3%, as measured by release of fluoride.

*α-Maltosyl fluoride as a substrate for beta amylase.* — The capacity of beta amylase to catalyze the complete hydrolysis of the C–F glycosylic bond of α-maltosyl fluoride was shown as follows. Test and control mixtures (0.2 ml), containing 2.5 μmol of α-maltosyl fluoride and 120 μg of active or heat-inactivated crystalline (α-D-glucosidase-free) sweet-potato beta amylase[13], were incubated at pH 5.6 for 5 h at 30°. Paper chromatography of the test digest (10 μl) versus appropriate standards revealed

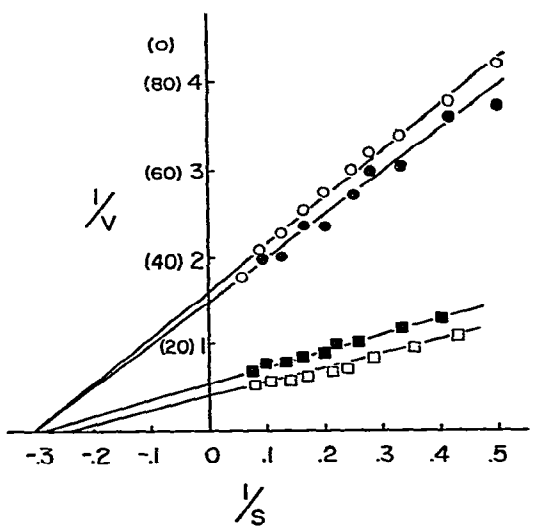


Fig. 1. Hydrolysis of α-maltosyl fluoride by beta amylase preparations at 30° and pH 4.8. Plots of the reciprocal of the reaction velocity (μmol of fluoride ion released/min/mg protein)$^{-1}$ versus the reciprocal of the substrate concentration (μmol α-maltosyl fluoride/ml)$^{-1}$ for crude soybean beta amylase (ordinate values in brackets), ○; partly purified soybean beta amylase, ●; crystalline, α-D-glucosidase-free, sweet-potato beta amylase, ■; commercial, crystalline, sweet-potato beta amylase, □. Fluoride release was determined with a specific ion-probe, except for digests with the commercial sweet-potato beta amylase, where a colorimetric method[2] was used with values corrected for hydrolysis of 4% of the α-maltosyl fluoride present during analysis.

TABLE I

BETA AMYLASE-CATALYZED HYDROLYSIS OF α-MALTOSYL FLUORIDE VERSUS OTHER SUBSTRATES

| *Hydrolysis* | *Substrate* | *Sweet potato amylase* | | *Soybean amylase* | |
|---|---|---|---|---|---|
| | | *Cryst. commercial* | *Cryst. repurified*[13] | *Crude commercial* | *Partly purified* |
| Rate, μmol/min/mg protein | Soluble starch[a] | 483. | 436. | 3.5 | 66. |
| | α-Maltosyl fluoride[b] | (2.62) | 1.88 | 0.031 | 0.67 |
| | Maltotriose[c] | 0.07 | 0.07 | | 0.01 |
| | Maltose[d] | 0.0105 | 0.0005 | | 0.0002 |
| $K_m$, mM | α-Maltosyl fluoride[b] | (4.2) | 3.6 | 3.3 | 3.3 |

[a]Initial rates of reducing-sugar release from 1% starch. [b]Maximal rates ($V_{max}$) or $K_m$ values based on fluoride release, as measured by specific ion-probe, or by the (less precise) colorimetric method (figures in parentheses). [c]Maximal rates ($V_{max}$) based on release of D-glucose, as measured with D-glucose oxidase. [d]Initial rates of D-glucose release from 10 mM maltose.

the essentially complete conversion of the substrate into maltose. A trace of glucose, accounting for less than 1% of the substrate, was the only other product detected; no residual material was seen at the substrate position, $R_{Glc}$ 1.15. No significant conversion of α-maltosyl fluoride into maltose was found in the control mixture.

Further understanding of the action of beta amylase on α-maltosyl fluoride was obtained through kinetic experiments in which the initial rates of fluoride release were determined as a function of substrate concentration. Data were obtained for two sweet-potato and two soybean beta amylase samples of different degrees of purity. In each case, a series of digests containing 2–15 mM α-maltosyl fluoride, a suitable, fixed concentration of beta amylase, and 0.08M pH 4.8 acetate buffer, was set up at 2-min intervals. Digests, and controls without enzyme were incubated for 40 min at 30°, and then analyzed to determine μmol of fluoride anion released/min/mg of enzyme. The results obtained with each enzyme preparation provided an essentially linear curve for the relationship, $1/v$ *vs* $1/S$ (Fig. 1). Kinetic constants were derived from the data by a least-squares method.

Table I lists the $V_{max}$ and $K_m$ values found for the hydrolysis of α-maltosyl fluoride by the four beta amylase preparations. Also listed, for comparison, are the rates at which the same enzymes catalyzed the hydrolysis of soluble starch, maltotriose, and maltose. It is evident that the initial rates of hydrolysis of 1% soluble starch (at 36° and pH 5.6) were in all cases at least 100 times greater than the calculated maximal rates of hydrolysis of α-maltosyl fluoride. On the other hand, α-maltosyl fluoride was much more rapidly hydrolyzed by beta amylase than maltotriose; the former had $V_{max}$ values 30–60 times greater than those found for the trisaccharide. The extremely low rates observed for hydrolysis of maltotriose (Table I) are in accord with similar findings reported by others[14–16]. According to the literature, only maltosaccharide chains longer than three glucose residues (and methyl α-maltotrioside[15])

have, hitherto, been found to be hydrolyzed at moderate or high rates by beta amylase. Thus, the present results with α-maltosyl fluoride show for the first time that beta amylase can exhibit substantial hydrolytic activity (~2 μmol/min/mg in the case of crystalline, sweet-potato enzyme) for an α-maltosyl compound containing only two D-glucose residues*.

This finding is of interest in the light of observations that beta amylase hydrolyzes malto-oligosaccharides at rapidly increasing rates with increasing chain-lengths for oligomers containing three to six D-glucose units[17]. Apparently, α-maltosyl fluoride can bind in the active site of beta amylase in such a manner as to position its glycosyl–fluorine bond in the region of the enzyme's catalytic groups, which subsequently promote hydrolysis of this bond. The higher $V_{max}$ for α-maltosyl fluoride relative to maltotriose suggests that the fluorine atom is a better leaving-group in this reaction than a D-glucose group. In addition, the similarity of the $K_m$ values found for α-maltosyl fluoride (3.6 mM) and maltotriose (3.9 mM) suggests that only the first two glucose groups of maltotriose significantly contribute to its binding.

The capacity of beta amylase to catalyze the cleavage of the C–F bond of α-maltosyl fluoride has a close parallel in the ability of another "inverting" exoamylase, glucoamylase, to effect the hydrolysis of α-D-glucosyl fluoride[2,18]. Moreover, beta amylase brings about the rapid condensation of β-maltose into maltotetraose; glucoamylase, the rapid condensation of β-D-glucopyranose to maltose[3]. These reactions from free sugars, like the hydrolyses of α-maltosyl fluoride and α-D-glucosyl fluoride, involve cleavage of a glycosylic (but not glycosidic) bond†. They differ in being reversals of hydrolysis, specific for the β-anomeric form of the substrate, and in involving a carbohydrate acceptor.

## EXPERIMENTAL

*General methods.* — T.l.c. was carried out with silica gel G according to Stahl (Brinkmann Instruments, Westbury, L.I.), with ethyl ether as the solvent for acetylated compounds and 2:5 abs. ethanol–ethyl acetate for nonacetylated compounds; spots were visualized by the sulfuric acid–char method. Paper chromatography was performed with Whatman no. 1 paper and two 22-h ascents in 6:4:3 1-butanol–pyridine–water. Staining was by a silver nitrate dipping-technique[19,20], with papers hung in air for 12 min following application of the sodium hydroxide reagent.

Concentrations were performed under diminished pressure in a rotary evaporator at or below 32°. Drying was done in a vacuum oven at or below 35°. Crystallinity was judged by use of a polarizing microscope with a first-order (red) retardation

*Maltose itself was found to be hydrolyzed to D-glucose at vanishingly low rates by the preparations tested, and there is also uncertainty that this action was due to beta amylase. The crystalline sweet-potato enzyme that had been specially treated to remove α-D-glucosidase[13] hydrolyzed maltose at ~5% of the rate found with the otherwise comparable commercial sample of beta amylase.

†A glycosylic bond may be defined as the union joining the anomeric carbon of a cyclic form of a sugar to the anomeric hydroxyl group or to any group replacing that hydroxyl group[1].

plate. Melting points were determined on a Mel-Temp block (Laboratory Devices, Cambridge, Mass.) and are uncorrected. Optical rotations were measured by using a Rudolph and Sons Model 70 polarimeter and 2 dm tubes. Total glucose was determined by the phenol–sulfuric acid method[21]. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

*Nuclear magnetic resonance spectra.* — $^{1}H$, $^{13}C$, and $^{19}F$ n.m.r. spectra were obtained by using a Jeol PFT-100 spectrometer interfaced with a Nicolet 1000 series computer. The instrument operated at 23.5 kGauss in the pulsed, Fourier-transform mode. N.m.r. spectra of the acetylated maltosyl fluoride preparations were recorded with benzene-$d_6$ as solvent. $^{19}F$-Chemical-shift assignments were made with respect to trichlorofluoromethane, which was used as the internal standard.

*Hepta-O-acetyl-α-maltosyl fluoride* (**1**). — Recrystallized β-maltose octaacetate (20 g), m.p. 160–161° and $[\alpha]_D^{23}$ +63.5° (*c* 3, chloroform), was dissolved in 65 ml of liquid, anhydrous hydrogen fluoride (Baker Chemical Co.) at −65°. After reaction for 15 min, the mixture was removed from the cold bath and the acid evaporated with a vigorous stream of dry nitrogen. The residue was taken up in 250 ml of ice-cold chloroform and shaken vigorously with an equal volume of 5% sodium hydrogencarbonate and ice. The chloroform phase was then repeatedly washed with ice-cold water, dried with sodium sulfate, and concentrated to a syrup (25 ml). On stirring with 75 ml of petroleum ether (b.p. 38–52°), a white, granular solid precipitated which, on drying, weighed 14.4 g. This comprised a small proportion of birefringent crystals in an amorphous matrix. T.l.c. revealed a large spot, $R_F$ ~0.5, and a smaller "fast" spot (the desired product), $R_F$ ~0.7. A number of trials were made, using various conditions of fluorination and product recovery, but none provided α-maltosyl fluoride heptaacetate as the preponderant product.

The foregoing, mixed material (14.4 g) was dissolved in 160 ml of hot (80°) ethanol and then cooled to 30°. Upon adjustment to pH 6 with 0.1 ml of ammonia, the solution was rapidly converted into a thick slurry of highly birefringent crystals. After 30 min at 4°, the product was collected on a sintered-glass filter and dried; yield, 9.2 g. T.l.c. showed a large spot, $R_F$ ~0.7, accompanied by a smaller spot, $R_F$ ~0.5; m.p. 172–175°.

In another preparative run, the crystals had m.p. 145–155°; moreover, a small second crop that formed from the ethanolic mother liquor at 4° showed m.p. 120–135°. The latter material, recrystallized after chromatography on silica gel 60 to remove components migrating at $R_F$ ~0.5, had m.p. 123–124°. Its $^{19}F$ n.m.r. spectrum showed $^{19}F$ chemical shifts of +136.4 and 147.6 p.p.m., with the former peak approximately four times more intense than the latter. These second-crop crystals evidently comprise a mixture of the β- and α-maltosyl fluoride acetates in a ratio of ~4:1. Authentic hepta-*O*-acetyl-β-maltosyl fluoride, m.p. 130–132° and $[\alpha]_D^{23}$ +80.1° (*c* 0.8, chloroform) (Hehre and Genghof, unpublished data), showed a $^{19}F$ chemical shift of +136.4 p.p.m.; $J_{1,F}$ 53.8 Hz, and $J_{2,F}$ 9.7 Hz.

Individual 1.5- to 2.5-g batches of the impure crystals, dissolved in chloroform, were admixed with 10 g of silica gel 60 (70–230 mesh, E. Merck, Darmstadt, Germany)

and dried under vacuum at 32°. The mixture was placed on a dry column (2.5 × 30 cm) of silica gel 60 and covered with 10 g of dry gel. The column was developed with ethyl ether, and 7-ml fractions were collected. Those showing only a single spot ($R_F$ ~0.7) on t.l.c. were pooled and dried. The product was recrystallized from benzene by addition of petroleum ether; total yield, from 20 g of maltose octaacetate, 5.26 g (28%).

The highly birefringent crystals had m.p. 172–174°, $[\alpha]_D^{23}$ +110.2° (*c* 0.8, chloroform) [lit.[7]: m.p. 174–175°, $[\alpha]_D^{20}$ +111.1° (chloroform)]. $^{19}F$ n.m.r. spectra showed $^{19}F$ chemical shift of +147.6 p.p.m. and $J_{1,F}$ $J_{2,F}$ coupling constants of 52.8 and 24.5 Hz, respectively. The $^1H$- and $^{13}C$-n.m.r. spectra of the product were in agreement with the assigned structure.

*Anal.* Calc. for $C_{26}H_{35}FO_{17}$: C, 48.91; H, 5.52; F, 2.98. Found: C, 48.80; H, 5.61; F, 3.08.

*α-Maltosyl fluoride.* — Deacetylation was routinely carried out by treating 0.26 g (400 μmol) of finely powdered α-maltosyl fluoride heptaacetate, suspended in 2.3 ml of cold, dry methanol, with 0.20 ml of fresh 0.2M sodium methoxide. The mixture was kept in a corked tube for 5.5 h at 0° with occasional shaking. Benzene (8 ml) was then added and, after rapid removal of solvent (Rotary EvapoMix, Buchler Instruments Co.), addition of benzene and evaporation were twice repeated. Final drying (1 h in a vacuum oven at 32°) provided a white, amorphous powder; average weight (13 trials) 155 mg. The product, which on t.l.c. (abs. 2:5 ethanol–ethyl acetate) regularly showed a large spot ($R_F$ ~0.3) accompanied by a smaller spot ($R_F$ ~0.5), was kept in a vacuum desiccator at −20° prior to further processing.

For final purification, the deacetylated product (~155 mg) was dissolved in 1.5 ml of chloroform, admixed with 1.5 g of silica gel 60, and dried. This mixture was placed on a column (1.6 × 30 cm) of dry silica gel 60 and covered with 2 g of dry gel. The column was developed with abs. 2:5 ethanol–ethyl acetate at the rate of 0.75 ml per min. Fractions of 3 ml were collected, and those showing a single spot ($R_F$ ~0.3) on t.l.c. were pooled and concentrated in a tared tube on a rotary evaporator. Addition of benzene (8 ml) and subsequent evaporation were carried out three times. The final product was dried in a vacuum oven at 32° and stored in a vacuum desicaator at −20°. The yield averaged 36 mg (250 μmol) from 400 μmol of the acetate (13 trials).

*Fluoride determination.* — Measurements of free fluoride ion (in the presence or absence of maltosyl fluoride) were made either by a modification[2] of the colorimetric method of Megregian and Maier (1962)[22], or with the aid of a specific fluoride-ion probe (Orion specific ion meter, Model 407A, and Combination fluoride electrode, Model 96-09). In each case, solutions of sodium fluoride of known concentration were employed as standards. In using the fluoride ion probe, standards and test samples were first diluted with an equal volume of a solution comprising M sodium acetate buffer (pH 5.2), M sodium chloride, and 0.4% 1,4-cyclohexanebis(dinitrilotetraacetic acid) monohydrate; and then poured into 5-ml polyethylene beakers.

Meter readings were recorded 45 sec after immersion of the electrode in the solution under test.

*Beta amylases.* — Two crystalline preparations of sweet-potato beta amylase (suspended in ammonium sulfate) were used. One was a commercial product (Lot 13C8090, Sigma Chemical Co.); the second, a similar product that had been further fractionated to remove α-D-glucosidase[13], was the generous gift of Drs. J. J. Marshall and W. J. Whelan. Immediately prior to use, these enzymes were separated from the suspension fluid by centrifugation (8000*g*, 15 min) and dissolved in ice-cold buffer to provide solutions of the desired concentration.

A crude, commercial preparation of soybean beta amylase (No. 1500, Nagase and Co., Amagasaki, Japan) was the gift of Dr. T. Komaki. A partly purified beta amylase was prepared from it as follows. Crude enzyme (0.2 g) was suspended in 15 ml of 0.05M acetate buffer (pH 5.6) and centrifuged at 100,000*g* for 30 min. The supernate (13 ml) was treated with 20 ml of a 3.9M solution of ammonium sulfate. After 24 h at 4°, the precipitate was collected by centrifugation and taken up in 2 ml of 0.05M acetate buffer (pH 5.6). The solution was placed on a column (2.5 × 64 cm) of Sephadex G-100 (Pharmacia) that had been equilibrated at 4° with the 0.05M buffer. Elution was carried out in the cold with the same buffer, at the rate of 0.25 ml/min. Individual 8-ml fractions were collected and assayed for capacity to hydrolyze soluble starch; tubes no. 20–22, which contained most of the amylase activity, were pooled. The solution, containing a total of 5.0 mg of protein[23], was treated with two volumes of 3.9M ammonium sulfate and kept at 4°. For use, the enzyme was sedimented by centrifugation and dissolved in buffer.

*Assays of beta amylase activity.* — Initial rates of starch hydrolysis were determined by using a soluble potato-starch (Mallinckrodt) having 0.5% of the reducing power of D-glucose (d.e. 0.5). A freshly prepared 2% solution of this substrate was treated with an equal volume of suitably diluted beta amylase (each in 0.05M acetate buffer (pH 5.6), and each attempered at 36°). The digest was incubated (10.0 min, 36°) with appropriate controls, and then the reducing sugars released were determined, against maltose standards, by using the Nelson[24] and Somogyi[25] reagents. Optical-density measurements at 540 nm, made after color stabilization and removal of particulate material, were used to calculate specific activity as μmol of maltose produced/min/mg of enzyme.

Specific hydrolytic activity for maltotriose was determined by using a preparation of the trisaccharide (U.S. Biochemicals Corp.) that behaved as a pure compound on paper chromatography (100-μg sample). Digests containing 2–30 mM of maltotriose, an appropriate (constant) concentration of beta amylase, and 0.08M acetate buffer (pH 4.8), were incubated for 5 h at 30°. The digests were assayed for D-glucose released, by the use of D-glucose oxidase–chromogen (Glucostat Special Reagent, Worthington Corp.), and the values used to calculate the specific, initial velocity of maltotriose hydrolysis at each concentration of substrate. Values of $V_{max}$ were derived by a least-squares method.

Rates of maltose hydrolysis were examined by using a highly purified prepara-

tion of the disaccharide that showed less than 0.1 % of D-glucose by D-glucose oxidase determination, and no trace of saccharide impurity as judged by paper chromatography (100 μg of sample). Test mixtures, containing beta amylase, 10 mM maltose, and 0.06M acetate buffer (pH 4.8) were incubated for 5 h at 30°. The amount of D-glucose liberated, determined by use of D-glucose oxidase, was used to calculate the specific rate of hydrolysis.

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