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STEREOCHEMISTRY OF THE HYDROLYSIS OF α,α-TREHALOSE BY TREHALASE, DETERMINED BY USING A LABELLED SUBSTRATE*

JACQUES DEFAYE, HUGUES DRIGUEZ, BERNARD HENRISSAT,
Centre de Recherches sur les Macromolécules Végétales, C.N.R.S. Grenoble, 68X F-38402 Saint-Martin d'Hères (France)

AND EDITH BAR-GUILLOUX
Laboratoire de Chimie Biologique, Université René Descartes, avenue de l'Observatoire, F-75006 Paris (France)

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ABSTRACT

(1,1′-^{13}C)α,α-Trehalose was obtained in 37% yield from the Pavia condensation of 2,3,4,6-tetra-*O*-benzyl-D-(1-^{13}C)glucopyranose, in dichloromethane in the presence of trifluoromethanesulfonic anhydride, followed by the usual deprotection techniques. The hydrolysis of this substrate by cockchafer trehalase was monitored at 37° by using ^{13}C-n.m.r. spectroscopy with short recording times. Equimolecular amounts of α- and β-D-glucopyranose are released simultaneously by the action of the enzyme. This result is consistent with a bimolecular substitution mechanism, taking into account previous results involving C-2 asymmetric participation in the catalytic step of hydrolysis of α,α-trehalose. For comparative evaluation of its accuracy, the usual polarimetric technique was also used for the determination of the anomeric configuration of the D-glucose released by the action of the enzyme on α,α-trehalose.

INTRODUCTION

An understanding of the mechanism of action of glycosyl hydrolases obviously requires knowledge of the anomeric configuration of the glycosyl unit released. Several methods have been used for this determination, including measurements and correlation of optical rotation and reducing power of the incubation medium[3], gas–liquid chromatography of the per(trimethylsilyl)ated, enzymic hydrolyzate[4], enzymic characterization of the catabolite[5], and proton n.m.r. spectroscopy[6]. Although these techniques have given accurate information for simple

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glycosidases and some glycanases, they gave inconsistent results for the hydrolysis of α,α-trehalose by trehalase†.

Labat *et al.*[7] observed the exclusive production of α-D-glucose by the action of pig-kidney trehalase on α,α-trehalose. Clifford[8], using an enzyme of flesh fly, and Hehre *et al.*[9], using either a rabbit renal-cortical trehalase or the enzyme from *Candida tropicalis*, found that equimolecular amounts of α- and β-D-glucopyranose were produced. Although these discrepancies may arise from differences in the mechanisms of action of enzymes from different species, they more probably reflect inadequacies in the methods used for the determinations.

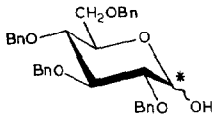
The wide, anomeric-carbon signal-dispersion associated with ^{13}C-n.m.r. spectroscopy makes it an attractive method for determination of the anomeric configuration of the hexose units released during the action of trehalase on α,α-trehalose. The acquisition time for spectral-data measurements must, however, be fast enough to compete with the expected mutarotation of the monosaccharide units released, and this was achieved by using 1,1′-^{13}C-labelled α,α-trehalose. A critical evaluation of the widely used polarimetric technique in its application in this area is also presented.

RESULTS AND DISCUSSION

(1,1′-^{13}C)α,α-Trehalose was prepared according to Pavia *et al.*[10] for the synthesis of α,α-trehalose, but starting from 2,3,4,6-tetra-*O*-benzyl-D-(1-^{13}C)glucopyranose (**1**). Owing to the cost of D-(1-^{13}C)glucose, a one-step technique was devised for the preparation of the benzylated derivative **1**. Glycosylation of the 90% isotopically enriched D-(1-^{13}C)glucose with methanol containing acetyl chloride led to an anomeric mixture of the D-glucosides in the ratio of 3:1 (^{1}H-n.m.r. spectrum). This mixture was then benzylated by using benzyl bromide and sodium hydride in *N,N*-dimethylformamide in the presence of tetrabutylammonium iodide. The resulting methyl 2,3,4,6-tetra-*O*-benzyl-α,β-D-(1-^{13}C)glucopyranosides were hydrolyzed, to afford 2,3,4,6-tetra-*O*-benzyl-D-(1-^{13}C)glucose (**1**) in an overall yield of 37% from D-(1-^{13}C)glucose.

Reaction of **1** in dichloromethane in the presence of trifluoromethanesulfonic anhydride[10] gave 2,3,4,6,2′,3′,4′,6′-octa-*O*-benzyl-(1,1′-^{13}C)α,α-trehalose (**2**) and the corresponding α,β anomer in 32 and 65% yield, respectively, after purification by column chromatography. Hydrogenolysis of **2** in the presence of palladium-on-charcoal, followed by acetylation of the deprotected disaccharide, gave crystalline 2,3,4,6,2′,3′,4′,6′-octa-*O*-acetyl-(1,1′-^{13}C)α,α-trehalose (**3**) in 88% yield from **2**. (1,1′-^{13}C)α,α-Trehalose (**4**) was then obtained by Zemplén *O*-deacetylation. Owing to the small scale of this preparation, no attempt was made to obtain crystalline (1,1′-^{13}C)α,α-trehalose, and lyophilization gave a microcrystalline foam of α,α-trehalose dihydrate. (1.1′-^{13}C)α,α-Trehalose (**4**) showed the expected ^{13}C-

†Trehalase or trehalose-1-glucohydrolase (EC 3.2.1.28).



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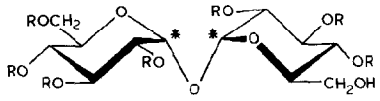


2 R = $PhCH_2$
3 R = Ac
4 R = H

n.m.r. singlet for C-1 at 94.0 p.p.m., and the *O*-acetylated precursor **3** gave a doublet of doublets for H-1 in the ^{1}H-n.m.r. spectrum, with $J_{C\text{-}1,H}$ 175 and $J_{H\text{-}1,H\text{-}2}$ 3.5 Hz, in support of the exclusive α,α configuration for disaccharide **4** and its *O*-acetylated precursor **3**.

Enzymic hydrolysis of (1,1′-^{13}C)α,α-trehalose (**4**) by cockchafer trehalase was monitored by ^{13}C-n.m.r. spectroscopy, using amounts of the isotopically enriched substrate compatible with spectral acquisition-times of 0.77 s and a spectral width of 4.400 kHz. This allowed continuous recording of the hydrolysis rate from 90 s reaction-time to completion. Four representative graphs are presented in Fig. 1 for incubations of 1.5, 7, 15, and 40 min of the labelled substrate with trehalase

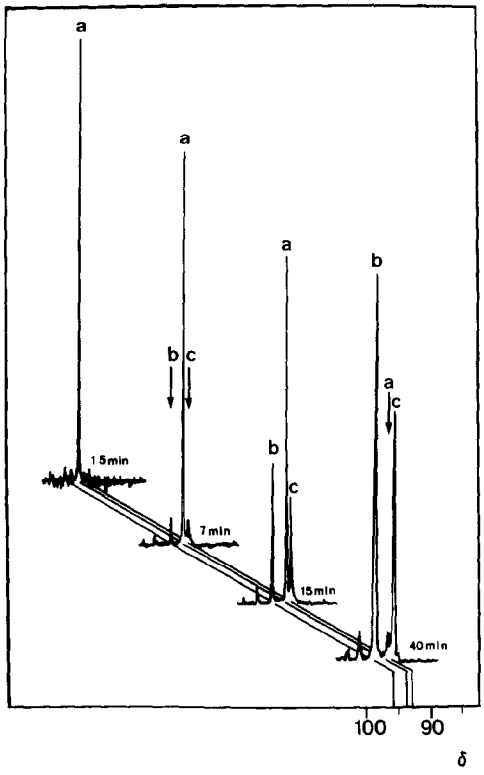


Fig. 1. ^{13}C-N.m.r. spectra, as a function of time, of (1,1′-^{13}C)α,α-trehalose (**4**; 7.5 μmol/mL) incubated with cockchafer trehalase (0.7 IU/mL). [Key: a, (94.0 p.p.m.) *C-1 α,α-trehalose; b, (96.8 p.p.m.) *C-1 β-D-glucopyranose; and c, (93.0 p.p.m.) *C-1 α-D-glucopyranose.]

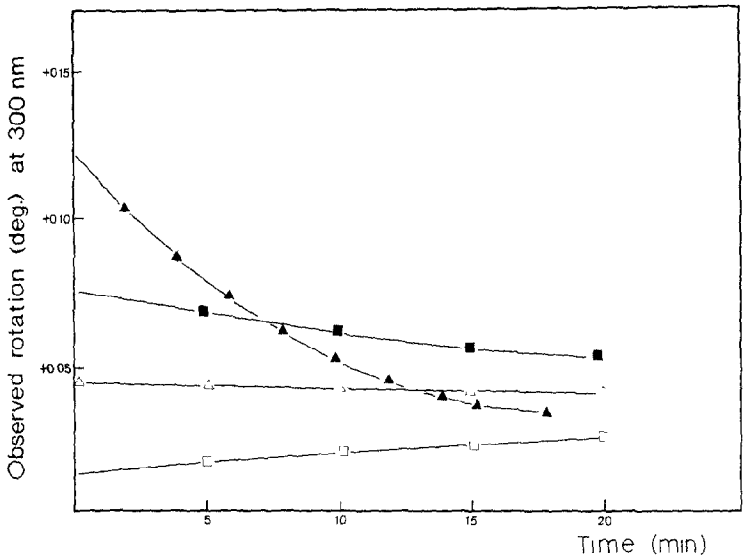


Fig. 2. Comparative variations in optical rotation, as a function of time, at 300 nm, in a Tris-maleate buffer solution (pH 6.4) containing 0.1M NaCl, at 37°. [Key: ▲, α,α-trehalose (7.5 μmol/mL) incubated with trehalase (1.0 IU/mL); ■, α-D-glucose (15 μmol/mL); △, equimolecular amounts of α- and β-D-glucose (7.5 μmol/mL for each compound); and □, β-D-glucose (15 μmol/mL)]

in a Tris-maleate buffer solution (pH 6.4), and the results are compatible with a half-life mutarotation time of 20 min for α- and β-D-glucopyranose. Acceptable accuracy for quantitation of the anomeric configuration of the D-glucose released was obtained by integration of the signals at 93.0 and 96.8 p.p.m. for the α and β anomers, using the anti-gate, ^{13}C-n.m.r. recording technique.

From the graphs in Fig. 1, which show the simultaneous appearance of both anomeric signals after an incubation time of 7 min, along with a decrease in the corresponding signal for α,α-trehalose, it is obvious that both α- and β-D-glucopyranose are produced in equimolecular amounts early in the reaction. Although the influence of mutarotation is perceptible (the β:α ratio increases from 1.18:1 at 7 min incubation to 1.54:1 at 40 min), it does not seem to interfere seriously with the accuracy of the determination. The β:α ratio of 1.63:1 expected for the mutarotation equilibrium is reached only after 60 min, in a comparable experiment involving equimolecular amounts of α- and β-D-glucose samples.

The simplicity and reliability of the foregoing methodology contrast with the conventional polarimetric technique (see Fig. 2). The polarimetric values recorded during the early stages of hydrolysis of α,α-trehalose by trehalase, using the previously described buffer medium, which gives a low mutarotation rate compared to that in phosphate buffer, are close to those obtained from mutarotation of an equimolecular mixture of α- and β-D-glucose. It must be pointed out, however, that, when 20 to 40% of α,α-trehalose is hydrolyzed (see Fig. 3), the differences in observed rotation for exclusive production of α-D-glucose compared to the present

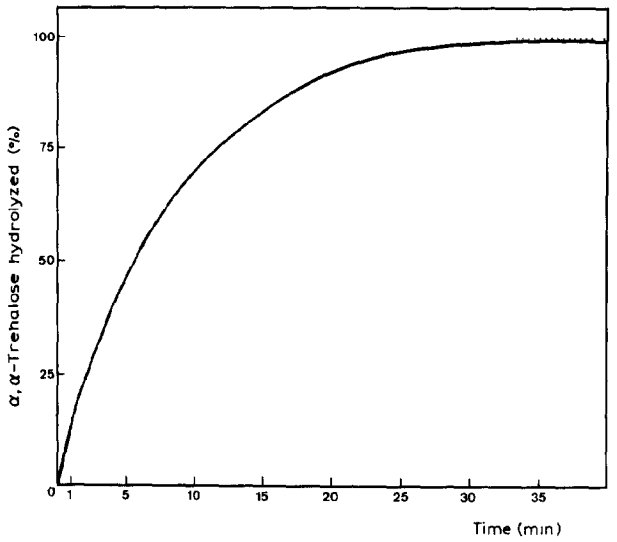


Fig. 3. Rate of hydrolysis of α,α-trehalose (7.5 μmol/mL) incubated with cockchafer trehalase (1.0 IU/mL), as estimated by the method of Nelson[20].

results for release of equimolecular amounts of α- and β-D-glucose would be in the range of 3.5 to 7.5%. This estimate takes into account the theoretical equation for optical rotation for a mixture of i compounds in a cell of 5.10^{-2} dm length: $[\alpha]_{obs} = 5.10^{-2}\ \Sigma_i C_i[M_i]^*$. This equation, when applied to the hydrolysis mixture from α,α-trehalose by the action of trehalase, would become: $[\alpha]_{obs} = 5.10^{-2}\ [(C_T^0 - C)[M_T] + 0.5C[M_\alpha] + 0.5C[M_\beta]]^*$, assuming that an equimolecular proportion of α- and β-D-glucose is released, or: $[\alpha]_{obs} = 5.10^{-2}\ [(C_T^0 - C)[M_T] + C[M_\alpha]]^*$, assuming that α-D-glucose is formed exclusively. Such slight changes in optical rotation arise from the important differences of specific molecular rotations [M] of α,α-trehalose, and α- and β-D-glucose, namely, 341,000°, 100,800°, and 15,500°, respectively, calculated from specific rotations of 997°, 560°, and 86° measured at 300 nm for these compounds. These results, which fall within the range of experimental error, make it clear that polarimetry cannot be used for determination of the anomeric configuration of the D-glucose released by the action of trehalase on α,α-trehalose.

CONCLUSION

From these results, ^{13}C-n.m.r. spectroscopy using a specifically ^{13}C-labelled α,α-trehalose appears to be an unequivocal technique for determination of the anomeric configuration of the enzymically released D-glucosyl units. This

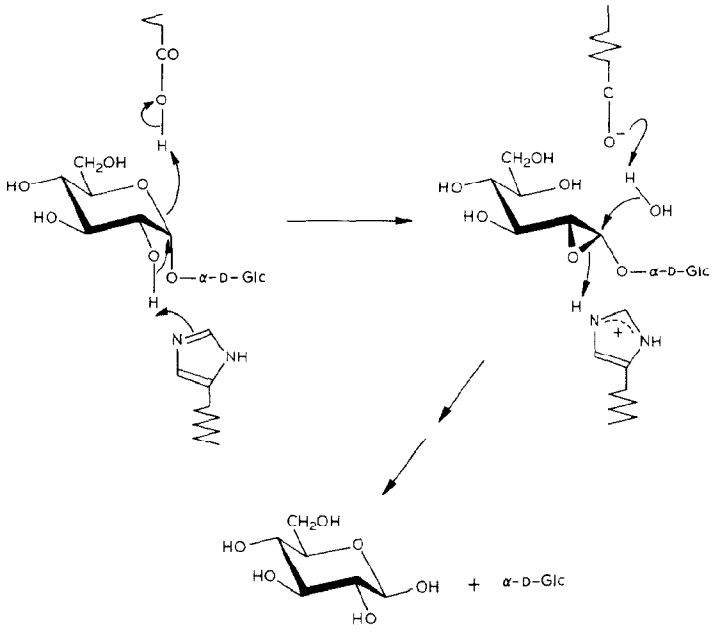
*C_T^0, = initial molecular concentration of α,α-trehalose; C, total molecular concentration of D-glucose; $[M_T]$, specific molecular rotation of α,α-trehalose; $[M_\alpha]$, specific molecular rotation of α-D-glucose; $[M_\beta]$ specific molecular rotation of β-D-glucose.

technique is especially useful when there is formed a complex mixture of optical isomers which may preclude the use of polarimetry.

In view of the unambiguous results provided by this technique for the action of cockchafer trehalase on α,α-trehalose, and the observed inversion at the anomeric carbon atom of one of the D-glucosyl units released, our hypothesis[2,11] for the mechanism of action of this enzyme has to be reevaluated.

This inversion of configuration, which is seldom observed for glycosyl-hydrolases[12], could be related to the absence of *trans*-glycosylating activity for this enzyme acting on its natural substrate[13]. However such an activity has been suggested[9] for *Candida* yeast trehalase, which forms a transient α,α-trehalose intermediate when acting on β-D-glucosyl fluoride.

Previous results[2,11] involving deuterated C-2 and 2-deoxy symmetrical and unsymmetrical derivatives of α,α-trehalose established the participation of the 2-hydroxyl group of one glucopyranosyl unit of α,α-trehalose in the catalytic mechanism of trehalase. Owing to the *cis*-relationship between the 2- and 2′-hydroxyl groups and the D-glucosidic oxygen atom of this substrate, participation of a C-2 oxyanion in breaking the linkage would fit well[2,11] with protonation of the corresponding O-5 atom, leading to a transient 1,2-epoxy acetal. This could be opened by water (see Scheme 1), to give inversion of configuration at the D-glucopyranosyl unit involved in the catalytic step of hydrolysis.



Scheme 1. Proposed mechanism for the hydrolysis of α,α-trehalose by trehalase.

EXPERIMENTAL

General methods for the chemical synthesis. — Solutions were dried with sodium sulfate, and evaporated *in vacuo* below 45°. Thin-layer chromatography (t.l.c.) was performed on silica gel (Merck F 254; Merck, Darmstadt, Germany) with 9:1 (v/v) chloroform–ether as the eluant. Preparative column-chromatography used silica gel (Merck 60; 70–230 mesh). Optical rotations were determined with a Quick polarimeter (Roussel and Jouan). ^{13}C-N.m.r. spectra (22.63 MHz) were recorded with a Bruker WP-100 spectrometer.

*2,3,4,6-Tetra-*O*-benzyl-*D*-(1-^{13}C)glucopyranose* (**1**). — D-(1-^{13}C)Glucose (Commissariat à l'Energie Atomique, Saclay, France; isotopic enhancement 90%; 1 g) was dissolved in anhydrous methanol (20 mL). Acetyl chloride (0.78 mL) was added, and the mixture was heated overnight at 70°. Neutralization of the acid with lead carbonate, followed by filtration through Celite, and evaporation of the filtrate, gave, in quantitative yield, a viscous mixture containing methyl α- and β-D-glucopyranoside, in the ratio of ~3:1, as determined by integration of the anomeric protons in the ^{1}H-n.m.r. spectrum. To a solution of this syrup in dry *N,N*-dimethylformamide (20 mL), cooled to 0°, were added sodium hydride (1.15 g; 50% dispersion in oil) and then tetrabutylammonium iodide (460 mg); benzyl bromide (2.95 mL) was now added dropwise, and the solution was stirred overnight at room temperature. The mixture was filtered through Celite, the filtrate evaporated, and the residue dissolved in dichloromethane (100 mL). The solution was washed with water (3 × 100 mL), and the washings were combined, and extracted with dichloromethane (2 × 100 mL). The organic solutions were dried, and evaporated to a syrup that was directly hydrolyzed with 50:7 (v/v) acetic acid–6M hydrochloric acid (57 mL) during 30 min at 85°. After evaporation, the residue was dissolved in chloroform (100 mL), and the solution was washed with cold water (2 × 100 mL), and evaporated to an oil that was purified by column chromatography with 24:1 (v/v) chloroform–ether as the eluant, to afford compound **1** (1.1 g, 37%), m.p. 148–150° (dichloromethane–ether) (lit.[14] m.p. 151-152°), which, without further purification, was used in the following step.

*2,3,4,6,2',3',4',6'-Octa-*O*-benzyl-(1,1'-^{13}C)-α,α-* (**2**) *and -α,β-trehalose.* — A solution of the tetra-*O*-benzyl derivative **1** (500 mg) in dichloromethane (5 mL) was cooled to −78°, and a solution of trifluoromethanesulfonic anhydride (0.20 mL) in dichloromethane (5 mL) at the same temperature was added. The temperature was gradually raised to 20° while the course of the reaction was monitored by t.l.c. After 35 min, the mixture was diluted with dichloromethane, washed with ice water, and evaporated; the residue was purified by chromatography on a column of silica gel with 9:1 (v/v) chloroform–ether as the eluant. The symmetrical α,α-disaccharide derivative **2** (146 mg, 32%), $[\alpha]_D^{20}$ +75° (*c* 1, chloroform) {lit.[15] $[\alpha]_D^{20}$ +84° (chloroform)}, was eluted first, followed by the α,β anomer (293 mg, 65%), m.p. 84–86° (ether–hexane), $[\alpha]_D^{20}$ +49° (*c* 1.9, chloroform); lit.[15] m.p. 100–101°, $[\alpha]_D^{20}$ +52.1° (chloroform).

2,3,4,6,2',3',4',6'-Octa-O-*acetyl-(1,1'-*^{13}*C)α,α-trehalose* (**3**). — A solution of the octa-*O*-benzyl-α,α-trehalose derivative **2** (73 mg) in ethyl acetate (5 mL) was diluted with ethanol (50 mL), and the mixture was hydrogenolyzed under hydrogen at a pressure of 1.6 MPa in the presence of 10% palladium-on-charcoal (200 mg) during 48 h. The catalyst was removed by filtration through Celite, and the filtrate was evaporated. To the residue were added pyridine (10 mL) and acetic anhydride (1 mL); the solution was stirred overnight, and then evaporated *in vacuo* to an oil which was purified by column chromatography with 4:1 (v/v) dichloromethane–ether, to give **3** (42 mg, 88%), m.p. 59–61° (ethanol), $[\alpha]_D^{20}$ +162° (*c* 0.85, chloroform); lit.[16] m.p. 78 to 103° according to refs. given $[\alpha]_D^{17-25}$ +162.2° to 164.5° (chloroform); ^{1}H-n.m.r. (acetone-d_6): δ 5.50 (t, $J_{3,4}$ 10 Hz, H-4), 5.30 (dd, $J_{C\text{-}1,H}$ 175 Hz, H-1), 5.06 (t, $J_{2,3}$ 10 Hz), 5.05 (dd, $J_{1,2}$ 3.5 Hz, H-2), 4.26 (dd, $J_{5,6a}$ 6 Hz, H-6a), 4.06 (m, $J_{4,5}$ 10 Hz, H-5), and 4.06 (m, $J_{6a,6b}$ 12 Hz, H-6b).

(1,1'-^{13}*C)α,α-Trehalose* (**4**). — To the peracetylated disaccharide **3** (40 mg) in dry methanol (20 mL) was added 0.1M sodium methoxide in methanol (0.2 mL). After 12 h, the base was neutralized with Amberlite IRN-77 (H^+) ion-exchange resin and the solution was evaporated. (1,1'-^{13}C)α,α-Trehalose was recovered as a foam (20 mg, 100%) by lyophilization of its aqueous solution; the ^{13}C-n.m.r. spectrum (deuterium oxide) showed a single signal at 94.0 p.p.m., with no detectable amount of α,β-trehalose contaminant.

Trehalase purification. — The purified enzyme from cockchafer (*Melolontha vulgaris*) was prepared according to ref. 17, but omitting the last purification step, and was used after chromatography on a column of acrylamide-agarose AcA 4/4. The specific activity was 9.2 units per mg of protein. Mutarotase activity of the enzyme preparation was tested by using α-D-glucose under the conditions used for the incubation of α,α-trehalose with the enzyme.

Polarimetric study of the hydrolysis of α,α-trehalose by trehalase. — Rates of change in optical rotation (see Fig. 2) were measured by using an optical rotatory dispersion instrument (Spectropol 1B Fica, Paris) in a quartz cell (0.05 dm) at 37° and 300-nm wavelength, which allows a greater sensitivity than the usual 589-nm wavelength. Incubations contained α,α-trehalose (dihydrate, Fluka, puriss., 7.5 μmol) and trehalase (1.0 IU) in a 25mM Tris-maleate buffer solution[18], pH 6.4, containing 0.1M NaCl, in a final volume of 1 mL.

A parallel experiment (same experimental conditions; final volume, 2 mL) was performed, in order to determine the amount of D-glucose released during the enzymic reaction. At given times (2, 8, 10, 12, 20, and 30 min), 250-μL aliquots were withdrawn, and diluted with the Somogyi reagent[19] to inactivate the enzyme. The amount of D-glucose released was then estimated by the method of Nelson[20] (see Fig. 3).

Control measurements of the rates of mutarotation were conducted using the same experimental parameters, with a 15 μmol/mL concentration of α-D-glucose (Prolabo R.P. Normapur) and β-D-glucose (N.B.C.), or their 1:1 mixture.

^{13}C-N.m.r. study of the enzymic hydrolysis of (1,1'-^{13}*C)α,α-trehalose* (**4**). —

The experiments (see Fig. 1) were conducted at 37°, using a Bruker WP-100 spectrometer and incubation solutions containing (1,1′-^{13}C)α,α-trehalose (**4**; 7.5 μmol), trehalase (0.35 IU), 4 drops of deuterium oxide, 25mM Tris-maleate buffer solution[18], pH 6.4, containing 0.1M NaCl, in a final volume of 0.5 mL. The n.m.r. spectra were recorded in the F.t. mode (acquisition time, 0.77 s; spectral width 4.400 kHz). Owing to the 90% isotopic enrichment, and the concentration used, the number of scans necessary to provide a sufficient signal-to-noise ratio was lowered to 36 for the first spectrum at 1.5 min of incubation time, and was raised to 120 for the other spectra.

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