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Determination of Maltase and Isomaltase Activities with a Glucose-Oxidase Reagent

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(*Received* 27 *February* 1961)

Glucose oxidase is a valuable tool for the assay of glycosidase activities, especially when the substrate itself is a reducing sugar, and so the common reductometric methods cannot be used for estimation of the hydrolysis products. However, the commercially available glucose-oxidase preparations also attack maltose and isomaltose in addition to glucose, probably as an effect of contaminant glycosidases. These contaminating enzymes prohibit the use of glucose oxidase for the assay of maltase and isomaltase activities, and necessitate the use of more complicated methods (Dahlqvist, 1960*c*).

This paper describes a modification of the glucose-oxidase reagent so that it may also be used for the determination of maltase and isomaltase activities.

MATERIALS AND METHODS

Glucose-oxidase preparations. The following preparations were used: glucose oxidase 'crude' and glucose oxidase 'purified' (Sigma Chemical Co., St Louis, U.S.A.), glucose oxidase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.), glucose oxidase–Dawes (obtained from Pfanstiehl Laboratories Inc., Waukegan, Ill., U.S.A.), glucose oxidase pure (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.), notatin Leo (Leo Pharmaceutical Prod., Copenhagen, Denmark) and glucose oxidase (C. F. Boehringer und Soehne, Mannheim, Germany).

Sugars. The commercial preparations used were of analytical-grade purity, glucose (dextrose) from Baker Chemical Co. (Phillipsburg, U.S.A.) and maltose monohydrate from Pfanstiehl Laboratories. Isomaltose was prepared in the laboratory by the method previously described (Dahlqvist, 1960*b*). α-Glucose refers to Baker Chemical Co. ordinary glucose (see above) dissolved in water at 20° less than 2 min. before use. Initial $[\alpha]_D^{20}$ +112° (5% in water). β-Glucose was obtained from Nutritional Biochemicals Corp. The sugar was dissolved in water at 20° less than 2 min. before use. Initial $[\alpha]_D^{20}$ +19° (5% in water). 'Equilibrium glucose' was obtained by keeping an appropriate solution of glucose at 20° for 18–20 hr. After that time $[\alpha]_D^{20}$ was +52·5° (10% in water). The solution

thus contained about one-third of the glucose present in the α-form and two-thirds in the β-form.

Other reagents. Ox-liver catalase was obtained from L. Light and Co. Ltd. (Colnbrook, Bucks.) horseradish peroxidase from Worthington Biochemical Corp. and 2-amino-2-hydroxymethylpropane-1:3-diol (tris), buffer grade, from Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Manometric determination of glucose-oxidase activity. The Warburg technique was used as described by Dixon (1951). Warburg flasks without centre cups were used. Each flask contained 2·0 ml. of acetate buffer–ethanol–catalase solution (50 mg. of ox-liver catalase and 1·0 ml. of 99·5% ethanol, made up with 0·1 M-sodium acetate–acetic acid buffer, pH 5·6, to a final volume of 100 ml.) and 1·0 ml. of an aqueous solution containing 9·85% (w/v) of equilibrium glucose. Into the side arm of each flask was introduced 0·5 ml. of glucose-oxidase solution containing 10–30 units of glucose oxidase. The reaction was performed at 25° in air; in oxygen the rate of oxygen uptake is 2·5 times that in air (Bentley, 1955). After equilibration the stoppers were closed and the flasks tipped. The absorption of oxygen was measured during 60 min. One unit of glucose-oxidase activity is the activity causing the consumption of 1 μl. of oxygen in 60 min. under these conditions.

The conditions used are essentially those recommended by Bentley (1955). The use of 0·1 M-β-glucose as substrate has been suggested, but the mutarotation of glucose then may cause difficulty since glucose oxidase does not oxidize α-glucose (Bentley, 1955). The author has therefore used 0·156 M-'equilibrium glucose' (which means 0·100 M-β-glucose and 0·056 M-α-glucose). Since α-glucose does not inhibit the oxidation of β-glucose (Bentley, 1955), this seemed to be a simple way to ensure the substrate concentration desired. The use of acetate buffer rather than phosphate buffer as suggested by Bentley (1955) does not alter the rate of oxidation (Keilin & Hartree, 1948).

Manometric determination of the maltase activity of glucose-oxidase preparations. Glucose formed by maltase in the presence of glucose oxidase will be oxidized to gluconic acid. For the determination of the maltase activity of glucose-oxidase preparations it was therefore impossible to use the reductometric method previously described (Dahlqvist, 1960*c*). For assay of the maltase activity of glucose-oxidase preparations a manometric method similar to that for determinations of glucose-oxidase activity was used, but the 'equilibrium glucose' solution was replaced by 1·0 ml. of 19·7% (w/v) maltose monohydrate in water, and the glucose-oxidase solution in the side arm of the flask contained 500–1000 units of glucose oxidase. The substrate solution was thus 0·146 M, pH 5·6, and the temperature 25°. The absorption of oxygen was measured during 60 min.

Under these conditions any glucose formed by the hydrolysis of maltose will be very rapidly oxidized, and each molecule of maltose hydrolysed will lead to the absorption of two molecules of oxygen. The relative maltase activity of the glucose-oxidase preparation is expressed as μl. of oxygen absorbed in 60 min. by 1000 units of glucose oxidase (see Table 1). Each 1 μl. of oxygen absorbed indicates the hydrolysis of 0·008 mg. of maltose. This way of expressing the maltase activity of glucose-oxidase preparations should not be confused with the unit for intestinal maltase activity specified below.

Spectrophotometric determination of glucose with a glucose-oxidase reagent. The method used was described by Huggett & Nixon (1957). The hydrogen peroxide formed during the dehydrogenation of glucose by glucose oxidase is catalysed by peroxidase at pH 7·0 to oxidize *o*-dianisidine to a brown compound. The intensity of the colour produced is measured spectrophotometrically.

The glucose-oxidase reagent of Huggett & Nixon (1957) was prepared in the following way: 125 000 units of glucose oxidase (i.e. 125 mg. of crude glucose oxidase, Sigma Chemical Co., or the corresponding amount of any other glucose-oxidase preparation; see Table 1), 0·5 mg. of horseradish peroxidase and 0·5 ml. of a 1% solution of *o*-dianisidine in 95% ethanol were taken and made up to 100 ml. with 0·5 M-sodium phosphate buffer, pH 7·0 [54·2 g. of $Na_2HPO_4,2H_2O$ and 23·4 g. of NaH_2PO_4 (or 26·9 g. of NaH_2PO_4,H_2O) dissolved in water and diluted to 1000 ml.; the pH was measured with a glass electrode and, if necessary, adjusted to 7·0]. The reagent was filtered through paper. On attempting to store this reagent in the refrigerator (at 4°) the phosphate buffer crystallized out.

For the determination of glucose, 0·5 ml. of the solution to be tested, containing 10–50 μg. of glucose, was mixed with 3·0 ml. of glucose-oxidase reagent in a test tube and placed in a water bath at 37° for 60 min. After that time the intensity of the colour produced was measured in a spectrophotometer at 420 mμ, with 1 cm. cuvettes, against a blank without sugar. At the same time a standard series was made, with tubes containing known amounts (10, 30 and 50 μg.) of glucose. The amount of glucose present should not exceed 50 μg., otherwise the Lambert–Beer law will not be obeyed.

The standard series may be prepared from a stock aqueous solution containing 100 mg. of glucose and 2·7 g. of benzoic acid in 1 l. This solution is stable for a long time at room temperature. The benzoic acid does not interfere with the enzymic determination of glucose.

Spectrophotometric determination of glucose with a modified tris–glucose-oxidase reagent. In this reagent the phosphate buffer has been replaced with 0·5 M-tris buffer, pH 7·0 (61·0 g. of tris is dissolved in 85 ml. of 5N-HCl and the solution is made up to 1 l. with water; the pH is measured with a glass electrode and, if necessary, adjusted to 7·0). Otherwise the preparation of the reagent and the determination of glucose are performed in the same way as described above for Huggett & Nixon's reagent.

The tris–glucose-oxidase reagent has an advantage over Huggett & Nixon's glucose-oxidase reagent, that it reacts considerably more slowly with maltose and isomaltose (see below). It may be stored for at least 3–4 days in the refrigerator.

RESULTS AND DISCUSSION

Glycosidase activities of different glucose-oxidase preparations

The analysis of a series of commercially available glucose-oxidase preparations (Table 1) showed them all to contain sufficient maltase activity to make them unsuitable for the determination of glucose in the presence of maltose. Isomaltose was hydrolysed, too, with a rate that was about one-third of that of maltose.

Many other disaccharides, however, such as turanose, palatinose (isomaltulose), lactose, cello-

Table 1. *Glucose-oxidase activity and relative maltase activity of some commercially available glucose-oxidase preparations*

Activities were measured manometrically, as described in the text.

Source and description	Glucose-oxidase activity (units/mg.)	Relative maltase activity (μl. of O_2 absorbed/1000 units of glucose oxidase)
Sigma Chemical Co; crude*	1 000	135
Sigma Chemical Co.; purified*	11 000	115
Worthington Biochemical Corp.*	1 000	135
Dawes (Pfanstiehl)*	10 000	50
Nutritional Biochemicals Corp.; pure*	17 600	113
Leo (Notatin Leo)†	18 600	180
C. F. Boehringer und Soehne†	13 850	35

* From *Aspergillus niger*.
† From *Penicillium notatum*.

biose, gentiobiose, sucrose and trehalose, were not hydrolysed by the glucose-oxidase preparations investigated, and therefore glucose oxidase has been used for the determination of the extent of hydrolysis of these disaccharides by glycosidases either by manometric (Dahlqvist, 1958, 1960*a*) or spectrophotometric (Dahlqvist, 1960*c*, 1961) methods.

That the action of glucose oxidase on maltose and isomaltose is caused by contaminating glycosidases rather than the oxidation of the disaccharides by glucose oxidase was shown by the fact that each molecule of maltose or isomaltose consumed two molecules of oxygen under the conditions used for manometric determinations, whereas one molecule of glucose consumed one molecule of oxygen. This fact also rules out the possibility that the oxygen consumption of maltose and isomaltose in the presence of glucose oxidase might be caused by contaminant glucose.

Attempts to separate glucose oxidase from the contaminant glycosidase activities

The purification of glucose oxidase from *Penicillium notatum* to yield a product free from maltase activity has been described by Coulthard *et al.* (1945). Attempts to use this procedure with one of the glucose-oxidase preparations studied (Worthington Biochemical Corp.) met with no success. Precipitation with tannic acid yielded a recovery of 30% of the original glucose-oxidase activity, but lowered the relative maltase activity by only 25%. Fractionation with ammonium sulphate (50–80% saturation) yielded a recovery of 26% of the glucose-oxidase activity, but did not lower the relative maltase activity at all. The negative results obtained may be explained by the fact that the enzyme preparations had been prepared from different species, since the Worthington Biochemical Corp. preparation is obtained from *Aspergillus niger*. The two glucose-oxidase preparations from *Penicillium notatum* (see Table 1) have not been subjected to fractionation by these methods.

Attempts to purify glucose oxidase by heat inactivation also gave negative results, and no encouraging results were obtained from preliminary experiments with cellulose ion-exchange columns (prepared and used as described by Peterson & Sober, 1956). Neither glucose oxidase nor maltase was adsorbed on carboxymethylcellulose in 0·01 M-maleate buffer, pH 5·3. Both enzymes were adsorbed on diethylaminoethylcellulose in 0·01 M-sodium phosphate buffer, pH 6·2, and they were eluted slowly and together by the addition of 0·10 M-NaCl to the buffer. The two enzymes thus seem to have similar chromatographic properties.

Selective inhibition of the glycosidase activities

Attempts were made to find an enzyme inhibitor which would inhibit the glycosidases without interfering with the glucose-oxidase activity. These experiments were performed with the spectrophotometric method of Huggett & Nixon (1957), with 50 μg. of glucose or 2 mg. of maltose (monohydrate) in each test tube. The substances to be tested for inhibitory action were added to the glucose-oxidase reagent. Tris buffer proved to be a suitable inhibitor.

The development of the colour with glucose and maltose as substrates in phosphate and tris buffers is seen in Fig. 1. When the reagent contained 0·5 M-phosphate buffer, pH 7·0 (i.e. the reagent of Huggett & Nixon 1957), intense colour was produced in 60 min. by 2 mg. of maltose, but when the buffer was replaced by 0·5 M-tris buffer, pH 7·0, the colour produced by maltose was strongly reduced. In the method for determination of the maltase activity of intestinal-glycosidase preparations which will be described below, each tube for glucose determination contains 1 mg. of maltose.

The development of colour with glucose was not significantly influenced by tris (Fig. 1). In other experiments, the presence of maltose did not influence the colour produced by varying amounts of glucose.

The isomaltase activity of glucose-oxidase preparations was found to be inhibited by tris in the same way as the maltase activity.

The selective inhibition of the glycosidase activities without interference with the glucose-oxidase activity has been demonstrated both with

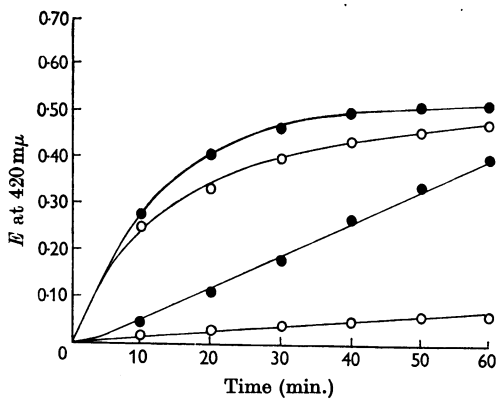

Fig. 1. Development of colour with glucose and maltose by Huggett & Nixon's (1957) glucose-oxidase reagent, containing 0·5 M-phosphate buffer, pH 7·0, and a modified reagent in which the phosphate buffer has been replaced by 0·5 M-tris buffer, pH 7·0 (for preparation of the reagent see text). ●, Phosphate buffer; ○, tris buffer. The upper two curves were obtained with glucose (50 μg.) present; the lower two curves were obtained with maltose (2 mg.) present.

preparations obtained from *Aspergillus niger* and with those obtained from *Penicillium notatum*.

During the conditions for manometric determination of the glucose-oxidase and maltase activities of glucose-oxidase preparations the inhibitory effect of tris on the maltase activity seemed weaker. In the presence of 0·57 M-tris (12·11 g. of tris dissolved in about 50 ml. of water, adjusted with acetic acid to pH 5·6 and diluted with water to 100 ml., this buffer replacing the acetate buffer used elsewhere for the manometric determinations) the maltase activity was reduced by about 50 %, and the glucose-oxidase activity was not affected.

The weaker inhibitory action of tris on the maltase activity in the manometric determination might have been caused by the lower pH used (pH 5·6 in the manometric method and pH 7·0 in the spectrophotometric method). In an earlier study on the inhibition of intestinal glycosidases by tris only the non-ionized tris molecule was found to be an inhibitor (Dahlqvist, 1958), and thus the degree of inhibition increased with increasing pH. In the present case, however, when the manometric determination was performed at pH 7·0, the degree of inhibition by 0·57 M-tris was as low as at pH 5·6. Likewise, when the spectrophotometric method was used at pH 5·6, the degree of inhibition was as high as at pH 7·0. The inhibition of the maltase activity by tris thus seems to be independent of pH within this interval, and either the positively charged tris ion (which is the predominating species at both these pH values), or both the ion and the uncharged molecule, act as inhibitors.

The weaker effect obtained in the manometric method was caused by the higher maltose concentration in this method (0·146 M in the manometric, and 1·6 mM in the spectrophotometric method). Variation of the maltose concentration in the spectrophotometric method at pH 7·0 between 0·4 and 40 mM revealed that tris acted as a competitive inhibitor for the maltase activity, so that the degree of inhibition was dependent on the substrate concentration. K_s for the maltase activity (i.e. the substrate concentration yielding half-maximum velocity of hydrolysis) was determined to be approximately 1×10^{-2}, and K_i for tris as a competitive inhibitor (i.e. the dissociation constant for the enzyme–inhibitor complex) about 1×10^{-1} (the enzyme kinetic constants have been calculated as described by Dixon & Webb, 1958). Thus if the test tube under the conditions used for the enzymic determination of glucose contains less than 2 mg. of maltose (1·6 mM), the maltase activity will be inhibited by more than 80 % (cf. Fig. 1).

Influence of the mutarotation of glucose

Glucose oxidase catalyses the oxidation of only β-glucose (Keilin & Hartree, 1952*a*), whereas the glucose liberated from maltose and isomaltose by animal enzymes is probably α-glucose (Ben-Gershom & Leibowitz, 1958). The spontaneous mutarotation of glucose at neutral or slightly acid pH is a rather slow process (Pigman, 1957), but it is strongly catalysed by a specific enzyme, mutarotase, which is present in crude glucose-oxidase preparations (Keilin & Hartree, 1952*b*). This enzyme may be important for the determination of glucose with glucose oxidase, and the possibility that mutarotase may be inhibited by tris has to be considered.

Investigation of the spontaneous mutarotation of α-glucose under conditions similar to those used for colorimetric determination of glucose (i.e. in 0·5 M-tris buffer, pH 7·0) showed, however, that more than 80 % of the reaction was completed in 15 min. at 20°, and hence supposedly even more at 37°.

When the development of the colour of the tris–glucose-oxidase reagent with freshly prepared solutions of α-glucose and β-glucose (50 μg. of each) was studied it was found that both forms of glucose were oxidized at about the same rate. Thus although it cannot be decided from these experiments whether or not mutarotase is inhibited by tris, it is evident that both α- and β-glucose react rapidly with the tris–glucose-oxidase reagent.

Assay of intestinal maltase and isomaltase activities with the tris–glucose-oxidase reagent

In accordance with the general conditions previously specified for intestinal glycosidase-activity

determinations (Dahlqvist, 1960*c*), the enzyme should act for 60 min. at 37° on 0·028 M-substrate in the presence of 0·05 M-maleate buffer at optimum pH. One unit of glycosidase activity under these conditions is defined as that causing 5 % of hydrolysis in 2·0 ml. of reaction mixture.

The substrate solutions are prepared by dissolving 0·20 g. of maltose monohydrate or 0·19 g. of isomaltose in 0·1 M-maleate buffer, pH 6·5 (prepared as described by Gomori, 1955), to a final volume of 10·0 ml. A few drops of toluene are added as a preservative.

The enzyme solution to be analysed is diluted so as to contain 0·2–1·0 unit of maltase or isomaltase activity/ml. Of this diluted solution 0·1 ml. is transferred to a small test tube (7 mm. × 120 mm.). The tube is placed in a water bath at 37°, 0·1 ml. of substrate solution is added and the contents of the tube are mixed. After 60 min. of incubation, 0·8 ml. of water is added and the enzymic reaction is immediately stopped by immersing the tube in a boiling-water bath for 2 min.

A blank is prepared, which has the same composition, but in which the enzymic activity is stopped by boiling immediately after the mixing of enzyme and substrate.

After the tubes are cooled with tap water, their contents are well mixed and 0·5 ml. is transferred to another test tube (16 mm. × 150 mm.). To each tube is added 3·0 ml. of the tris–glucose-oxidase reagent (prepared as described above) and the tubes are placed in a water bath at 37° for 60 min. for development of the colour. A standard series with glucose (see above) is prepared for each set of determinations. The intensity of the colour is measured in a spectrophotometer at 420 mμ in 1 cm. cuvettes, against a sample without sugar.

The production of 50 μg. of glucose during these conditions corresponds to 1 unit of maltase or isomaltase activity/ml. of the diluted enzyme solution used for the determination. The degree of hydrolysis then is 5 %.

Use of the tris–glucose-oxidase reagent for assay of other intestinal-glycosidase activities

The tris–glucose-oxidase reagent may replace the Huggett & Nixon (1957) reagent in the method described for the assay of intestinal-turanase, -lactase, -cellobiase and -gentiobiase activity (Dahlqvist, 1960*c*), as well as for measuring the activity towards non-reducing substrates such as sucrose and trehalose. The number of glucose molecules liberated per substrate molecule must, however, be taken into account, since glucose oxidase does not react with fructose or galactose (Dahlqvist, 1960*c*).

This reagent thus may be used as a general tool for measuring glycosidase activities which liberate glucose from a disaccharide or an oligosaccharide. It is not suitable, however, for the assay of phenylglucosidase activities, since free phenol interferes with the production of the colour (Dahlqvist, 1960*c*).

SUMMARY

1. Commercially available glucose-oxidase preparations contain also maltase and isomaltase activity. Attempts to separate glucose oxidase from these contaminating glycosidase activities were unsuccessful.

2. The presence of 0·5 M-tris buffer, pH 7·0, selectively inhibits these glycosidase activities, without interfering with the production of colour with free glucose by a glucose-oxidase reagent containing *o*-dianisidine as a chromogen.

3. A method for the determination of intestinal maltase and isomaltase activities based upon a tris–glucose-oxidase reagent is described.

4. The tris–glucose-oxidase reagent may be used as a general reagent for the determination of glucose liberated by glycosidases from di- and oligo-saccharides. It is not suitable for the assay of the hydrolysis of phenylglucosides.

This investigation has been supported by a grant from the Swedish Medical Research Council. Miss A. Hansson and Miss E. Josefsson are thanked for skilful technical assistance.

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