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CARBOHYDRATE OXIDASE, A NOVEL ENZYME FROM *POLYPORUS OBTUSUS**

II. SPECIFICITY AND CHARACTERIZATION OF REACTION PRODUCTS

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SUMMARY

1. Carbohydrate oxidase, an enzyme obtained from the mycelium of the Basidiomycete *Polyporus obtusus*, has been shown to catalyze the oxidation of several carbohydrates at the second carbon atom to yield 2-keto products. The reactions catalyzed are as follows:

a. D-glucopyranose + O_2 → D-glucosone + H_2O_2
b. D-xylopyranose + O_2 → D-xylosone + H_2O_2
c. L-sorbose + O_2 → 5-keto-D-fructose + H_2O_2
d. δ-D-gluconolactone + O_2 → 2-keto-D-gluconic acid + D-araboascorbic acid + H_2O_2

2. Other carbohydrates were tested and found not to be substrates. The structural requirements for substrate reactivity were studied.

3. The oxidation of δ-gluconolactone to araboascorbic acid was shown to be inhibited by D-glucose, D-xylose and L-sorbose.

4. The specificity of carbohydrate oxidase was compared with that of enzymes reported to occur in other microorganisms.

INTRODUCTION

The Basidiomycete *Polyporus obtusus* produces an enzyme which catalyzes the oxidation of D-glucose, D-xylose, L-sorbose and δ-D-gluconolactone. The isolation and purification of this enzyme, which has been named "carbohydrate oxidase", were reported in the preceding paper[1]. The present publication describes the specificity of the enzyme, the nature of the oxidation reaction and the identification of the oxidation products.

MATERIALS

Crystalline beef liver catalase suspension (152 000 units/ml) was purchased

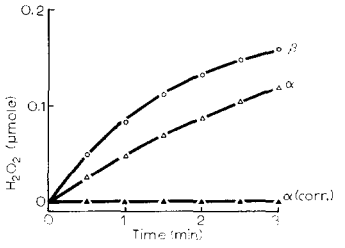
* This paper is dedicated to the memory of RICHARD KUHN.

from Worthington Biochemical Corporation, Freehold, N.J. γ-Gluconolactone was prepared by the method of PASTERNACK AND CRAGWALL[2] and recrystallized from dioxane as described by ISBELL AND FRUSH[3]. Glucosazone and xylosazone were prepared as described for glucosazone in HAWK, OSER AND SUMMERSON[4]. Glucosone and its diphenylhydrazone were prepared by the method of HENSEKE AND LIEBENOW[5]. All other substrates and reagents were obtained in the highest purity available from commercial suppliers.

METHODS

Assay and purification of carbohydrate oxidase. Assay of the enzyme by a peroxidase-chromogen procedure and purification by fractional precipitation with polyethylene glycol were performed as described in the first report[1].

Specificity of carbohydrate oxidase. The activity of the enzyme in the oxidation of the anomeric glucoses and the isomeric gluconolactones was determined as described in Figs. 1 and 2, respectively. Mutarotation rates were determined at 25° for α- and



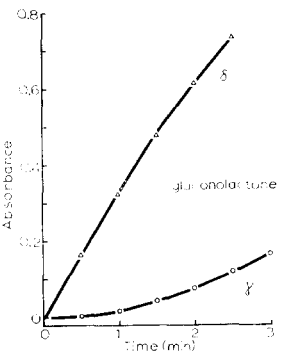


Fig. 1. Action of carbohydrate oxidase upon α- and β-glucose. Solutions of α- and β-glucose (50 μg/ml H_2O) were prepared immediately before use. A 1.0 ml aliquot was added to a test tube and mixed rapidly with 2.7 units of carbohydrate oxidase in 4.0 ml of peroxidase–dianisidine reagent[1] buffered at pH 7.0. The contents of the tube were immediately transferred to a cuvette and the absorbance read at 30-sec intervals at 460 mμ in a Beckman DB spectrophotometer at 25°. Correction of α-D-glucose oxidation for mutarotation is described in the text. ○—○, β-glucose; △—△, α-glucose; ▲—▲, α-glucose corrected for mutarotation.

Fig. 2. Action of carbohydrate oxidase upon γ- and δ-gluconolactones. The reaction rates of carbohydrate oxidase in the oxidation of γ- and δ-gluconolactones were determined in the same manner as for the anomeric glucoses except that the substrates were dissolved in 0.02 M acetic acid (1 mg/ml), the enzyme level was increased to 4.2 units and the reagent was buffered at pH 6.5. △—△, δ-gluconolactone; ○—○, γ-gluconolactone.

β-glucose in 0.04 M sodium phosphate (pH 7.0), the buffer used in the oxidation studies. The curve for observed oxidation of α-D-glucose was corrected for mutarotation by determination of the real velocity constant as described by KEILIN AND HARTREE[6]. The curve for oxidation of β-D-glucose was not corrected. The enzyme was tested for activity against several substrates as described in Table I.

Preparation of the oxidation product of glucose and its 1,1-diphenylhydrazone. The glucose oxidation product was prepared by allowing 4 mmoles of D-glucose (equilibrium mixture), 29 units of carbohydrate oxidase, 12 000 units of catalase and 200 mg of

$CaCO_3$ to react for 2 h under an O_2 atmosphere in a shaker water bath at 25° in a total volume of 40 ml water. Excess $CaCO_3$ was removed by filtration and the solution was stored in the refrigerator under N_2. The 1,1-diphenylhydrazine derivative was prepared from the aqueous reaction mixture as described by HENSEKE AND LIEBENOW[5]. No crystals formed upon standing, but an oil which gradually solidified was obtained by addition of H_2O to the ethanolic solution. Colored impurities were removed from the derivative by extraction of an aqueous suspension with toluene. The derivative was then crystallized from ethanol–water. Melting points, mixed melting points and infrared spectra were compared for the 1,1-diphenylhydrazine derivatives of the glucose oxidation product and of authentic glucosone.

TABLE I

SPECIFICITY OF CARBOHYDRATE OXIDASE

Solutions of the substrates were prepared at 0.22 M concentrations. To 1.0 ml of substrate solution was added 3.0 ml of peroxidase–dianisidine reagent prepared as described in the first report[1]. At zero time, 1.0 ml of a solution containing 0.06 unit of carbohydrate oxidase was added. The reaction was stopped by addition of 0.2 ml of 4.0 M HCl and the color was read on a Klett–Summerson colorimeter using a No. 42 filter. The four substrates were allowed to react 5 min at 25° and the unreactive substances (1% or less activity) were allowed to react for 15 or 30 min.

Substrate	*Activity (% of glucose)*	*Substrate*	*Activity (% of glucose)*
D-Glucose	100	D-Glucosamine	<1
L-Sorbose	59	*N*-Acetyl-D-glucosamine	<1
D-Xylose	38	3-*O*-Methyl-D-glucose	<1
δ-D-Gluconolactone	14	D-Fucose	<1
D-Galactose*	<1	D-Gulono-γ-lactone	<1
D-Maltose*	<1	D-Glucose -6-phosphate	<1
D-Mannose	<1	D-Ribose	<1
2-Deoxy-D-glucose	<1	*meso*-Inositol	<1
D-Fructose	<1	D-Trehalose	<1
D-Sorbitol	<1	D-Lactulose	<1
D-Mannitol	<1	Levoglucosan	<1
D-Arabinose	<1	Methyl-β-D-glucoside	<1
D-Lactose	<1	D-Gluconoheptonic lactone	<1
D-Glucuronolactone	<1	D-Saccharic acid	<1
Sodium D-gluconate	<1	*N*-Methylglucosamine	<1
Dulcitol	<1		

* Two apparent substrates, maltose and galactose, were shown to contain glucose. When the maltose preparation was subjected to paper chromatography and sprayed with a reagent consisting of carbohydrate oxidase and peroxidase-chromogen (*cf.* PAZUR AND KLEPPE[11]) the maltose spot was negative, but a spot having the R_F of glucose yielded a positive reaction. The galactose preparation was subjected to oxidation for 1 h by carbohydrate oxidase in the presence of catalase. After destruction of the residual enzymes, the galactose was tested in the peroxidase–chromogen system. No reaction was observed.

Preparation of the phenylhydrazine derivative of the glucose oxidation product. Glucose was oxidized as described above except that the reaction period was 20 h and the reaction mixture was buffered at pH 6.0 with 0.1 M sodium succinate buffer. The reaction mixture (5.5 ml) was acidified with 0.34 ml of 4.0 M HCl and was then added to 10 ml of a 0.3% solution of phenylhydrazine acetate[4] in methanol. The solution was kept at room temperature. The crystals, which began to form in about 3 min, were filtered after 2 h and washed with 25% acetic acid followed by 50% ethanol. Infrared

spectra were obtained (KBr pellets) on the phenylhydrazine derivative of the oxidation product and on authentic glucosazone.

Preparation of the L-sorbose oxidation product and its phenylhydrazine derivative. L-Sorbose was oxidized in the presence of $CaCO_3$ under the same conditions as described for glucose. After the reaction had proceeded for 4 h, the phenylhydrazine derivative was prepared by addition of 400 mg of phenylhydrazine base to 15 ml reaction solution. The mixture was kept at room temperature for 4 h. The suspension of crystals was then placed in the refrigerator overnight. The derivative was collected on a filter and washed with cold ethanol and ether. Melting point, optical rotation and elemental analyses were carried out on the derivative after recrystallization from ethanol–water.

Preparation of the xylose oxidation product and its phenylhydrazine derivative. Xylose was oxidized in the following system: A mixture of 5 mmoles of D-xylose, 80 units of carbohydrate oxidase, 7500 units of catalase and 50 ml of 1% phenylhydrazine base in water at pH 7.8 was shaken for a total of 4 h at 25° under an atmosphere of O_2 in a shaker water bath. After 2 h the pH of the reaction mixture, which had decreased from 7.8 to 5.6, was readjusted to its original value with 0.1 M NaOH. The precipitate which formed during the reaction was recovered by filtration and dried under vacuum. It was then dissolved in ethanol and filtered to remove insoluble material. The ethanol solution was evaporated to dryness and the colored impurities were removed by extraction of the residue with ether. The residue was then redissolved in ethanol and a crystalline derivative was obtained by careful addition of water. Infrared spectra of the derivative and of authentic xylosazone were obtained (KBr pellets).

Preparation and paper chromatography of the oxidation product of δ-gluconolactone. Gluconolactone was oxidized and the products chromatographed as described in Table II.

Inhibition of δ-gluconolactone oxidation. The carbohydrate oxidase-catalyzed oxidation of δ-gluconolactone was studied in the presence of D-glucose, D-xylose and L-sorbose. The enzyme reactions were set up as described in Fig. 4.

RESULTS

Specificity of carbohydrate oxidase. The action of carbohydrate oxidase upon several substrates is summarized in Table I. The results are based upon the amount of H_2O_2 formed per min and are expressed as percentages of the glucose reaction.

Action of carbohydrate oxidase upon α- and β-D-glucose. The curves for reaction rate of the two anomeric forms show that only β-glucose is a substrate (Fig. 1). Although the uncorrected curve for α-glucose indicates appreciable oxidation, the real velocity constant (*cf.* KEILIN AND HARTREE[6]) was found to be zero after correcting for mutarotation. The mutarotation constant under our experimental conditions is 0.289.

Action of carbohydrate oxidase on γ- and δ-gluconolactones. Fig. 2 shows the action of carbohydrate oxidase on γ- and δ-gluconolactones. The initial rate of reaction with the δ-lactone is linear. For γ-gluconolactone the initial reaction is negligible; the reaction begins only after a lag period of nearly 1 min and the rate increases slowly over the next 2 min. Apparently only the δ-lactone is a true substrate.

Identity of the glucose oxidation product. The 1,1-diphenylhydrazine derivative of the oxidation product of glucose had m.p. 162–163°. (Found: C, 62.99; H, 6.05;

N, 7.99. $C_{18}H_{20}O_5N_2$ requires C, 62.78; H, 5.86; N, 8.14.) Authentic glucosone-1,1-diphenylhydrazone had m.p. 163–164°, mixed m.p. 162–163°. The infrared curves were identical. These data establish the identity of the 1,1-diphenylhydrazones of glucosone and of the glucose oxidation product. The infrared spectrum of the phenylhydrazine derivative of the glucose oxidation product was identical to that of glucosazone. Glucose does not yield the osazone at room temperature under the conditions used for preparation of the oxidation product derivative. Glucosone, however, has been shown to form the osazone at room temperature[7].

Identity of the L-*sorbose oxidation product.* The phenylhydrazine derivative of the L-sorbose oxidation product had m.p. 132–133.5° (lit. value for the bis-phenylhydrazone of 5-keto-D-fructose, 133–135°; see ref. 8), $[\alpha]_D$ −170 (*c* 1.2 in pyridine), (lit. $[\alpha]_D$ −164 [*c* 1.2 in pyridine][8]). (Found: C, 61.3; H, 6.44; N, 15.03. $C_{18}H_{22}O_4N_4$ requires C, 60.5; H, 5.9; N, 15.7.) These data indicate that L-sorbose is oxidized to 5-keto-D-fructose.

Identity of the xylose oxidation product. The infrared spectra of xylosazone and the phenylhydrazine derivative of oxidized xylose are essentially identical (Fig. 3),

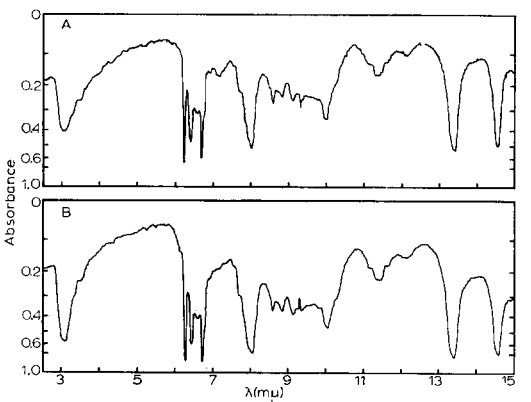


Fig. 3. Infrared spectra of xylose phenylosazone (A) and of the phenylhydrazine derivative of the xylose oxidation product (B). Spectra were obtained on derivatives prepared as described in the text.

thus indicating that xylosone is the product formed from xylose by the action of carbohydrate oxidase. Xylose does not yield the osazone under the conditions of this experiment.

Identity of the gluconolactone oxidation products. The paper chromatography of the gluconolactone oxidation products is summarized in Table II. Spots having the same R_F as araboascorbic acid were detected on paper chromatograms of the reaction product after treatment with 2,6-dichlorophenolindophenol or silver nitrate.

Phenylenediamine reacts with 2-ketogluconic acid to give a hydroxyquinoline which is fluorescent and can be detected under short wave ultraviolet light. This reagent reveals one main spot at the same R_F as 2-ketogluconic acid on paper chromatograms of the gluconolactone reaction product; therefore, the main phenylenediamine positive spot appears to be 2-ketogluconic acid. Minor fluorescent reaction products were not identified.

TABLE II

PAPER CHROMATOGRAPHY OF THE OXIDATION PRODUCT OF GLUCONOLACTONE

Gluconolactone was oxidized in the following system: 1 mmole of δ-D-gluconolactone, 15 units of carbohydrate oxidase, 15 000 units of catalase and 1 mmole of $CaCO_3$ in 10 ml of water was shaken under an O_2 atmosphere for 2 h. 1 ml samples were removed at 1 and 2 h, cooled in an ice bath and centrifuged at 12 800 × *g* for 5 min at 4°. 5 μl aliquots of the supernatants and standards were chromatographed on No. 1 Whatman paper in the three solvents listed below. The chromatograms were then treated with the indicated reagents.

	Reaction mixture	*2-Keto-gluconic acid*	*Arabo-ascorbic acid*
R_F in Solvent 1*	0.11[a] 0.36[b,c]	0.11[a]	0.36[b,c]
R_F in Solvent 2**	0.16[a] 0.47[c]	0.19[a]	0.47[c]
R_F in Solvent 3***	0.10[a]	0.09[a]	

[a] Spots revealed by *o*-phenylenediamine[9].
[b] Spots revealed by 2,6-dichlorophenolindophenol (0.04% in 50% ethanol).
[c] Spots revealed by silver nitrate (0.6% in 98% acetone).
* Solvent 1: *n*-butanol–acetic acid–water (4:1:5, by vol.), H_2S atmosphere.
** Solvent 2: *n*-propanol–methyl benzoate–acetic acid–water (7:3:2:5, by vol.).
*** Solvent 3: pyridine–ethyl acetate–acetic acid–water (5:5:1:3, by vol.).

Inhibition of δ-gluconolactone oxidation. D-Glucose completely inhibits the formation of araboascorbic acid from gluconolactone. D-Xylose and L-sorbose partially suppress gluconolactone oxidation. No araboascorbic acid-like products are obtained from oxidation of D-glucose, D-xylose or L-sorbose. These results are summarized in Fig. 4.

DISCUSSION

Of the many substances tested, significant reaction occurred with only four:

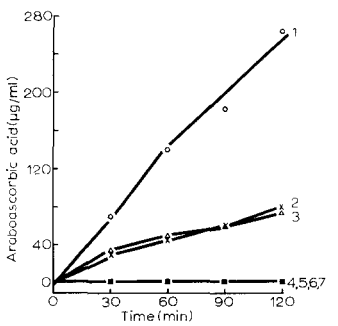


Fig. 4. Inhibition of δ-gluconolactone oxidation. All flasks contained 3.2 units of carbohydrate oxidase, 1500 units of catalase and 50 mg of $CaCO_3$ in a final volume of 10 ml H_2O. In addition, control flask (1) contained 0.5 mmole of δ-gluconolactone and flasks (2), (3) and (4) also contained 1 mmole of L-sorbose, D-xylose and D-glucose, respectively. Flasks (5), (6) and (7) were the same as (2), (3) and (4), respectively, except that δ-gluconolactone was omitted. All flasks were shaken for 2 h in a 25° water bath shaker. 1 ml aliquots were transferred at 30-min intervals to test tubes containing 1.0 ml of 3% metaphosphoric acid. The tubes were stored in an ice bath until assayed for araboascorbic acid by the method of OWEN AND IGGO[10].

D-glucose, D-xylose, L-sorbose and δ-D-gluconolactone. The other sugars, sugar alcohols, sugar acids and amino sugars were not substrates.

Glucose, the best substrate, is oxidized at the second carbon atom to glucosone. This substance is known to be produced by a mollusk[12], a mold of the genus Aspergillus[13] and an alga[14], but its formation from glucose by means of a purified enzyme has not been previously reported. Indeed, the present publication appears to be the first report on a purified enzyme that specifically catalyzes the oxidation of an aldose in the 2-position. The reactive anomer is β-D-glucose. The apparent oxidation of the α-anomer was found to be negligible after correction for mutarotation.

D-Xylose is also oxidized at the second carbon to yield D-xylosone. The enzymatic formation of this substance has not been reported previously. Attempts to isolate the phenylhydrazone, after the termination of the reaction as was done for glucose, resulted in the recovery of a small amount of an unidentified phenylhydrazine derivative. Spontaneous degradation of xylosone (the initial reaction product) to reducing fragments is considered the possible explanation for this finding. The addition of phenylhydrazine to the reaction apparently traps the xylosone as it forms and prevents its degradation. In analogy to the glucose oxidation, the β-anomer of D-xylose is probably the reactive form.

L-Sorbose is oxidized at the fifth carbon to form 5-keto-D-fructose. YAMADA *et al.*[15] reported the formation of this substance from L-sorbose by an oxidase obtained from *Trametes sanguinea*, a Basidiomycete. Also, a particulate dehydrogenase which catalyzes the oxidation of L-sorbose to 5-keto-D-fructose has been isolated from *Acetobacter suboxydans*[16].

The oxidation of gluconolactone is apparently more complex than the oxidation of the three other substrates. Two main products, *i.e.*, 2-ketogluconic acid and araboascorbic acid, are formed. An enzyme which oxidizes gluconic acid to 2-ketogluconate has been described[17].Ara boascorbic acid formation from glucose, gluconolactone (γ and δ) and gluconate anion by dried cells of *Penicillium notatum* has been reported by TAKAHASHI AND MITSUMOTO[18]. Of the various forms of gluconic acid—*i.e.*, gluconate anion, undissociated gluconic acid, δ-gluconolactone and γ-gluconolactone—only the δ-lactone is oxidized by carbohydrate oxidase to a significant extent. Apparent oxidation of γ-gluconolactone may be attributed to spontaneous formation of the δ-lactone from the γ-lactone[19].

Formation of the two reaction products from δ-gluconolactone could arise as shown in Scheme I. We postulate the intermediate formation of δ-2-ketogluconolactone followed by spontaneous conversion to 2-ketogluconic acid and araboascorbic acid. With carbohydrate oxidase the intermediate formation of γ-gluconolactone is not a prerequisite for araboascorbic acid production as proposed by TAKAHASHI AND MITSUMOTO[18] for *Penicillium notatum*. Furthermore, no araboascorbic acid was formed with carbohydrate oxidase acting on glucose, which is the best substrate for araboascorbic acid production from *Penicillium notatum*[18].

The inability to separate enzymic entities by several fractionation methods suggests that all four substrates are oxidized by the same enzyme. This view is supported by the inhibition experiment described in Fig. 4. The oxidation of gluconolactone to araboascorbic acid is completely inhibited by glucose and is partially inhibited by L-sorbose and D-xylose. These results can best be explained by competition between gluconolactone and the other substrates for the same active site on a single enzyme.

Once the specificity of carbohydrate oxidase and the nature of the reaction products had been established, the relation of this enzyme to previously reported sugar oxidases was examined.

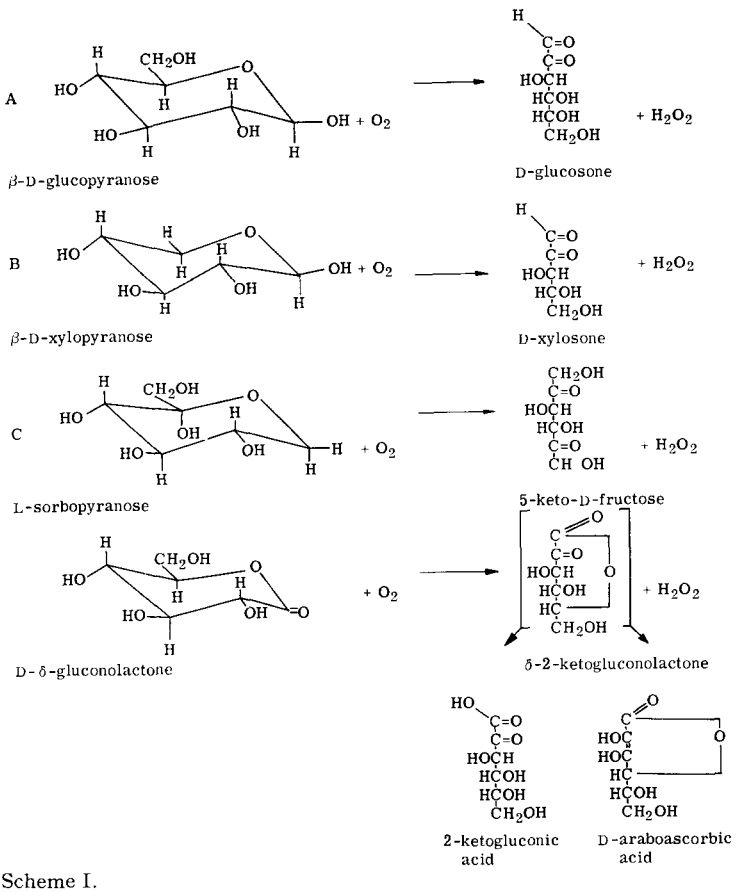
YAMADA *et al.*[15] recently described an enzyme from *Trametes sanguinea* which catalyzed the oxidation of L-sorbose to 5-keto-D-fructose. Their preparation also catalyzed the oxidation of D-glucose, D-galactose, D-xylose and maltose, but the reaction products from these substrates were not identified. The specificities of this "L-sorbose oxidase" are remarkably similar to those of carbohydrate oxidase except for the activity on galactose and maltose. Although both of these sugars were apparent substrates for carbohydrate oxidase, removal of glucose by paper chromatography or a preliminary carbohydrate oxidase oxidation revealed that neither maltose nor galactose was a substrate. Purified galactose has been shown to contain glucose[20]. Oxidation of gluconolactone would not have been detected by YAMADA *et al.*, since they used the sodium salt of gluconic acid in their experiments. Sodium gluconate, it may be noted, was also not a substrate for carbohydrate oxidase. While this manuscript was in preparation a second report by YAMADA *et al.*[21] described the purification and properties of L-sorbose oxidase. The lack of separation of this preparation during its purification and its stability to heat suggests that here also one enzyme may be acting on all substrates. However, the authors still seem to have reservations about the homogeneity of L-sorbose oxidase, especially with respect to the glucose oxidizing activity. If, indeed, glucose and L-sorbose are oxidized by the same enzyme in their preparation, then glucose is a better substrate than L-sorbose for "L-sorbose oxidase". Furthermore, it would then appear likely that the preparation described by YAMADA *et al.* may contain carbohydrate oxidase.

LYR[22] reported an enzyme in crude extracts of *Trametes versicolor* and *Phellinus igniarius* which catalyzed the oxidation of xylose. Xylonic acid was identified as a product only by paper chromatography of the reaction mixture. The same organisms contained a glucose oxidase which was always found together with the xylose oxidase but was claimed to be more sensitive to heat and to have a lower optimal pH than the xylose oxidase. The data presented by LYR are insufficient to exclude the possibility of a relationship between his "xylose oxidase", the L-sorbose oxidase reported by YAMADA *et al.*[15] and carbohydrate oxidase.

A summary of the reactions known to be catalyzed by carbohydrate oxidase is shown in Scheme I.

While at first glance the substrate specificity of carbohydrate oxidase appears to be of a low order, the formulae in Scheme I illustrate the close relationship of the four substrates. They are presented in the chair form (C1 conformation) because this is the most stable arrangement: β-D-glucopyranose in the C1 conformation contains no elements of instability[23]. D-δ-Gluconolactone is depicted similarly since Dreiding stereo models show that this compound can assume an almost stress-free structure which is very close to the C1 conformation. L-Sorbose is shown in the inverted pyranose form. A comparison of the structures of the four substrates with those of the unreactive substances suggests the following substrate requirements:

1. The 6-membered ring is the reactive form. This conclusion is based upon the specificity of carbohydrate oxidase for δ-gluconolactone as compared with γ-gluconolactone. The conclusion is supported by the finding that a freshly prepared solution of β-D-glucopyranose is rapidly oxidized. Little or no furanose is present under these



Scheme I.

conditions[24]. The other two substrates are assumed to be also in the pyranose form[25].

2. The substituents on C-5 are relatively unimportant. Thus, glucose and gluconolactone have equatorial -CH_2OH groups at C-5, whereas xylopyranose has 2 H atoms attached at this position. L-Sorbose, which is probably in the α-L-sorbopyranose form[26], has an -OH as well as a -CH_2OH group. The -CH_2OH may be assumed to be equatorial, which would make for a more stable structure than if this bulky group were axial[27].

3. The -OH group on C-4 must be equatorial, since galactose, which has an axial -OH group at this position but otherwise has the same conformation as glucose, is not a substrate.

4. The -OH group on C-3 must be equatorial and unsubstituted, since ribose and 3-*O*-methylglucose are unreactive.

5. The -OH group on C-2 must be equatorial, a conclusion based on the lack of reactivity with mannose. Removal of an -OH group or substitution with an amino group such as in 2-deoxy-D-glucose or D-glucosamine, respectively, results in loss of reactivity.

6. Considerable variation in the substituents on C-1 is compatible with reactivity: *e.g.*, C-1 may have carbonyl oxygen (gluconolactone), hydroxyl (glucose and xylose)

or 2H (L-sorbose). When -OH is one of the substituents it must be equatorial and unsubstituted, since α-glucopyranose and β-methylglucopyranoside are unreactive. These findings suggest that the best arrangement of substituents on C-1 is the β -OH, but that smaller equatorial substituents, such as the H atoms in L-sorbose, still allow a good fit between the substrate and the enzyme. Increasing the size of the equatorial group as in β-methylglucopyranoside or increasing the size of the axial group as in α-D-glucopyranose prevents the close approach of substrate to the active site of the enzyme.

The findings may be summarized as follows: All of the substrates have in common a 6-membered ring and the same structural and conformational features on carbons 2, 3, and 4.

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