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# OXIDATION OF GLUCOSE TO γ- AND δ GLUCONOLACTONE BY MICROBIAL ENZYMES

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## SUMMARY

The product of glucose oxidation by three particulate and two soluble glucose-oxidizing enzymes was determined. The particulate enzymes yield glucono-γ-lactone, whereas the soluble ones produce glucono-δ-lactone.

## INTRODUCTION

A variety of enzymes has been described that are capable of catalyzing the oxidation of glucose to gluconate. In general, the evidence is clear that the actual product of oxidation is a lactone of gluconic acid. However, because of the lability of both γ- and δ-gluconolactones under the conditions of the reaction, most investigators have not determined which lactone was produced. In those few cases where the configuration of the lactone was determined, it was concluded that it was the δ-lactone[1-3].

In a study of the specificity of an aldose dehydrogenase in *Pseudomonas fragi*, it was found that L-arabinose, xylose, ribose, galactose, and glucose were oxidized to the corresponding aldonolactones[4]. Since the products from the first four sugars were easily identified as γ-lactones and since the oxidation of all sugars was catalyzed by a single enzyme system, it seemed logical to assume that the product of glucose oxidation should be the γ-lactone also.

Because oxidation of glucose to glucono-γ-lactone by biological systems has not been reported previously, determining the product of this oxidation by enzymes from several microbial sources seemed worthwhile. Although of limited utility, an assay was developed to distinguish between the γ- and δ-lactones of gluconic acid, based on JERMYN's kinetic studies of the hydrolysis of these two isomers[1]. This assay method was used to determine the products generated by the particulate enzymes from *P. fragi* and from *Pseudomonas fluorescens* and by the extracellular soluble enzyme, glucose oxidase. *Acetobacter suboxydans* contains two glucose dehydrogenases—one particulate and the other an intracellular soluble enzyme. The product of oxidation by each of these two enzymes was determined also.

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## EXPERIMENTAL

### *Methods and materials*

*P. fragi* NRRL B25 was grown on a medium composed of 0.25% glucose, 0.5% yeast extract and 0.5% tryptone in 0.033 M phosphate buffer (pH 6.8). *P. fluorescens* A3.12 was grown in 0.25% glucose, 0.1% $NH_4Cl$, 0.05% $MgSO_4$, 0.01% ferric ammonium citrate and 0.005% $CaCl_2$ in 0.033 M phosphate (pH 6.8). After 18 h growth at 30°, the cells were harvested by centrifugation and washed in 0.003 M phosphate (pH 6.8). The washed pellet was suspended in an amount of 0.003 M phosphate buffer that was three times the wet weight of the cells and then was irradiated 10 min in a 10 kc Raytheon* sonic oscillator. The preparation was centrifuged 10 min at 10 000 × *g*. The supernatant was centrifuged again for 60 min at 140 000 × *g*. This clear second supernatant was removed; the particles were washed two times and finally resuspended in a half volume of 0.003 M phosphate (pH 6.8).

*Acetobacter suboxydans* NRRL B72 was grown in Roux bottles in a medium previously described[5]. After 3 days' growth at 30°, the cells were washed off the agar surface with 0.003 M phosphate (pH 6.8). Cell-free extracts were obtained exactly as described for *P. fragi* and *P. fluorescens*.

Glucono-γ-lactone was prepared from calcium gluconate. The calcium salt was dissolved in water and the calcium removed by precipitation with oxalate. The solution was concentrated by distillation under vacuum, and the last traces of water were removed by adding butanol-1 and concentrating by vacuum distillation several times. The γ-lactone crystallized from the concentrated butanol solution. All other chemicals and enzymes were commercial preparations.

The glucono-δ-lactonase used in this work was present in commercial glucose-oxidase preparations. All glucose oxidase preparations tested contained large amounts of the lactonase. The preparations varied only slightly in the relative content of lactonase to oxidase.

### *Production and assay of lactone*

Gluconolactone was produced enzymically as follows: 0.1 ml of 0.3 M glucose was mixed with 2.0 ml of 1.0 M acetate buffer (pH 5.5), 0.1 ml of 0.05 M $MgSO_4$ and 0.8 ml enzyme in sufficient concentration to oxidize all the substrate completely in 30 min. The reaction was followed by measuring $O_2$ uptake in a Warburg respirometer. When the oxidation was over, the flask contents were chilled in an ice bath. If more than one flask was used, the contents of all flasks were pooled.

The configuration of the lactone was ascertained by following the rate of hydrolysis in the presence and absence of a glucono-δ-lactonase at 0° and 30°. Two portions of 2.5 ml each were removed from the pooled reaction mixtures. To one was added 0.5 ml of lactonase and to the other, a like amount of water. The concentration of lactonase was such that 0.5 ml would completely hydrolyze 30 μmoles of glucono-δ-lactone in approx. 5 min at 0° and pH 5.5. With a glucose oxidase preparation purchased from Nutritional Biochemical Corporation**, for example, this hydrolysis was accomplished with 0.1% protein solution.

* Raytheon, Waltham, Mass. Products mentioned are not endorsed by the U.S. Department of Agriculture over other products of the same quality.

** Nutritional Biochemical Corporation, Cleveland, Ohio. Products mentioned are not endorsed by the U.S. Department of Agriculture over other products of the same quality.

Samples were removed at intervals and assayed for lactone[6]. After development of color, the insoluble material was removed by centrifugation and the absorbancy measured on the clear supernatant.

## RESULTS

### *Glucose oxidation by particulate enzymes*

While glucose is rapidly oxidized by particles from *P. fragi*, *P. fluorescens* and *A. suboxydans*, larger quantities of $O_2$ than the theoretical amount for the oxidation of the substrate to gluconate were consumed. This is probably due to a gluconate dehydrogenase also present in the particles. However, since the rate of gluconate oxidation is only 20–30% of the rate for glucose oxidation, the presence of this enzyme should not interfere with the assay for gluconolactone. The lactonases present in these three organisms are all specific for glucono-δ-lactone as JERMYN reported for *P. fluorescens*[1]. The enzyme, however, is found only in the soluble fraction of cell-free extracts. The washed particles are free of lactonase activity.

The two lactones of gluconic acid are unstable in solution even at pH 5.5. In reconstructed experiments where 30 μmoles of each lactone are mixed with particles and incubated at 30° for 30 min at pH 5.5, about 50% and 66% of the γ-lactone and δ-lactone, respectively, are hydrolyzed in that length of time. When glucose is completely oxidized by the particles in a 30-min period, the product is recovered as lactone in a 40–50% yield. The configuration of the lactone was determined as described in EXPERIMENTAL.

The results of several experiments with the different particulate preparations are presented in Figs. 1 and 3. The concentrations are corrected for endogenous values, which were large for the *P. fluorescens* enzyme system. At 30°, the rate for the spontaneous hydrolysis of the lactone produced enzymically followed that of glucono-γ-lactone fairly well (Fig. 1).

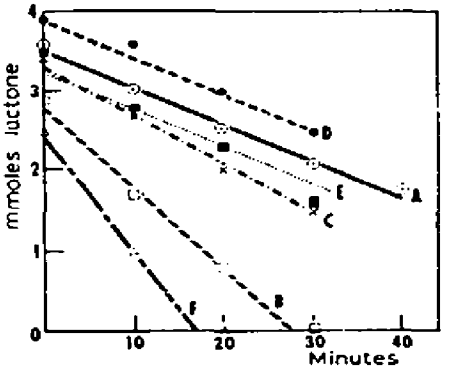


Fig. 1. Hydrolysis at 30° of glucono-γ- and glucono-δ-lactone and of the lactones produced by the oxidation of the glucose by glucose dehydrogenases from various microbial sources. Curve A, 0.1 ml of 0.1 M glucono-γ-lactone; 2.0 ml of 1.0 M acetate (pH 5.5); 0.1 ml of 0.05 M $MgSO_4$ and 0.8 ml *P. fragi* particles; Curve B, same as curve A, except that substrate is glucono-δ-lactone; Curve C, lactone produced by dehydrogenase in *P. fragi* particles; Curve D, lactone produced by dehydrogenase in *P. fluorescens* particles; Curve E, lactone produced by dehydrogenase in *A. suboxydans* particles; Curve F, lactone produced by glucose oxidase.

The effect of the enzyme lactonase on the hydrolysis was studied at 0° since the enzyme had no measurable effect on glucono-$\gamma$-lactone during the time of the experiment at this temperature and, also, because the rate of spontaneous hydrolysis of the lactones was greatly reduced (Fig. 2). Results similar to those in Fig. 2 were

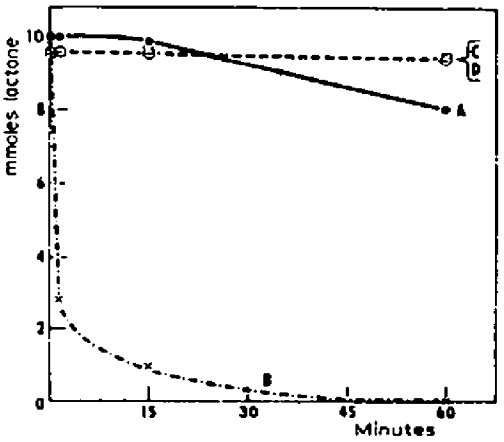


Fig. 2. Hydrolysis of $\gamma$- and $\delta$-gluconolactone in the presence and absence of a $\delta$-gluconolactonase at 0°. Curve A, 2.25 ml of 1.0 M acetate (pH 5.5); 0.05 ml of 0.5 M glucono-$\delta$-lactone and enough water to bring volume to 2.5 ml; Curve B, same as curve A, except that 0.1 ml of 0.1% glucose oxidase was added; Curve C, same as curve A, except that substrate was glucono-$\gamma$-lactone; Curve D, same as curve C, except that 0.1 ml of 0.1% glucose oxidase was added.

obtained when known samples of the two lactones were added to washed particles.

When the experiment is repeated with lactone produced enzymically, there is some rapid hydrolysis of the lactone in the presence of lactonase that accounts for a few percent of the total amount (Fig. 3). The remaining lactone is unaffected by

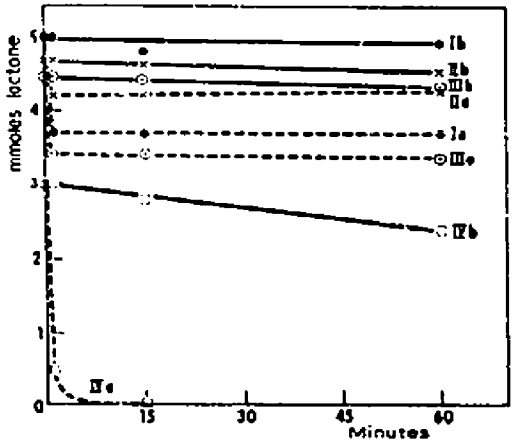


Fig. 3. Hydrolysis at 0° of the lactones produced by the oxidation of glucose by glucose dehydrogenases from various microbial sources. Conditions are as described in EXPERIMENTAL. The "a" curves are the hydrolysis of the lactone in the presence of lactonase and the "b" curves are the hydrolysis in its absence. Curve I, lactone produced by particles from *P. fragi*; Curve II, lactone produced by particles from *P. fluorescens*; Curve III, lactone produced by particles from *A. suboxydans*; Curve IV, Lactone produced by glucose oxidase.

the enzyme. Thus it appears that while a small amount of lactone is present as the δ-lactone, the major product of glucose oxidation is the γ-lactone.

The results of these kinetic assays were confirmed chromatographically. A reaction mixture which contained 10 μmoles/ml of the enzymically produced lactone was treated with Dowex-50 ($H^+$) to remove cations and then centrifuged to remove particulate matter. Known samples of the two lactones and gluconate were mixed with enzyme and treated exactly the same way. The supernatants were subjected to paper chromatography using three solvent systems (Table I). The products were

TABLE I

CHROMATOGRAPHY OF GLUCONIC ACID AND ITS TWO LACTONES

Solvent 1, propanol-1–HCOOH–$H_2O$ (6 : 3 : 1); solvent 2, ethyl acetate glacial acetic acid–$H_2O$ (3 : 1 : 3); solvent 3, butanol : HCOOH (95 : 5).

| | Solvent | | | | | |
|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | |
| | Acid spray ($R_f$) | Lactone spray ($R_f$) | Acid spray ($R_f$) | Lactone spray ($R_f$) | Acid spray ($R_f$) | Lactone spray ($R_f$) |
| Gluconic acid | 0.30 | 0.35 | 0.12 | 0.11 | 0.34 | 0.34 |
| Glucono-γ-lactone | 0.35 | 0.51 | 0.12 | 0.36 | 0.33 | 0.55 |
| | 0.55 | | 0.36 | | 0.55 | |
| Glucono-δ-lactone | 0.35 | 0.30 | 0.12 | 0.11 | 0.34 | 0.34 |
| Reaction mixture | 0.35 | 0.52 | 0.12 | 0.37 | 0.33 | 0.55 |
| | 0.55 | | 0.36 | | 0.55 | |

located either with a hydroxylamine spray for lactones[7] or with bromocresol blue for acids[8]. Both gluconic acid and glucono-δ-lactone have the same $R_f$ in these solvents. A small amount of the acid lactonizes under these conditions but only to the δ-lactone.

When the reaction mixture was chromatographed, the only lactone detected was glucono-γ-lactone. As expected, the acid spray revealed the presence of gluconic acid also. No spot corresponding to the δ-lactone was observed probably because the amount spontaneously formed from the free acid was too low to be detected.

*Glucose oxidation by glucose oxidase*

Commercial preparations of glucose oxidase all contained a lactonase in high concentration and thus could not be directly assayed as were the particles. The lactonase could be removed by passing a 5% solution of glucose oxidase through a column of Amberlite XE-64. By this procedure about 20% of the oxidase was washed through the column with water while only approx. 2% of the lactonase failed to be absorbed to the resin. The procedure was repeated. The yield of oxidase was extremely low, but it was free of lactonase. This eluate was used to oxidize a sample of glucose. A much smaller amount of lactone was present in the reaction mixture at the end of 20 min than when a similar amount of glucose was oxidized by particles of the bacterial preparations (Fig. 1). Upon addition of the specific glucono-δ-lactonase, the lactone that was produced by the purified glucose oxidase was rapidly hydrolyzed

(Fig. 3). This hydrolysis confirms the earlier results obtained by BENTLEY AND NEUBERGER[2].

*Glucose oxidation by a soluble glucose dehydrogenase from Acetobacter suboxydans*

The soluble glucose dehydrogenase from *A. suboxydans* utilizes TPN as the hydrogen acceptor[5]. The pH optimum of the oxidation with this enzyme is near pH 8. Under these conditions both lactones hydrolyze so rapidly that neither would accumulate in the reaction mixture. Therefore, the specificity of the reaction was determined by studying the reverse reaction, *i.e.* reduction of lactone by TPNH. The reverse reaction was studied at pH 6.0. The soluble fraction from *A. suboxydans*

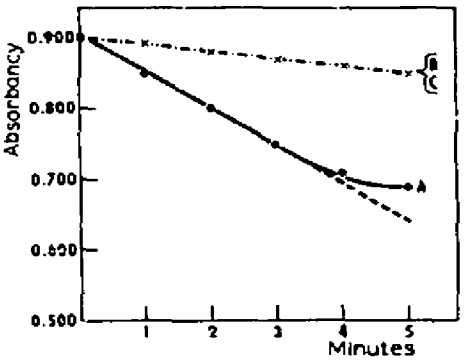


Fig. 4. Reduction of glucono-δ-lactone by TPNH in the presence of glucose dehydrogenase from *A. suboxydans*. Curve A, 0.1 ml of enzyme, 0.05 ml of 0.05 M $MgSO_4$, 0.02 ml of 0.1% TPNH, 0.9 ml of 0.1 M phosphate, (pH 6) and 5 mg of glucono-δ-lactone; Curve B, same as curve A, except that substrate was glucono-γ-lactone; Curve C, same as curve A, except that substrate was omitted.

contains a glucono-δ-lactonase which interferes with the assay; however, the effect of the lactonase could be reduced by using a dilute solution of enzyme. The results illustrated in Fig. 4 show that it is glucono-δ-lactone which is reduced under these conditions and by inference, must be the product of glucose oxidation by the soluble glucose dehydrogenase.

## DISCUSSION

The oxidation of sugars by bromine invariably yields the δ-lactones of the sugar acids[9,10]. This reaction has been taken to mean that sugars exist in the pyranose form in aqueous solution. Consequently, it has generally been assumed that glucose is oxidized to glucono-δ-lactone in biological systems also. Supporting evidence usually offered is that cell-free extracts contain a lactonase active on this product[11–13]. More definite characterization of the compound was undertaken in only a few cases[8,3]. Glucose 6-phosphate oxidation has been studied in much the same way with the conclusion that it, too, is oxidized to a δ-lactone[14–16]. On the other hand, studies with sugars other than glucose have shown the product of enzymic oxidation is the γ-lactone of the corresponding aldonic acid[17–21]. This suggests that it is the furanose form of the sugar that is oxidized.

The results reported here show that there are two different kinds of enzymes for oxidizing glucose based upon product of the reaction. One group of enzymes oxidizes glucose to glucono-δ-lactone, while another group produces the γ-lactone. While it may possibly be fortuitous, it should be noted that the three enzyme systems oxidizing glucose to glucono-γ-lactone are particulate and that the two oxidizing the sugar to glucono-δ-lactone are soluble. Though there is no direct evidence, it may well be that, since the predominate form of glucose in solution is the pyranose structure, an enzyme might be associated with the particulate fraction that converts the glucose into a form acceptable to the oxidizing enzyme, *i.e.* into glucofuranose.

The source of the small amount of δ-lactone also present in the reaction mixture with particulate enzymes requires further investigation. It has been claimed[1,22] that there is a non-enzymic interconversion of the two gluconolactones without any opening of the ring. Such a mechanism may be operating here. TAKAHASHI AND MITSUMOTO[22] have reported, recently, that glucono-γ-lactone is an intermediate in the formation of D-araboascorbic acid in *Penicillium notatum*. They infer, however, that the γ-lactone is formed by such a mechanism from the δ-lactone rather than directly by the oxidation of glucose.

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