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The Oxidation of Glucose and Related Compounds by Glucose Oxidase from *Aspergillus niger**

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Glucose oxidase from *Aspergillus niger* was purified by ammonium sulfate fractionation and chromatography on DEAE-cellulose. The purified enzyme was homogeneous on ultracentrifugation, density-gradient centrifugation, and starch-gel and paper electrophoresis. The enzyme was capable of oxidizing D-aldohexoses, monodeoxy-D-glucoses and *O*-methyl-D-glucoses at varying rates. Differences in the rates of oxidation of these compounds have been interpreted to indicate that the structural features of the substrate of particular importance in the enzymatic reaction are a pyranose ring in the chair or Cl conformation, an equatorially orientated hydroxyl group at position 1, and an equatorially orientated hydroxyl group at position 3. These structural features are probably involved in the formation of the enzyme-substrate complex.

Since the detection of glucose oxidase in extracts from *Aspergillus niger* (Müller, 1928), significant progress has been made on methods for the purification of this enzyme from various fungal sources (Franke, 1944; Keilin and Hartree, 1952; Underkofler, 1958; Kusai *et al.*, 1960) and on the elucidation of the mode of action of the enzyme (Keilin and Hartree, 1952; Bentley, 1955; Gibson *et al.*, 1963). In our laboratory a rapid and effective method based on ammonium sulfate fractionation and chromatography on DEAE-cellulose (Pazur and Ando, 1959) has been utilized for the preparation of glucose oxidase from *Aspergillus niger* in a highly purified form. The chromatography step was found to be particularly effective for separating the glucose oxidase from catalase, a separation difficult to achieve by other techniques (Underkofler, 1958). The purified glucose oxidase was homogeneous on ultracentrifugation, density-gradient centrifugation, and starch-gel and paper electrophoresis. Like glucose oxidase from other organisms (Keilin and Hartree, 1952; Kusai *et al.*, 1960), the enzyme from *Aspergillus niger* contains two flavin adenine dinucleotide (FAD) moieties per molecule and has a molecular weight of approximately 150,000. The *p*H optimum for the enzyme is 5.5 and the isoelectric point is 4.2.

Conflicting reports have appeared in the literature on the ability of glucose oxidase to oxidize hexoses other than D-glucose (Adams *et al.*, 1960; Hlaing, *et al.*, 1961). Consequently the action of the purified enzyme has been examined on some fifteen glucose isomers and derivatives with the view of determining which compounds are oxidized by the enzyme and which structural features of the substrate are of importance in the enzyme reaction. Included in the list of compounds were the monodeoxy derivatives, the epimers, a sulfur-containing derivative, and several *O*-substituted derivatives of D-glucose. A new procedure has been used for the preparation of 4-deoxy-D-glucose and 6-deoxy-D-glucose via mesyl- and iodotetraacetyl-D-glucose. Since the replacement of a secondary hydroxyl group with an iodo group is difficult to achieve, the reaction conditions are described in detail. Most of the compounds tested in this study were found to be slowly oxidized by pure glucose oxidase. Differences in the rates of oxidation of the compounds have been interpreted to indicate that certain structural features of the hexose molecule are of particular importance in the enzyme reaction. These structural features are a pyranose ring most probably in the chair or Cl conformation (Reeves, 1951), an equatorially orientated hydroxyl group at position 1, and an equatorially orientated hydroxyl group at position 3. Alteration of the hexose structure in any of these aspects has a greater effect on the rate of enzyme action than an alteration at other parts of the D-glucose molecule.

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MATERIALS

Enzyme Source.—A preparation of glucose oxidase (Dee-O) was provided by the Miles Chemical Co., Elkhart, Ind. This enzyme preparation was isolated from a strain of *Aspergillus niger* by extraction of the enzyme with water and precipitation with a nonaqueous solvent. Examination of the preparation by density-gradient centrifugation showed that it contained several ultraviolet-absorbing components of different molecular size (Pazur *et al.*, 1962). Assays for enzyme activities showed also that the preparation contained catalase and hydrolytic carbohydrases as well as glucose oxidase. The starting material for the purification work contained approximately 100 units of glucose oxidase activity per mg of nitrogen. A unit of glucose oxidase activity is defined as that quantity of enzyme which will cause the uptake of 11.2 μl of oxygen at STP per minute in a Warburg manometer at 30° and 760 mm Hg in the presence of excess catalase and oxygen with a 3% solution of D-glucose in 0.1 M potassium phosphate buffer, *p*H 5.9. This defini-

tion is in accord with the recommendation of the Commission on Enzymes of the International Union of Biochemistry. The new unit is equivalent to 1.12 of the units defined by Scott (1953) and widely used in the literature.

4-Deoxy-D-glucose.—1,2,3,6-Tetra-*O*-acetyl-4-*O*-mesyl-β-D-glucose, mp 175°, was prepared by methods described in the literature (Helferich and Gruechtel, 1938). Samples of 2 g of the tetraacetyl-mesyl derivative and 3 g of sodium iodide were dissolved in 25 ml of acetone in an airtight steel cylinder and the resulting solution was heated at 138° for 48 hours. A white precipitate which formed in the reaction mixture was removed by filtration and the filtrate was concentrated to a syrup. The ethyl ether–soluble product in the syrup was recovered by extraction with ether and evaporation of the solvent. The product was dissolved in a minimum amount of warm ethyl alcohol. A crystalline material obtained from this solution was recrystallized twice from ethyl alcohol–petroleum ether solution. The yield was 0.5 g. The broad melting point range (155–170°) of the crystalline sample indicated that the preparation was a mixture of compounds, presumably 1,2,3,6-tetra-*O*-acetyl-4-iodo-4-deoxy-β-D-glucose and 1,2,3,6-tetra-*O*-acetyl-4-iodo-4-deoxy-β-D-galactose. A halogen value of 27.4% (theoretical for the above compounds, 27.7%) showed that the preparation was relatively free of other impurities. A sample of 0.5 g of the iodo compounds was dissolved in 40 ml of absolute alcohol, 1 g of freshly prepared Raney nickel catalyst was added, and the mixture was subjected to a hydrogen atmosphere for 2 hours. Concentration of the clear filtrate from the reaction mixture yielded 0.3 g of crystalline compound which melted at 108–110°. Deacetylation of this compound in 0.01 M sodium methoxide and methanol yielded a solution from which 4-deoxy-D-glucose was isolated by preparative-paper chromatography. The compound was crystallized from an ethyl ether–petroleum ether solution. On paper chromatography in a solvent system of 9 parts *n*-butyl alcohol, 5 parts pyridine, and 7 parts water the compound migrated with an R_F value typical of deoxy-D-glucoses. The melting point of the 4-deoxy-D-glucose was 128°, literature value 130° (Hedgley *et al.*, 1960); the specific rotation was +47.7° (*c*, 1 water), literature value +47.9° (Dahlgard *et al.*, 1962), and +60° (Hedgley *et al.*, 1960).

6-Deoxy-D-glucose.—6-Deoxy-D-glucose was prepared from 2 g of 1,2,3,4-tetra-*O*-acetyl-6-*O*-mesyl-β-D-glucose by a series of reactions similar to those described above. The 1,2,3,4-tetra-*O*-acetyl-6-iodo-6-deoxy-β-D-glucose was obtained in crystalline form, mp 147–149°, in a yield of 1.8 g. Reduction of this product with hydrogen and Raney nickel catalyst, yielded 1.2 g of crystalline 1,2,3,4-tetra-*O*-acetyl-6-deoxy-β-D-glucose, mp 143–145°. Deacetylation of this compound in sodium methoxide and methanol yielded a reaction mixture from which 6-deoxy-D-glucose was obtained in approximately 80% yield. The 6-deoxy-D-glucose melted at 145–146° and possessed a specific rotation of +29° (*c*, 2 water).

5-Thio-5-deoxy-D-glucose.—A sample of 3,6-di-*O*-acetyl-1,2-*O*-isopropylidene-5-thio-5-deoxyacetyl-D-glucofuranose (Feather and Whistler, 1962) was kindly provided by R. L. Whistler, Purdue University, Lafayette, Ind. The removal of the blocking groups was effected by a slight modification of the procedure of the above authors. A sample of 0.2 g of the compound was dissolved in 5 ml methyl alcohol and warmed slightly. To this solution 5 ml of 2 N HCl was added dropwise and the reaction mixture was allowed to stand at room temperature for 64 hours. The hydrolysate was neutralized by passing the solution through a column of amberlite IR-45 (OH). Concentration of the neutral effluent yielded the sulfur-containing reducing sugar 5-thio-5-deoxy-D-glucose.

Other Deoxyhexoses.—2-Deoxy-D-glucose was obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio. It was recrystallized twice from methyl alcohol. 3-Deoxy-D-glucose was kindly provided by N. K. Richtmyer, National Institutes of Health, Bethesda, Md. 5-Deoxy-D-glucose was a gift of W. G. Overend, Birkbeck College, University of London, England. 1-Deoxy-D-glucose (1,5-anhydro-D-glucitol) was provided by R. Barker, University of Tennessee, Memphis.

Aldoses.—D-Glucose used in these studies was a product of the Mallinckrodt Chemical Works. D-Mannose free of D-glucose was obtained by treatment of a solution of reagent D-mannose with glucose oxidase and crystallization of the D-mannose from the reaction mixture on addition of ethyl alcohol. The enzymatic treatment converted D-glucose to D-gluconic acid which was separated from the D-mannose by the crystallization step. Recrystallization of the D-mannose from ethyl alcohol yielded a preparation which was suitable for our purposes. D-Galactose was purified by a procedure similar to that described for the purification of D-mannose. D-Allose and L-glucose were kindly provided by N. K. Richtmyer, National Institutes of Health, Bethesda, Md., and M. L. Wolfrom, The Ohio State University, Columbus.

Derivatives of D-Glucose.—4,6-*O*-Benzylidene-D-glucose was prepared from D-glucose and benzaldehyde with the aid of zinc chloride catalyst. The product was crystallized from hot water; the melting point of the crystalline compound was 187–188°, literature value 188° (Bates, 1942). 3-*O*-Methyl-D-glucose and 4-*O*-methyl-D-glucose were provided by F. Smith, University of Minnesota, St. Paul, and 6-*O*-methyl-D-glucose was provided by A. S. Perlin, Prairie Regional Laboratory, Saskatoon, Sask., Canada.

Methods and Results

Purification of Glucose Oxidase.—A sample of 100 g of the glucose oxidase preparation was dissolved in 150 ml of water and the insoluble material was removed by centrifugation. An aliquot of the solution was assayed for glucose oxidase activity by the procedure described in an earlier section and for nitrogen by a micro-Kjeldahl method. The remaining solution was dialyzed against distilled water for 25 hours with two transfers to new dialysis tubing. Fractionation of the protein material in the dialyzed sample was effected with ammonium sulfate. Addition of ammonium sulfate to 60% saturation yielded a protein fraction which contained little glucose oxidase activity and consequently was discarded. Further addition of ammonium sulfate to 87% saturation yielded a fraction which contained a high percentage of the original glucose oxidase activity. This material was dissolved in 100 ml of distilled water and dialyzed against 0.01 M sodium acetate buffer, *p*H 4.5, for 12 hours at 3°. A glass column (450 × 35 mm) was packed with 30 g of DEAE-cellulose and washed with 300 ml of 0.1 M sodium hydroxide, 300 ml of 0.5 M acetic acid, and 3 liters of 0.05 M sodium acetate buffer, *p*H 4.5. The dialyzed enzyme sample was introduced slowly from a separatory funnel on the DEAE-cellulose column. After adsorption of the protein material in the sample on the DEAE-cellulose, the column was washed with 1 liter of 0.07 M sodium acetate buffer, *p*H 4.5, and

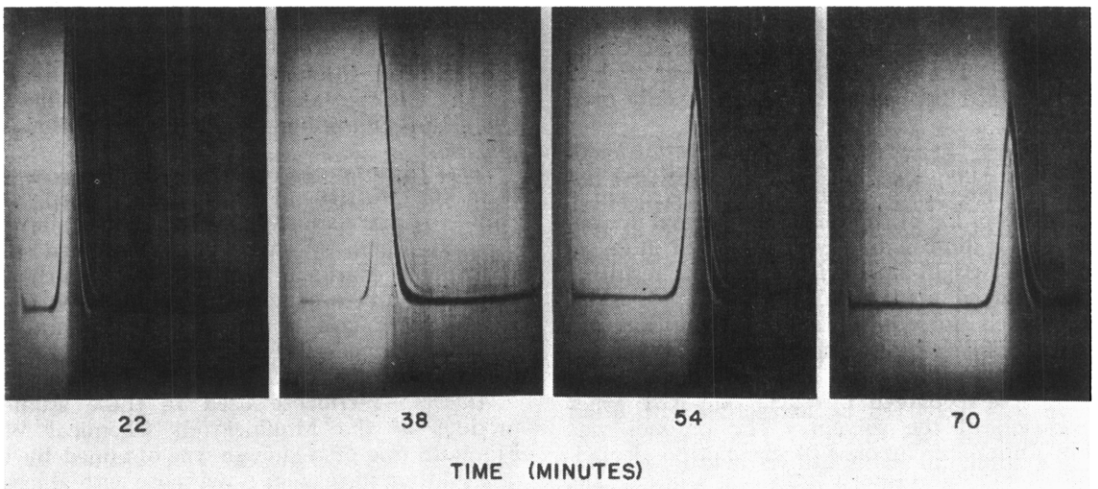

FIG. 1.—Photographs of the ultracentrifuge patterns of purified glucose oxidase.

with 0.5 liter of 0.1 M sodium acetate buffer, *p*H 4.5. The latter treatment eluted the carbohydrases and catalase from the DEAE-cellulose. The glucose oxidase was subsequently eluted with 0.1 M acetate buffer, *p*H 3.7. During the elution step a yellow compound was visible moving slowly down the column. The fraction containing the yellow compound was collected and on analysis was found to contain the glucose oxidase activity. This sample was rechromatographed on a smaller column (400 × 20 mm and containing 10 g of DEAE-cellulose) by the procedure described. The glucose oxidase solution which was obtained from the second column contained 0.7% protein and assayed approximately 1200 units per mg N. Recovery values and specific activities of the fractions at various steps of the purification procedure are presented in Table I. The purified enzyme can be stored for many months at 2° in the presence of thymol without loss of activity.

Starch-Gel and Paper Electrophoresis.—Gel electrophoresis of a sample of the purified glucose oxidase was performed as described by Smithies (1959). Starch-gel strips of 20.3 × 2.5 × 0.2 cm and a buffer solution of 0.1 M Tris–EDTA–borate–acetic acid were used in these experiments. The electrophoresis was conducted at four different *p*H values, 4.0, 6.0, 7.5, and 8.6, and was continued for 16 hours at room temperature at 50 ma and at the voltage of 60–90 d-c volts. On completion of the experiment the gel strips were stained with amido black dye and washed with appropriate solvents (Smithies, 1959). The protein in the preparation appeared as blue bands in the faintly blue gel. At each *p*H value only one protein band was visible in the glucose oxidase preparation. Duplicate unstained gel strips were sprayed with reagents for detecting glucose oxidase as described below for the paper electrophoretic strips. The band of enzymatic activity in the gel corresponded in position to the protein band at all the *p*H values tested. At *p*H 4.0 the glucose oxidase had migrated slightly toward the cathode while at the three other *p*H values the enzyme migrated from 1 to 3 cm toward the anode.

Paper electrophoresis of glucose oxidase was carried out in a Spinco Model R cell with Spinco B-2 buffer, *p*H 8.6, ionic strength 0.075. The electrophoresis was effected at a potential of 200 d-c volts and a current of 8 ma for 14 hours at 2°. The protein in the sample was detected by appropriate staining procedures. The glucose oxidase activity was detected by spraying the strips first with a solution of 2% D-glucose and 0.01% horseradish peroxidase in 0.3 M acetate buffer, *p*H 5.6, and then with a solution of 1% *O*-tolidine in 80% ethyl alcohol (White and Secor, 1957). A blue band appeared on the paper strip at the areas of glucose oxidase activity. Under the conditions of the experiments a single band located approximately 5 cm toward the anode was obtained. This band of enzymatic activity corresponded identically with the band of protein material in the preparation. Two comparable paper electrophoretic strips were sprayed in a similar manner but using pure D-mannose or D-galactose as the carbohydrate constituent of the sprays. The band of protein material which oxidized the D-glucose also effected a slow oxidation of the D-mannose and D-galactose. This observation was interpreted to indicate that one enzyme was responsible for the oxidation of the three hexoses. When the electrophoresis was conducted in a citrate-phosphate buffer, *p*H 4.2, the glucose oxidase remained at the origin, indicating an isoelectric point of 4.2 for the enzyme.

TABLE I
GLUCOSE OXIDASE ACTIVITIES OF FRACTIONS OBTAINED AT VARIOUS STAGES OF PURIFICATION

	Volume (ml)	Units/ ml	Total Units	Units/ mg N
Enzyme sample	205	541	112,000	104
After dialysis	563	186	105,000	323
Ammonium sulfate fractionation	100	680	68,000	
1st DEAE-cellulose chromatography	77	766	59,000	
2nd DEAE-cellulose chromatography	60	992	53,000	1192

Ultracentrifugation.—Samples of the original enzyme preparation and of the purified enzyme were examined in the Spinco Model E ultracentrifuge and in the Model L preparative centrifuge. Photographs of ultracentrifugation patterns from one experiment are reproduced in Figure 1. In this experiment the centrifugation was carried out in the Model E centrifuge at 59,780 rpm. The temperature was 22° and the protein concentration was approximately 1% in 0.1 M acetate buffer, *p*H 4.1. The patterns in Figure 2 indicate a single component in the purified enzyme sample. Patterns for the nonpurified enzyme material

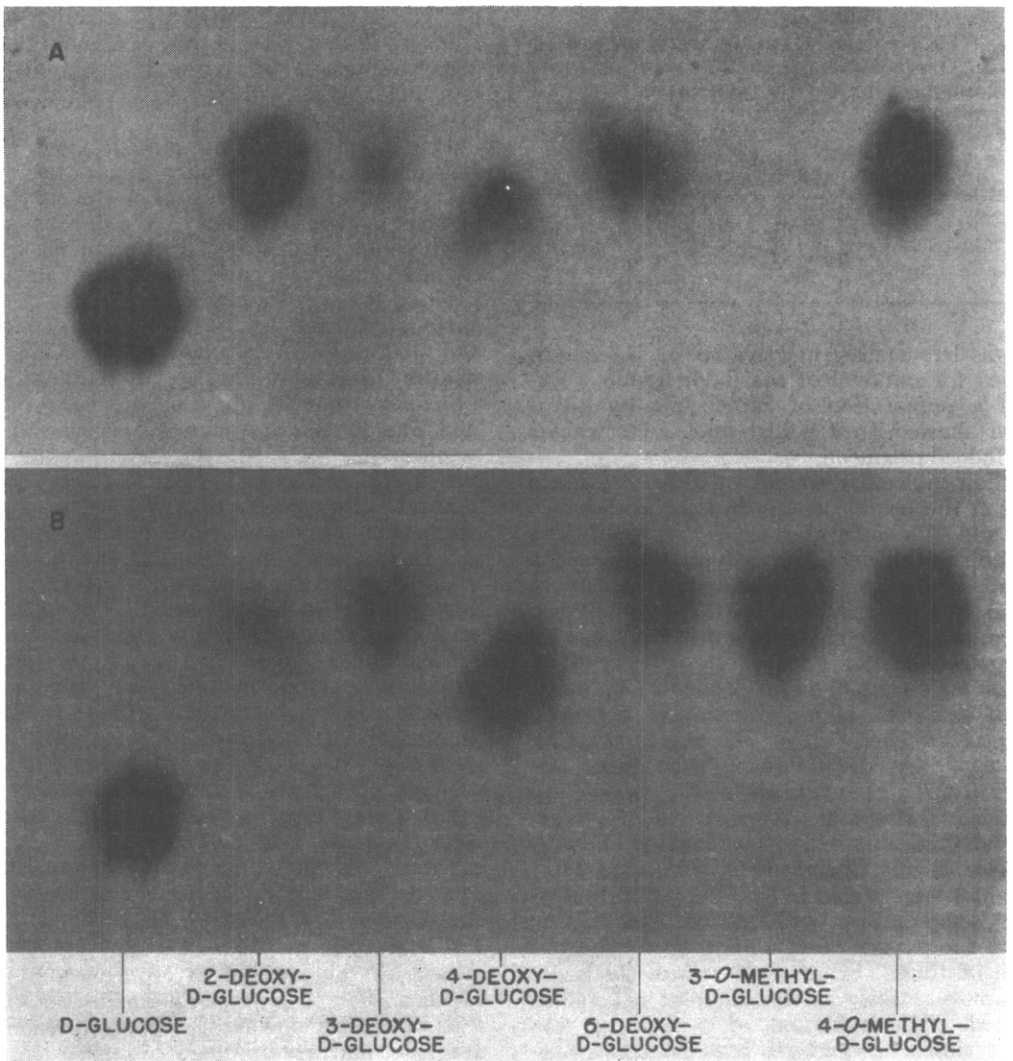

FIG. 2.—A photograph of paper chromatograms of carbohydrates sprayed with glucose oxidase and appropriate dye(A) and with aniline oxalate (B).

obtained in a similar manner showed that four distinct components were initially present. The sedimentation coefficient of glucose oxidase was evaluated from the data and found to be 7.7 S. From the S value the molecular weight of the glucose oxidase was estimated to be 150,000.

A sample of 0.2 ml of the purified glucose oxidase was subjected to density-gradient centrifugation (Pazur *et al.*, 1962). A single ultraviolet-absorbing component was detected in the preparation by this technique. The enzymatic activity was present in the fraction containing this component.

The Flavin Group of Glucose Oxidase.—A sample of 7 mg of lyophilized enzyme was dissolved in 0.1 ml of water and 0.9 ml of freshly distilled pyridine was added. The mixture was heated at 45° for 15 minutes and allowed to stand at room temperature for 20 minutes. The protein which precipitated was removed by centrifugation and the filtrate was evaporated under vacuum. The residue was dissolved in 0.1 ml of water and aliquots of the solution and of reference compounds were chromatographed in two solvent systems (*n*-butyl alcohol–methyl alcohol–5% disodium acid phosphate, 4:1:2, v/v; *n*-butyl alcohol–acetone–acetic acid–water, 5:2:1:3, v/v). The reference compounds consisted of flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN), and riboflavin. In each solvent the ultraviolet-absorbing material obtained from the enzyme migrated at a rate identical to that for flavin adenine dinucleotide.

The flavin group of glucose oxidase could also be separated from the protein at *p*H 2.8 by saturation of the solution to 85% with ammonium sulfate. A sample of 5.7 mg of glucose oxidase in 1 ml of acetate buffer, *p*H 4.1, was mixed with 0.3 g of ammonium sulfate at 0°. After solution of the ammonium sulfate, 0.3 ml of 1 N hydrochloric acid and 0.26 g of ammonium sulfate were added. The mixture was stirred vigorously and the material which precipitated was removed by centrifugation and washed with phosphate buffer, *p*H 5.9. The final product was dissolved in 1 ml of phosphate buffer, *p*H 5.9. Aliquots of 0.1 ml of this solution were added to 0, 1×10^{-4}, 2×10^{-4}, and 5×10^{-4} μmoles of flavin adenine dinucleotide or flavin adenine mononucleotide. The tubes were maintained at 2° for 12 hours and then assayed for glucose oxidase activity by the manometric method. Glucose oxidase activity was detected only in the tubes containing flavin adenine dinucleotide and the apoenzyme (Table II). In the experiment recorded in the table only about 15% of the original enzyme activity was restored. In other experiments up to 35% of the activity could be restored but a 100% reconstitution of the enzyme was not achieved. Apparently some of the

TABLE II

RECONSTITUTION OF GLUCOSE OXIDASE BY ADDITION OF FLAVIN ADENINE DINUCLEOTIDE AND FLAVIN ADENINE MONONUCLEOTIDE TO THE APOENZYME

μMoles of Coenzymes × 10^4	FMN (μl oxygen/min)	FAD (μl oxygen/min)
0	0	0
1	0	2.8
2	0	3.1
5	0	3.1

apoenzyme was irreversibly inactivated by the conditions employed for removal of the flavin group. Examination of a preparation of apoenzyme by ultracentrifugation showed that a high molecular weight material was indeed produced.

The minimum molecular weight of glucose oxidase calculated from the protein concentration, absorbance at 458 mμ, and the molecular extinction coefficient of flavin adenine dinucleotide is 73,000. Since the sedimentation coefficient of the enzyme indicates a molecular size of approximately 150,000, the enzyme apparently contains two flavin adenine dinucleotide moieties per molecule.

pH Optimum for D-Glucose and D-Mannose Oxidation.—The rate of oxidation of D-mannose and D-glucose was determined at 12 different *p*H values, ranging from *p*H 2 to 9, by the following procedure. An aliquot of 2 ml of 0.1 M D-glucose in the appropriate buffer was added to the main compartment of a Warburg flask, and 0.24 unit of glucose oxidase and 500 units of catalase (Sigma Chemical Co., St. Louis, Mo.) in 0.5 ml of water were added to the side-arm compartment. After temperature equilibration and mixing of the sample, the oxygen consumption was measured as a function of time. From these values the units of glucose oxidase activity at the different *p*H values were calculated. The oxidation of D-mannose was followed by a similar procedure, but the concentration of glucose oxidase was increased 100-fold since the mannose was oxidized at a much slower rate. A significant oxidation of both D-glucose and D-mannose occurred over a *p*H range of 4–7 with maximum oxidation of both substrates occurring at *p*H 5.5.

Substrate Specificity of Glucose Oxidase.—The action of glucose oxidase on some fifteen different hexoses and derivatives was tested first qualitatively by using paper chromatography and a spraying technique, and subsequently by quantitative methods. In the first procedure, aliquots of 0.1 M solution of the substrates were placed on paper chromatograms and developed in a solvent system of *n*-butyl alcohol–pyridine–water (6:4:3 or 9:5:7, v/v). The dried chromatograms were sprayed with the glucose oxidase, peroxidase, and *o*-tolidine reagents (White and Secor, 1957). Areas of D-glucose on the chromatograms appeared as blue spots immediately while areas of other compounds reacting more slowly with glucose oxidase appeared at later intervals. This technique was very useful for substrates which may be contaminated with D-glucose since R_F values for many of the compounds were different from the R_F value of D-glucose. A photograph indicating the utility of the method is reproduced in Figure 2. Also reproduced in this figure is a duplicate chromatogram which was sprayed with aniline oxalate reagent for locating the reducing sugars. It will be noted in the figure that the 2-deoxy-D-glucose and 3-deoxy-D-glucose did not react as readily with the aniline oxalate as the other compounds. Differences in the rates of oxidation of the compounds by glucose oxidase are also readily apparent. D-Glucose, 2-deoxy-D-glucose, 4-*O*-methyl-D-glucose, 6-deoxy-D-glucose, 4-deoxy-D-glucose, 3-deoxy-D-glucose, and 3-*O*-methyl-D-glucose were oxidized at decreasing rates and in the order listed.

For quantitative rate measurement extensive use was made of the Warburg apparatus. In a typical assay 2 ml of 0.1 M solution of the substrate in 0.1 M phosphate-citrate buffer, *p*H 5.6, was added to the main compartment of the reaction flask and 1 ml of enzyme solution was added to the side arm. For D-glucose, 2-deoxy-D-glucose, and 6-deoxy-D-glucose the enzyme solution contained 0.3 unit of glucose oxidase and 500 units of catalase per ml. For other substrates (3-deoxy-D-glucose, D-mannose, D-galactose, 4,6-*O*-benzylidene-D-glucose) the enzyme concentration was 30 units of glucose oxidase and 500 units of catalase per ml. All the experiments were carried out in duplicate and with D-glucose as a control. The rate of oxygen uptake was followed as a function of time and a linear relationship between oxygen uptake and time was obtained with all compounds. A maximum of about 8% of the total substrate was oxidized during the observation period. The rate of oxidation of D-glucose was also measured in the presence of 1,5-anhydro-D-glucitol and in all such experiments no inhibition of glucose oxidation was obtained.

With some substrates a procedure based on the production of hydrogen peroxide was employed for measuring the oxidation rates. In this procedure aliquots of 0.2 ml of 0.01 M substrates, buffered at *p*H 5.6, were equilibrated at 30° for 10 minutes in a rotary shaker, and subsequently 0.1 ml of enzyme solution was added to the substrate. The reaction mixture was shaken at 30° for specified periods and the enzyme was inactivated by addition of 4.7 ml of 1 N sulfuric acid. The hydrogen peroxide was determined by the method of Savage (1951). The final concentration of hydrogen peroxide was less than 0.002 M in all experiments and this concentration of hydrogen peroxide had no measurable effect on the glucose oxidase. The concentration of enzyme was 0.3 unit per ml in the experiments with D-glucose and 30 units per ml in the experiments with the other substrates (4-deoxy-D-glucose, 5-deoxy-D-glucose, 5-thio-5-deoxy-D-glucose, D-allose, D-mannose, and D-galactose). D-Mannose and D-galactose were used as controls to check for the agreement between the rates obtained by the two methods. In the assays the variation between the two methods was less than 10%. The rate of oxidation of D-glucose has been assigned an arbitrary value of 100 and the rate of oxidation of the other compounds has been compared to that for D-glucose with a proper correction for the differences in the enzyme concentrations. These data are presented in Table III. Quantitative measurements were also made for some of the compounds with the original nonpurified enzyme preparation. The relative rates of oxidation of D-glucose, D-mannose, D-galactose, and 2-deoxy-D-glucose were identical when determined with the pure glucose oxidase and the nonpurified enzyme sample.

DISCUSSION

Glucose oxidase has been obtained in a high degree of purity from *Aspergillus niger* by the use of ammonium sulfate fractionation and chromatography on DEAE-cellulose. Ultracentrifuge patterns (Fig. 1) and density-gradient centrifugation data for the purified enzyme were typical for a preparation of uniform molecular size and a single protein component.

TABLE III
RELATIVE RATE OF OXIDATION OF HEXOSES BY GLUCOSE OXIDASE

Compound	Apparent R_F Value[a]	Relative Rate
D-Glucose	0.52	100
L-Glucose	0.52	0
1,5-Anhydro-D-glucitol	0.65	0
2-Deoxy-D-glucose	0.71	20
D-Mannose (2-epimer)	0.59	1
3-Deoxy-D-glucose	0.70	1
D-Allose (3-epimer)	0.54	0.02
3-O-Methyl-D-glucose	0.71	0.02
4-Deoxy-D-glucose	0.63	2
D-Galactose (4-epimer)	0.47	0.5
4-O-Methyl-D-glucose	0.70	15
4,6-O-Benzylidene-D-glucose	0.93	1
5-Deoxy-D-glucose	0.68	0.05
5-Thio-5-deoxy-D-glucose	0.61	0.01
6-Deoxy-D-glucose	0.71	10
6-O-Methyl-D-glucose	0.68	1

[a] The apparent R_F value is obtained by dividing the distance traveled by the compound by the total height of the paper. Two ascents of the solvent *n*-butyl alcohol–pyridine–water, 9:5:7 (v/v) were employed in the chromatographic procedure.

Electrophoresis on starch gels and paper strips under several different conditions revealed the presence of a single band of protein material in the purified sample. As noted in Figure 2, the purified enzyme preparation oxidized glucose and a number of glucose derivatives. That a single enzyme was responsible for the oxidation of the various compounds was substantiated by a number of lines of evidence. First, on paper electrophoresis the enzymatic activity responsible for the oxidation of D-glucose, D-mannose, and D-galactose was found at the same point on the paper strips. Second, the *p*H optimum for the oxidation of D-glucose and D-mannose was 5.5. Third, the relative rates of oxidation of D-glucose, D-mannose, D-galactose, and 2-deoxy-D-glucose were identical with the highly purified and the nonpurified enzyme samples.

Analytical data thus far obtained for the *Aspergillus niger* enzyme show that this enzyme is similar to the glucose oxidase isolated from other organisms (Keilin and Hartree, 1952; Kusai *et al.*, 1960) in respect to molecular size (150,000), isoelectric point (4.2), and flavin adenine dinucleotide content (2 FAD per mole). The flavin group of the *Aspergillus niger* enzyme was separated from the enzyme by extraction with pyridine or by dissociation from the enzyme at a low *p*H and precipitation with ammonium sulfate. The protein portion of the enzyme without the prosthetic group rapidly aggregated to a high molecular weight material and only a portion of the glucose oxidase activity could be restored to the sample by the addition of flavin adenine dinucleotide.

The data in Table III have been arranged to indicate the effect of alteration of structure at the various carbon atoms of D-glucose on the rate of oxidation. It will be noted that alterations at certain positions (3, 5) of the molecule have a more pronounced effect on the rate of oxidation than alterations at other positions (2, 4, 6). Thus, D-allose (the 3 epimer of D-glucose), 3-O-methyl-D-glucose, 5-deoxy-D-glucose, and 5-thio-5-deoxy-D-glucose were oxidized at barely measurable rates. Apparently the pyranose ring oxygen and a hydroxyl group at position 3, equatorial to the pyranose ring, are important structural aspects of the substrate for glucose oxidase. As might be expected for an enzymatic reaction the L isomer of glucose was not oxidized. Likewise, 1,5-anhydro-D-glucitol was not oxidized by the enzyme. The latter compound when tested as a competitive inhibitor for the oxidation of D-glucose was found to be ineffective. This finding indicates that the hydroxyl group at position 1 is probably involved in the formation of the enzyme-substrate complex as well as in the oxidative process. It has been shown earlier (Keilin and Hartree, 1952; Adams *et al.*, 1960) that the beta isomer of D-glucose, in which the hydroxyl group at position 1 is equatorial to the ring, is oxidized more rapidly than the alpha isomer. In view of the above considerations it is suggested that equatorially orientated hydroxyl groups at positions 1 and 3 and the pyranose ring oxygen are possible sites of combination of the substrates with glucose oxidase.

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