persisting there. This remnant is of mesodermal origin. That part of groove protein, which resists exposure to Schweitzer's solution, is probably a fragment of Nasmyth's membrane which existed in histological continuity with the groove protein, and was inadequately separated from it by the use of a pipette, and the gentle suction used.

In its resistance to chemical and bacterial attack, and to pepsin and trypsin, some resemblance between Nasmyth's membrane and silk fibroin had been pointed out to the writer by the late Sir Patrick Laidlaw prior to 1939. Later, the work of Takamatsu (1933) came to notice, describing the solution of silk in cupriethylenediamine, after which a test of the effect of this substance on enamel proteins seemed to be of interest. Clinical trial of local application of cupriammonium solutions for caries prevention is contemplated, when a suitable technique has been evolved.

SUMMARY

1. Human dental enamel protein has been shown to consist of two proteins, Nasmyth's membrane and groove protein; results of some tests on these are given.

2. Both contain tyrosine, and both resist enzymic attack. Groove protein contains carbohydrate, possibly glucose. In cupriammonium solutions the two structures are sharply differentiated, Nasmyth's membrane resisting solution and staining black, while groove protein readily dissolves.

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Properties of Glucose Oxidase (Notatin)

By D. KEILIN AND E. F. HARTREE, *Molteno Institute, University of Cambridge*

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A glucose oxidase was discovered by Müller (1928, 1936) in *Aspergillus niger* and *Penicillium glaucum*. He found that this enzyme catalyzes the oxidation of glucose to gluconic acid by means of molecular oxygen, and that at a much slower rate it also oxidizes mannose and galactose but not other sugars. Since the activity of this enzyme was found by him not to be affected by cyanide he concluded that it does not depend upon the presence of an active heavy metal.

The enzyme was reinvestigated by Franke & Lorenz (1937) and by Franke & Deffner (1939) who, working with a more purified preparation, demonstrated that during the catalytic oxidation of glucose to gluconic acid, O_2 is reduced to H_2O_2 . They found also a certain proportionality between the activity of different preparations and their flavine content and concluded that the enzyme must be a flavo-protein. Although their enzyme preparation was 80 times more active than that of Müller, we now realize

that it was not more than 10% pure. Unlike Müller they found that in addition to O_2 other substances such as indophenols may also act as hydrogen acceptors although their efficiency is much lower.

Müller (1941), from a study of the oxidation of various sugars by his enzyme preparations both in O_2 and anaerobically using 2:6-dichlorophenolindophenol, concluded that two enzymes were present, one reacting directly with O_2 and a second which reacts only with certain other H acceptors. Franke (1944) has criticized this conclusion of Müller and also those of Ogura (1939) who claimed to have isolated from *Aspergillus oryzae* a glucose-oxidizing enzyme which could not utilize O_2 as H acceptor. According to Franke (1944) the glucose oxidases from *Penicillium glaucum*, *Aspergillus niger* and *A. oryzae* are virtually identical and consist of a single aerobic oxidase. The findings of Müller (1941) and Ogura (1939) may have resulted from the low purity of their preparations; their Q_{0_2} values being of the

order of 100 at 25° in air, whereas Franke was able to prepare a much purer material ($Q_{O_2} = 8000$ at 30° in O_2). Moreover, the highly purified oxidase ($Q_{O_2} = 130,000$) that we have used for the experiments described in this paper can utilize both O_2 and 2:6-dichlorophenolindophenol as H acceptors.

The almost complete purification of glucose oxidase from culture media of *Penicillium notatum* was achieved by Coulthard, Michaelis, Short, Sykes, Skrimshire, Standfast, Birkinshaw & Raistrick, (1942, 1945). They demonstrated that the antibiotic 'notatin', which is thermolabile and only effective in presence of glucose and O_2 , is identical with the glucose oxidase of Müller and that its bactericidal action is due to H_2O_2 produced during the oxidation of glucose: $C_6H_{12}O_6 + O_2 + H_2O = C_6H_{12}O_7 + H_2O_2$. The same conclusion was arrived at independently by van Bruggen, Reithel, Cain, Katzman, Doisy, Muir, Roberts, Gaby, Homan & Jones (1943) who purified this enzyme and described it under the name Penicillin B. Their experiments suggested that the prosthetic group of the enzyme was the alloxazine-adenine dinucleotide. Working with the highly purified enzyme we were able to demonstrate that this dinucleotide is indeed the prosthetic group of notatin (Keilin & Hartree, 1946).

Glucose oxidase, notatin and penicillin B therefore designate the same enzyme which catalyzes the aerobic oxidation of glucose to gluconic acid. As a matter of convenience we shall use in our descriptions the short term notatin.

The first part of this paper will deal with the constitution and physical properties of notatin while the second part will consist of a general account of the kinetics of the pure enzyme.

EXPERIMENTAL

A. Physical properties and constitution of the enzyme

Notatin* was obtained as a pale yellow powder which dissolved freely in water to give a bright yellow solution with a faint opalescence. On centrifuging, a perfectly clear solution was obtained plus a small purplish residue constituting < 10% of the preparation.

Absorption spectrum and equivalent weight. A solution of notatin in water containing 10.0 mg./ml. was centrifuged to remove insoluble impurities. It then contained 9.12 mg./ml. This solution was diluted with half its volume of 0.1M-phosphate buffer pH 6.5 and the absorption curve determined in a 1 cm. layer (Curve I, Fig. 1) using a Beckman photoelectric quartz spectrophotometer. A typical flavine type of absorption spectrum was obtained with maxima at 377 and 455 m μ . van Bruggen *et al.* give the positions of these maxima as 375 and

452 m μ . Addition of glucose to the solution in a closed vessel gives rise to the autoxidizable dihydro or leuco form of the enzyme which is practically colourless (curve II). A more complete reduction to the leuco form was obtained in presence of $Na_2S_2O_4$ (curve III) though the strong absorption by this salt in the ultraviolet region precludes any determination of absorption below 400 m μ .

Fig. 1. Absorption spectra of a 0.008% solution of notatin in 0.03M-phosphate buffer pH 6.5. I, without reducing agent. II, +glucose. III, + $Na_2S_2O_4$.

The absolute value of the height of the absorption band near 450 m μ . is fairly constant for all flavoproteins and the average value of the absorption constant is $\beta = 2.4 \times 10^7$ (Straub, 1939). From this figure it is possible to estimate the minimum molecular weight of the enzyme; i.e. the weight of enzyme containing 1 mol. of the alloxazine-adenine dinucleotide. Thus $\beta = (2.303 DM)/cl$, where D = optical density ($0.76 = \log_{10}$ incident light/transmitted light), M = molecular weight, c = concentration in g./ml. (0.00608), and l = optical depth in cm. The calculated value of M is 83,000 assuming the enzyme to be completely pure. The correction for colourless impurities will lower this figure while flavoprotein impurities will probably not change it. From the experiments described below it appears that the flavine-free impurities do not exceed 10%, hence the equivalent weight is about 75,000 and the molecular weight a simple multiple of this figure. As the molecular weight of this enzyme determined by Ogston & Cecil (see addendum to this paper) was found to be about 152,000, each molecule of enzyme contains two prosthetic groups.

Coulthard *et al.* determined the absorption spectrum of a similar highly purified preparation and deduced a purity of 90% from the absorption at 450 m μ . Whereas their value of the height of

* The notatin used in these experiments was kindly supplied by Dr W. F. Short (Coulthard *et al.* 1945).

this band agrees very closely with ours, they obtained a considerably higher figure for the second band near 380 m μ .

Electrophoresis. Fig. 2 summarizes the results obtained in the Tiselius electrophoresis apparatus. The material is seen to be almost homogeneous. A small fraction migrates more rapidly than the oxidase and a trace of a more slowly moving fraction can just be detected. Further evidence on the homogeneity of the oxidase was obtained from its behaviour in the ultracentrifuge (see addendum to this paper).

Fig. 2. Migration of glucose oxidase in the Tiselius electrophoresis apparatus. 1.67% solution. 0.05M-phosphate buffer pH 6.42. Current 19 milliamp. at 250 V. Temp. = 1.5°.

Exposure no.	Time (min.)	Schlieren angle
1	28	65°
2	49	65°
3	80	55°

Nature of the prosthetic group. From the work of van Bruggen *et al.* (1943) it can be concluded that the prosthetic group is probably the alloxazine-adenine dinucleotide (A.A.D.) common to diaphorase, D-amino-acid oxidase and most other flavoproteins. It has not so far been possible to split off the prosthetic group of glucose oxidase and at the same time obtain the protein in a native state. Were it possible to obtain the native protein, then a restoration of catalytic activity on addition of A.A.D. would identify the latter as the prosthetic group. The alternative method, as was suggested by Coulthard *et al.* (1945), is to add the prosthetic group of notatin to the native protein of D-amino-acid oxidase. The prosthetic group of D-amino-acid oxidase is known to be A.A.D. (Warburg & Christian, 1938), hence, as the addition of boiled notatin to the amino-acid oxidase protein restores the specific catalytic activity of the latter (Keilin & Hartree, 1946) the two enzymes must have a common prosthetic group.

The D-amino-acid oxidase protein was separated from its prosthetic group according to the method

of Negelein & Brömel (1939) and the enzyme was then reconstructed from 5 mg. free protein (fraction B of Negelein & Brömel) and varying volumes of a standard solution of A.A.D. containing 0.65 mg. riboflavin phosphate/ml. The catalytic activities of the mixtures were estimated manometrically by the rates of oxidation of DL-alanine in pyrophosphate buffer pH 8.3 at 39° in presence of catalase. The relationship between the volume of A.A.D. solution added and the catalytic activity of the system is shown in Fig. 3. The activity of the amino-acid oxidase protein was now tested under the same conditions with the prosthetic group of notatin in place of the A.A.D. solution. For this purpose a solution of notatin in water containing (after centrifuging) 2.77 mg./ml. was heated at 100° for 15 min. and cooled. The addition of 0.02 and 0.04 ml. of this solution to the above oxidizing system brought the amino-acid oxidase activity to the same level as would the additions of 0.00049 and 0.00102 ml. respectively of the standard A.A.D. solution (Fig. 3).

Fig. 3. Rates of O₂ uptake obtained on adding various amounts of alloxazine-adenine dinucleotide or of boiled notatin to the system: 5 mg. D-amino-acid oxidase protein, 12 mg. DL-alanine, 0.1 ml. catalase, 0.07M-pyrophosphate buffer pH 8.3 to 3.3 ml. Temp. = 39°. Standard dinucleotide solution for calibration (circles) contained 0.65 mg. riboflavin phosphate/ml.

Hence 1 ml. boiled notatin solution containing 2.77 mg. notatin is equivalent to 0.025 ml. of the standard A.A.D. or to 0.0163 mg. riboflavin phosphate. The sample of notatin therefore contains 0.59% riboflavin phosphate. Pure diaphorase contains 0.66% riboflavin phosphate (Straub, 1939), hence if we assume that the riboflavin contents of diaphorase and notatin are the same, and that all the

flavine in the preparation is present in the form of the enzyme, then the notatin is about 90% pure. On the other hand, if the notatin contains flavoprotein impurities this estimate is too high. Thus Ogston & Cecil (addendum to this paper) estimate the purity to be 80% or possibly more, whereas the agreement (above) between their value of the molecular weight and our estimate of the equivalent weight, based on the assumption that there are no flavoprotein impurities, is very close. The purity of the notatin is therefore between 80 and 90%.

Effect of temperature and pH on the fluorescence of notatin. Pure notatin does not fluoresce in ultra-violet light although the strong green fluorescence of A.A.D. appears when the enzyme is denatured. In this respect it differs from diaphorase which fluoresces both before and after denaturation. At temperatures below 60° no fluorescence can be detected while at 70° it develops very rapidly. Experiments were carried out at intermediate temperatures in an attempt to reverse the phenomenon by sudden cooling. In no case was there a decrease in the fluorescence produced by the higher temperature.

The effect of pH on the fluorescence is parallel to its effect on enzyme activity (see below). The solution becomes fluorescent at pH > 8 or < 2, i.e. outside the range of catalytic activity. Here again there was no evidence of reversibility of the appearance of fluorescence or of enzyme activity on rapid neutralization to pH 5. Coulthard *et al.* (1945) showed that the bactericidal properties of notatin rapidly disappear outside the pH range 2–8.

B. Kinetics of the enzyme reaction

It was shown by previous workers that oxidation of glucose to gluconic acid catalyzed by glucose oxidase is accompanied by reduction of O_2 to H_2O_2 (p. 221). The formation of H_2O_2 in this primary oxidation reaction has been confirmed by the addition of azide-catalase which rapidly turns from greenish brown to red and changes the pattern of its absorption spectrum (Keilin & Hartree, 1945a). It can also be demonstrated by the utilization of this peroxide in a secondary or coupled oxidation of alcohols catalyzed by catalase (Keilin & Hartree, 1945b).

Notatin was made up as a 0.1–0.2% aqueous solution, which was stable for 1 week at 5°, and suitable dilutions were prepared as required. Measurements of O_2 uptake were carried out in air in Barcroft differential manometers at 39° (unless otherwise stated). As a general rule the right-hand flasks of the manometers received 0.3 ml. notatin, 0.3 ml. glucose, 0.1 ml. catalase (Keilin & Hartree, 1945a) and buffer to 3.3 ml. The left-hand flasks received 3.3 ml. buffer.

Influence of pH and of buffer concentrations. The O_2 uptake of the system composed of 15 μ g. notatin,

0.3 ml. 10% glucose and 0.1 ml. catalase was determined in various buffers over the pH range 2.3–10.3 (Fig. 4). The buffer concentrations were about 0.2M

Fig. 4. Effect of pH on the activity of glucose oxidase. 15 μ g. notatin, 1% glucose, 0.1 ml. catalase. 0.2M-buffers, Temp. = 39°. $\odot$ phosphate-citrate; $\oplus$ phosphate borate; $\triangle$ acetate; $\times$ pyrophosphate; $\square$ phosphate; $\bullet$ phosphate-NaOH.

and the pH's of the reaction mixtures were determined with a glass electrode. The optimum pH is 5.6 which is in close agreement with the figure given by Müller (1936) for the enzyme from *Aspergillus niger*. Variations of phosphate and acetate buffer concentrations between 0.03 and 0.25M do not influence the rates of O_2 uptake. The loss of activity above pH 8 is noteworthy and the effect of high pH was further investigated as follows:

Three borate buffers were prepared as shown in Table 1. Notatin was incubated for various periods in these buffers, and then brought to pH 5.3 by addition of the previously determined volume of 0.2M-acetate buffer pH 3.6. The activity of the enzyme was then determined under the standard conditions with glucose and catalase at pH 5.3. Table 1 gives the volumes of acetate buffer required to neutralize the borate buffers to pH 5.3. Thus in one set of experiments an aqueous solution of notatin (1.25 mg./ml.) was diluted 50 times in the mixture borate I + acetate (Table 1) and 0.5 ml. tested in presence of catalase and 1% glucose at 39°; the volume being made up to 3.3 ml. with the mixed buffer (Exp. 1, Table 2). A second sample of the aqueous notatin was similarly diluted with borate I alone. At intervals up to 40 min. 0.5 ml. was pipetted into a Barcroft flask containing 1.5 ml. borate I + 1.0 ml. acetate buffer. The enzyme was then tested with glucose and catalase as before (Exps. 2–5, Table 2).

Table 1. Acidities of borate buffers used to test destruction of notatin at high pH

Buffer	Constitution	pH	Volume of 0.2M-acetate buffer pH 3.6 added to 10 ml. of the borate buffer to give final pH 5.3 (± 0.1) (ml.)
Borate I	0.05M-borax	9.1	5
Borate II	100 ml. I + 50 ml. 0.1M-KH ₂ PO ₄	8.1	3
Borate III	100 ml. I + 75 ml. 0.1M-KH ₂ PO ₄	7.6	2.5

Table 2. Destruction of notatin by incubation at room temperature at pH 9.1

(12.5 μ g. notatin, 1% glucose, 0.1 ml. catalase.)

Exp. no.	Time of incubation at pH 9.1	Activity after neutralization to pH 5.3 (μ l. O ₂ /25 min.)
1	0	296
2	2	25
3	7	24
4	23	7
5	40	0

Table 2 shows the rapid disappearance of enzyme activity that follows incubation at the high pH. These results are plotted in Fig. 5 (line A) together with similar experiments in which the incubation pH was 8.1 and 7.6. Experiments in which glucose was present during the incubation period (dashed lines) show that the substrate can protect the enzyme to a considerable extent from destruction at pH > 8.

Fig. 5. Loss of activity of glucose oxidase following incubation at room temperature at pH 7.6, 8.1 and 9.1 for various periods prior to neutralization to pH 5.3 and testing with glucose at 39°. 12.5 μ g. notatin, 1% glucose, 0.1 ml. catalase. ●—● incubation in absence of glucose. ×—× incubation in presence of glucose.

Unless otherwise stated, all subsequent experiments were carried out in 0.2M-phosphate buffer pH 5.6.

Effect of substrate concentration and oxygen tension at 39°. The effect of glucose concentrations was determined with 5 μ g. notatin and glucose concentrations up to 7.3%. From the results obtained (Fig. 6) the Michaelis constant is found to be

Fig. 6. Effect of substrate concentration on glucose oxidase. 5 μ g. notatin, 0.1 ml. catalase, 0.2M-phosphate buffer pH 5.6. Temp. = 39°.

$K_m = 0.0042M$ which is considerably lower than the value 0.026 found by Franke & Lorenz (1937) with a crude enzyme preparation.

The influence of O₂ tension is summarized in Fig. 7 from which it is seen that the activity in pure O₂ is nearly 2½ times higher than in air. In these experiments 10 μ g. notatin was used. From Fig. 6 the Q_{O_2} of the enzyme at maximum glucose concentration is found to be 82,000 while from Fig. 7 where less glucose was present the figures are 60,000 and 140,000 in air and O₂ respectively. Coulthard *et al.* estimate the Q_{O_2} of their purest enzyme to be 120,000 at 30° in O₂, a figure which is corroborated by our results. As the purity of our sample of notatin was 80–90% the activity of the completely pure enzyme may be expressed by increasing these Q_{O_2} values by about 10%. In comparing these figures with those of previous workers (e.g. Franke & Deffner (1939) reach $Q_{O_2} = 8000$) it should be remembered that the earlier experiments were carried out in O₂ at 30° in absence of catalase. The absence of catalase will tend to increase the O₂ uptake

(Keilin & Hartree, 1945b) while the lower temperature will slightly decrease it (see below) hence the old and new figures can be considered as reasonably comparable. Franke & Deffner's preparations were therefore only 5–10 % pure.

Fig. 7. Effect of O₂ tension on the activity of glucose oxidase. 10 μg. notatin, 1 % glucose, 0.1 ml. catalase, 0.2M-phosphate buffer pH 5.6. Temp. = 39°.

Effect of temperature. Fig. 8 shows the relationship between enzyme activity and temperature using 12.5 μg. notatin under the standard conditions. The temperature coefficient of the reaction falls off rapidly with rising temperature.

Fig. 8. Effect of temperature on the activity of glucose oxidase. 12.5 μg. notatin, 1 % glucose, 0.1 ml. catalase, 0.2M-phosphate buffer pH 5.6.

catalase and 300 μg. notatin. For comparison purposes glucose was tested under the same conditions with 10 μg. notatin from which the O₂ uptake with

Table 3. *Specificity of glucose oxidase*

Not oxidized			
Sugars	Methylated sugars		Methyl glucosides
Arabinose	2-Methylglucose		α -Methylglucoside
Fructose	3-Methylglucose		2-Methyl- α -methylglucoside
Fucose	2:3-Dimethylglucose		2:3-Dimethyl- α -methylglucoside
Glucoheptose	2:3:6-Trimethylglucose		2-Methyl- β -methylglucoside
Lactose	2:4:6-Trimethylglucose		2:3:6-Trimethyl- β -methylglucoside
Melizitose	2:3:4:6-Tetramethylglucose		
Raffinose	2:4-Dimethyl- α -galactose		Acetyl glucoses
Rhamnose	4:6-Dimethyl- α -galactose		2:3:4:6-Tetraacetylglucose
Ribose	2:4:6-Trimethyl- α -galactose		Pentaacetylglucose
Sorbose	2-Methyl- β -galactose		3:4:6-Triacetyl-2-deoxyglucose
Sucrose	2:6-Dimethyl- β -galactose		
Tagatose	3:4-Dimethyl- β -galactose		Phosphorylated sugars
	3:4:6-Trimethylfructofuranose		Glucose-1-phosphate
			Hexose-6-phosphate
			Fructosediphosphate
Oxidized (glucose = 100)			
6-Methylglucose	1.85	Melibiose	0.11
4:6-Dimethylglucose	1.22	Cellobiose	0.09
Mannose	0.98	Glucuronic acid	<0.05
Xylose	0.98	Galacturonic acid	
Trehalose	0.28	2-Aminoglucose	
Maltose	0.19	2-Aminogalactose	
Galactose	0.14		

300 $\mu\text{g.}$ could be calculated. Although the rate of O_2 uptake is not strictly proportional to the quantity of enzyme taken, the figures obtained for the various sugars are nevertheless comparable among themselves. Franke & Deffner (1939), using an enzyme preparation of $Q_{\text{O}_2} = 2000$, give the ratio of O_2 uptakes as follows: glucose : mannose : galactose = 1 : 0.07 : 0.14. Similar ratios are recorded by Müller (1941). From Table 3 it is seen that of the commoner sugars, apart from glucose, only mannose and xylose are oxidized though at a rate $> 1\%$ that of glucose. Galactose is much less readily attacked and the previously recorded higher activities are undoubtedly due to the glucose which is invariably present in samples of galactose prepared in the normal way by hydrolysis of lactose. This point is illustrated in Fig. 9, which shows the O_2 uptake under the standard conditions with 300 $\mu\text{g.}$ notatin with a commercial sample of 'pure' galactose, and a specially purified sample kindly provided by Dr D. J. Bell. With all commercial galactoses there is an initial rapid O_2 uptake followed by a slow but steady oxidation of the galactose. By extrapolation to zero time the glucose content may be estimated. Thus in the upper curve of Fig. 9 the glucose content $\approx 80 \mu\text{l.}$

Fig. 9. Oxidation of pure galactose, and commercial galactose containing glucose, by glucose oxidase. 300 $\mu\text{g.}$ oxidase, 0.1 ml. catalase. pH = 5.6, Temp. = 39°.

O_2 , i.e. 1.29 mg. or 4.0%. It is impossible to be certain that all phosphorylated sugars are un-attacked as the very slight O_2 uptakes frequently observed may be due to a slow hydrolysis. The slow oxidations of 6-methyl- and 4:6-dimethylglucose, however, appear to be genuine since the rates of oxidation remain constant over long periods.

Inhibitors of glucose oxidase. Working with enzyme preparations of Q_{O_2} 20–30 Franke & Lorenz (1937) found that narcotics (alcohols, urethanes) had a marked inhibitory effect comparable in

magnitude to that observed with, for example, succinic dehydrogenase. However, we have found the pure oxidase not to be affected by this class of substances. The respiratory inhibitors HCN, H_2S , HN_3 do not inhibit glucose oxidase, a fact which follows from the flavine nature of the enzyme, but may even give rise to an increased O_2 uptake. This increase is due to the poisoning of the catalase present in all but the purest samples of notatin and the consequent accumulation of H_2O_2 . In his earliest experiments Müller (1928) records a slight inhibition of his enzyme by cyanide although as high a concentration as 0.5M was required in order to obtain 50% inhibition.

In attempts to find a powerful, specific and reversible inhibitor of glucose oxidase we have tested a large range of substances but without positive results. In a few cases a very slight inhibition was observed. Thus under the standard conditions 0.01M concentrations of 8-hydroxyquinoline, sodium nitrate and semicarbazide gave rise to 11, 13 and 20% inhibition respectively.

Oxidation of glucose by *Penicillium notatum*. The mould (strain 4222 of the National Collection of Type Cultures) was grown in plugged Barcroft manometer

Fig. 10. Oxidation of glucose by washed 10-day mycelia (Myc.) of *Penicillium notatum*. Effect of cyanide. 2% glucose, 0.004M-HCN, 0.2M-phosphate buffer pH 5.6. Temp. = 39°. The dry weights of mycelia, grown in manometer flasks, were 21–25 mg.

flasks on 5 ml. Czapek-Dox medium (Coulthard *et al.* 1945). After 3–10 days growth at 24° the medium was sucked off and replaced with successive lots of 0.2M-phosphate buffer pH 5.6. In this way all traces of the medium were removed. The respiration

of the mycelia was tested at pH 5.6 in presence of 2% glucose and/or 0.002M-HCN. The centre wells of the manometers contained a roll of filter paper soaked in 10% NaOH or, when cyanide was present, with the appropriate NaOH-KCN mixture (Umbreit Burris & Stauffer, 1945). The results of such experiments with a 10-day culture are shown in Fig. 10. The O_2 uptakes are given for mg. dry weight of mycelium, the dry weight of the latter (21–25 mg.) being determined at the end of the experiment. Experiments with a 3-day culture, showing a much higher Q_{O_2} , gave essentially the same results. Although the endogenous respiration is cyanide-sensitive, the oxidation of glucose is not. Hence it must be accepted that the intracellular oxidation of glucose to gluconic acid, like the extracellular reaction, does not involve the cytochrome system.

Cultivation of Penicillium notatum on a fructose medium. An attempt to affect the specificity of glucose oxidase was made by repeated subcultivation on a Czapek-Dox medium in which glucose had been replaced by fructose. Small plugged test tubes were used, each containing 1 ml. medium. The organism grew well at 24°, and was subcultivated every 4 days within which time a thick crop of spores was produced under these conditions. After 20 passages the culture was inoculated into two culture dishes, 15 cm. diameter, each containing 200 ml. of the fructose medium. Ten days later the yellow culture medium was removed and tested in the usual way with glucose and fructose. It was found to be still completely specific for glucose.

SUMMARY

1. Mould glucose oxidase of Müller obtained in the highly purified state (notatin) by Coulthard *et al.* from *Penicillium notatum* was investigated in respect to its structure, physical properties and enzyme kinetics.

2. The enzyme, as was previously shown, catalyzes the oxidation of glucose to gluconic acid by molecular O_2 , which is reduced to H_2O_2

3. The enzyme, which is a flavoprotein, shows in the oxidized state the characteristic absorption

spectrum with bands at 377 and 455 m μ . On addition of glucose the enzyme is almost completely decolorized and the bands become hardly perceptible. They disappear completely in presence of $Na_2S_2O_4$. That the prosthetic group is the alloxazine-adenine dinucleotide was demonstrated by removing the prosthetic group from D-amino-acid oxidase, and regenerating the activity of the latter by the addition of boiled notatin.

4. From the absorption constant at 455 m μ . the minimum molecular weight is estimated to be about 75,000. The molecular weight calculated from the sedimentation velocity and diffusion constant is 152,000 (see addendum), which shows that the molecule of enzyme contains two prosthetic nuclei.

5. From the behaviour of the most active enzyme preparation in the ultracentrifuge and in the Tiselius electrophoresis apparatus, it would appear to be between 80 and 90% pure.

6. The enzyme does not fluoresce in ultraviolet light within the pH limits of its catalytic activity (pH 2–8). Outside this range it loses its activity and becomes fluorescent.

7. The enzyme shows a pronounced specificity for glucose. Of about 50 sugars and their derivatives tested, four are oxidized at about 1% of the rate of glucose oxidation (mannose, xylose, 6-methylglucose and 4:6-dimethylglucose), whereas the remainder are virtually or completely unattacked. The previously recorded oxidations of galactose are due to glucose impurities.

8. In experiments with mycelia of *P. notatum* grown in manometer flasks it was found that the endogenous respiration of the mycelium was cyanide-sensitive, but that the oxidation of glucose by the mycelia was not inhibited by cyanide. Thus the cytochrome system is not involved in the intracellular oxidation of glucose to gluconic acid.

9. Attempts to modify the specificity of glucose oxidase by repeated subcultivation on a fructose medium were unsuccessful.

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Addendum. Sedimentation and Diffusion of Glucose Oxidase (Notatin)

By R. CECIL AND A. G. OGSTON, *Department of Biochemistry, Oxford*

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A sample of glucose oxidase (Coulthard *et al.* 1945) was examined with the Svedberg oil-turbine ultracentrifuge by the method of Philpot (1938), and its diffusion constant was measured by a new method (Coulson, Cox, Ogston & Philpot, 1948). There was a main sedimenting component (Fig. 1 (a)) which accounted for at least 80 % of the total refracting material and which appeared to be homogeneous: in addition, there were small quantities of a faster and a slower component (Fig. 1 (b) and (c)). The whole of the absorption at 495–515 m μ . was associated with and sedimented at the same speed as the main component. The buffer was NaCl 0.2M; KH₂PO₄ 0.057M; NaH₂PO₄ 0.0095M: the speed of rotation was 1015 rev./sec. The results are given in the table.

The values of *M* and of *f*/*f*₀ are obtained assuming a value of 0.75 for the partial specific volume of the protein. The value of *D*₂₀ is an average for all the protein present; since this is not strictly monodisperse, the value may not be accurate for the main component. However, the value of *M* is in reason-

able agreement with that of the equivalent weight as estimated in the preceding paper.

Fig. 1. Diagrams of glucose oxidase boundaries 15 and 25 min. after reaching 1015 rev./sec.

Refractive increment × 10 ⁴	Concentration (g./100 ml.)	<i>S</i> ₂₀ × 10 ¹³	<i>S</i> ₂₀ (corr.) × 10 ¹³	<i>D</i> ₂₀ × 10 ⁷	<i>M</i>	<i>f</i> / <i>f</i> ₀
13.5	0.75	7.25	7.86	—	—	—
6.4	0.36	7.62	8.27	5.13	152,000	1.16

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