GLUCOSE OXIDATION AND CYTOCHROMES IN SOLUBILIZED PARTICULATE FRACTIONS OF ACETOBACTER SUBOXYDANS*

BY TSOO E. KING AND VERNON H. CHELDELIN
(From the Department of Chemistry and the Science Research Institute, Oregon State College, Corvallis, Oregon)

(Received for publication, July 18, 1956)

Microbial terminal oxidases are usually associated with particulate fractions in the cell. These fractions, however, are difficult to resolve into smaller active molecules that are suitable for enzymatic studies in vitro. This fact led Rittenberg (1) to propose that these particles are surrounded by a special membrane.

The present paper will report a method of "solubilization" of particulate oxidase systems from Acetobacter suboxydans. This method can also be applied to other organisms, such as Acetobacter pasteurianum. With the extracts obtained, cytochrome spectra and a glucose oxidase have been observed.

EXPERIMENTAL

A. suboxydans ATCC 621 cells were grown as previously described in a medium of glycerol-yeast extract (2). Methods for disintegration of cells by alumina grinding (2) and by sonic vibration (3) were used.

Protein was determined by a biuret method (4) with the following modification: 1 ml. of sample was treated with 1.5 ml. of 60 per cent urea and 2.5 ml. of the biuret reagent containing 0.3 per cent CuSO₄·5H₂O, 0.9 per cent Rochelle salt, 0.5 per cent KI, and 0.2 N NaOH. Readings were taken at 550 mp after the solution stood at room temperature for 5 minutes. Crystalline bovine serum albumin was used as the standard.

Acylhydroxamic acids were determined by the method of Hestrin (5). Gluconolactone was employed as the standard, those experiments being

---

* This work was supported by grants from the Nutrition Foundation, Inc., and the Division of Research Grants and Fellowships, National Institutes of Health, United States Public Health Service. Published with the approval of the Monographs Publications Committee, Research paper No. 304, School of Science, Department of Chemistry.

1 "Solubilization" is defined as the production from particulate fractions of extracts that remain in the supernatant layer after centrifuging for 100 minutes at top speed in the Spinco model L ultracentrifuge, with rotor No. 40.3. Performance index (PI) = (RPM)²/(log R_{max} - log R_{min}), where R = centrifugal force × g. PI at top speed of the Spinco L centrifuge with rotor No. 40.3 is 31.3 × 10⁸ (see the Spinco ultracentrifuge technical manual, model L).
included in which glucose was not the substrate. The method of Isbell et al. (6) was used for the isolation of barium gluconate and its conversion to the δ-lactone. The change of optical rotation of the lactone was studied in aqueous solution.

Deoxycholic acid (California Foundation for Biochemical Research) was crystallized twice from 95 per cent ethanol. Difference spectra were read in a Beckman DK-2 spectrophotometer in matched 1 sq. cm. Corex cuvettes at temperatures from 18-22°. Reduced mammalian cytochrome c was used in every experiment for the standardization of wave length readings.

RESULTS AND DISCUSSION

Preparation and Solubilization of Particulate Fractions

Particulate fractions prepared by either grinding with alumina (2) or by sonic disintegration (3) showed similar behavior with respect to glucose oxidation, "solubilization," and cytochrome spectra of the solubilized extracts.

The procedures (3, 7, 8) used previously in this laboratory for preparing particulate fractions furnished active enzymes, but were tedious and gave low yields. Several modifications of these methods were therefore explored.

It was found that the magnitude of the decrease in light scattering after solubilization was inversely proportional to the contamination of the pink gel by white-colored materials. The latter were presumably cell walls, debris, together with partially broken and unbroken cells. When a "pure" pink gel was used, the optical density at wave length 550 μ could be reduced nearly to zero after treatment with deoxycholate. However, the contaminating materials were unaffected by deoxycholate. Likewise, whole cells were not solubilized. This fact greatly simplified the operation, since the white materials were removed at the final step by centrifugation.

The method described below for preparation of particulate fractions and their "solubilization" was found satisfactory. The separation scheme, with the terminology employed in this paper, is summarized in the accompanying diagram.

3.5 gm. of lyophilized cells were suspended in 35 ml. of 0.02 M phosphate buffer, pH 6.0, and disintegrated as described previously (2). All operations henceforth were performed at about 4°. The mixture was centrifuged for 20 minutes at performance index (PI)1 of 1.40 × 10⁸ in a Spinco model L ultracentrifuge. The supernatant liquid was separated from the loosely packed precipitate and was further centrifuged at PI = 22.4 × 10⁸ for 60 minutes. The clear supernatant extract (non-particulate) was saved.
Fractionation of A. suboxydans

Lyophilized cells

- Disintegrated in PO₄ buffer; then centrifuged 20 min.; PI = 1.40 × 10⁸
- Centrifuged 60 min.; PI = 22.4 × 10⁸
- Debris, etc.
  - Washed 3 times by centrifugation for 30 min.; PI = 22.4 × 10⁸
  - Cell-free extract (non-particulate)
  - Particulate fraction treated with deoxycholate; then centrifuged 30 min.; PI = 22.4 × 10⁸
  - Washings
  - Debris, etc.
  - "Solubilized" extract

for other studies. The upper pink layer of the precipitate, which constituted approximately 0.8 of the total pellet, was removed and washed with about 30 ml. of the buffer. Dispersion of the gel in this and all subsequent steps was carried out in a Potter-Elvehjem homogenizer. The second centrifugation at the same speed usually gave a very small amount of white materials.

The pink gel was removed and washed as before. A third washing was performed in the same manner. The pellet from the final centrifugation was suspended in 0.02 M glycylglycine buffer, pH 8.0, to a volume of 17 ml. At this point the preparation was free from soluble enzymes and inorganic materials derived from the sonic oscillator. It was also essentially free from the white particles described above; however, contamination by the latter was not critical.

The suspension obtained in the previous step was homogenized in two portions for 1 minute with a total volume of 17 ml. of 2 per cent deoxycholate in 0.1 M glycylglycine at pH 8.0 to 8.4. The decrease of optical density at 550 nm by the deoxycholate treatment was usually more than 1.0 after a 5-fold dilution. The mixture was then centrifuged for 30 minutes at PI = 22.4 × 10⁸. The inactive precipitate was discarded. The supernatant liquid, which is called the "solubilized" extract in this paper, contained glucose oxidase and cytochrome. Glucose oxidase was not pre-

2 The pink color of the gel usually became paler after washings. This was apparently due to the oxidation of cytochrome in the slightly acidic medium, once separated from cell-free extracts. The latter presumably contained endogenous reducing matter.
GLUCOSE OXIDATION AND CYTOCHROMES
cipitated by further centrifugation at PI = 31.3 \times 10^8 \text{ (the highest speed attainable in the centrifuge)} for 100 minutes. However, in dialyzed extracts, about 50 per cent of the enzymatic activity was sedimented under the same conditions.

In thirty or more experiments, the average yield was about 34 ml. of extract with a protein content of 8 mg. per ml. This constituted an 8 per cent over-all yield, based on the lyophilized cells. The protein yield in the soluble (non-particulate) fraction was approximately 50 to 60 per cent. Of the remainder, up to 20 per cent can be accounted for by larger particles, including unbroken cells, observed under the electron microscope (2).

During the last 3 years a number of methods, enzymatic, chemical, and physical, have been tested for increasing the solubility of particulate systems from A. suboxydans. Of these, only the isobutanol method of Kun (9) was at all effective, and this only slightly. Thus far, only deoxycholate has proved useful and practical. Treatment with cholate gave rise to no increase in solubility. Deoxycholate has been used by several investigators for solubilization of particles derived from animal tissues (see, for example, Wainio and Cooperstein (10)), but it has not been widely applied to microbial particles. The mechanism of the “solubilization” in either system is not clear.

“Glucose Oxidase” in Deoxycholate-Treated Extracts

A. suboxydans particulate enzymes can oxidize glucose, ethanol, propanol, mannitol, sorbitol, erythritol, glycerol (8), and butanol. All these activities, with the exception of glucose oxidation, disappeared in aged preparations. The “solubilized” extracts of fresh or aged particulate fractions oxidized only glucose. As shown in Fig. 1, the consumption of oxygen catalyzed by the deoxycholate-treated preparations was rapid up to a limit of 1 atom per molecule of glucose. However, it was necessary to dialyze the extracts for about 48 hours at pH 8.0 against 0.02 M glycylglycine or phosphate in order to remove deoxycholate. Oxidation in the presence of this reagent was greatly retarded, as may be noted in Fig. 1. Concentrations of deoxycholate as low as 0.02 per cent were inhibitory, and therefore all preparations were exhaustively dialyzed before use.

Electron Acceptors—Triphenyltetrazolium chloride or methylene blue were unable to replace oxygen as electron acceptor. However, 2,6-dichlorophenol-indophenol was effective under the same conditions as those under which oxygen was the final acceptor. In view of the oxidation-reduction potentials of these dyes, it is evident that the potential for electron transfer from glucose to the primary acceptor must be rather high. No evidence was found that pyridine nucleotides were required in the oxidation.

Exogenous flavin adenine mononucleotide or dinucleotide did not stimu-
late the rate of oxidation in either fresh or aged preparations. The activity of flavoproteins is specifically inhibited by quinacrine. Complete or nearly complete inhibition has been shown at $10^{-3}$ M in diphosphopyridine nucleotide (DPN) cytochrome c reductase (11, 12) and triphosphopyridine nucleotide (TPN) cytochrome c reductase (13), and at $10^{-4}$ M in aldehyde oxidase (14). The present glucose oxidase was inhibited less than 50 per cent by quinacrine at concentrations as high as $10^{-2}$ M with an incubation period of 20 minutes prior to the determination. If a flavoprotein is involved in the electron transfer from glucose to oxygen, it must possess a higher potential than any of the known free flavins. Moreover, the apo-enzyme must have low affinity for the inhibitor.

No exogenous cofactors or cations were found to be essential or stimulatory. Phosphate was not required. As might be expected in view of the failure of methylene blue or tetrazolium to accept electrons, substrate

**Fig. 1. Glucose oxidation in A. suboxydans.** Curve A particulate fractions, Curve B solubilized extracts before dialysis, and Curve C solubilized extracts after dialysis. The systems contained 50 μmoles of glucose, 250 μmoles of phosphate buffer, 9.0 mg. of crystalline bovine serum albumin, and 1.0 mg. of enzyme. pH 6.0; volume = 2.8 ml.; temperature, 29°; atmosphere, oxygen.
amounts of DPN and TPN were likewise ineffective. No reduction took place upon the addition of glucose, glucose-6-phosphate, 6-phosphogluconate, or any intermediates of the Krebs cycle, in the presence or absence of cyanide. The mechanism of oxidation by this glucose oxidase in the solubilized extracts is thus entirely different from that of the non-particulate, soluble enzymes of the pentose cycle in this organism (15).

**Oxidation Products**—The product of glucose oxidation was identified as gluconic acid by direct isolation as its barium salt, which possessed the same melting point and mixed melting point as an authentic sample, viz. 145-146°. The barium salt was converted to the δ-lactone.

### Table I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Time</th>
<th>Oxygen consumption</th>
<th>Hydroxamic acid formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min.</td>
<td>micromols</td>
<td>µmoles</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ-Gluconolactone</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>30</td>
<td>32</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>150†</td>
<td>103</td>
<td>84</td>
</tr>
<tr>
<td>δ-Gluconolactone</td>
<td>150</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0</td>
<td>52</td>
</tr>
<tr>
<td>Galactose</td>
<td>30</td>
<td>16</td>
<td>30§</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>45</td>
<td>90</td>
</tr>
</tbody>
</table>

* The systems contained 100 µmoles of substrate, 150 µmoles of succinate buffer, pH 6.0, 9 mg. of crystalline bovine albumin, and 1 mg. of dialyzed, “solubilized” extract, except where otherwise indicated. The values for hydroxamic acid were corrected with the use of appropriate blanks.

† Oxidation stopped at about 110 minutes.

‡ With boiled enzyme.

§ δ-Gluconolactone was used as the standard in the colorimetry.

**Chemical Analysis**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Calculated</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₆H₁₂O₆.</td>
<td>C 40.5, H 5.60</td>
<td>&quot; 40.3, &quot; 5.70</td>
</tr>
<tr>
<td></td>
<td>&quot; 40.4, &quot; 5.60</td>
<td>&quot; 40.4, &quot; 5.60</td>
</tr>
</tbody>
</table>

The lactone showed the same kinetic behavior as an authentic sample in the change of optical rotation in water.

As shown in Table I, the primary product of the glucose oxidation was, however, a lactone. Since the rate of hydrolysis of the lactone was approximately the same in the presence and absence of enzyme, the lactone cleavage enzyme may not have been present in the solubilized extracts. The position of the lactone ring is not certain.

Since the solubilized extracts contained an enzyme which catalyzed the decomposition of hydrogen peroxide, it was not possible to determine whether hydrogen peroxide or water was the primary product. However,
neither the rate of oxidation nor the total amount of oxygen was changed by addition of ethanol, high concentrations of acetate, or crystalline catalase. These results may point to water formation by this oxidase.

**Properties of Enzyme**—The oxidation possessed an optimal pH of 5.5, as shown in Fig. 2. The Michaelis constant was determined by the Lineweaver-Burk plot at $29^\circ$, pH 6.0, with glucose concentrations ranging from 20 to 600 μmoles per 2.8 ml., and found to be $8.5 \times 10^{-2}$ mole per liter. The activation energy of the oxidation was found to be 11.4 kilocalories per mole from the Arrhenius plot at temperatures of 14.7, 24.0, and 40.0°.

![Graph showing the effect of pH on glucose oxidation](image)

**Fig. 2.** The effect of pH upon glucose oxidation by "solubilized" particulate enzymes of *A. suboxydans*. The systems contained 100 μmoles of glucose, 250 μmoles of the buffer indicated, 5.0 mg. of crystalline bovine serum albumin, and the "solubilized" particulate enzyme (2 mg. of protein content). Volume = 2.8 ml.; temperature, $29^\circ$; atmosphere, oxygen.

Temperatures above $40^\circ$ rapidly inactivated the enzyme. At $49^\circ$, no oxygen consumption could be registered in a Warburg apparatus.

**Inhibitors**—Cyanide at $5 \times 10^{-4} \text{ M}$, pH 7.0, and azide at $5 \times 10^{-4} \text{ M}$, pH 6.0 or 7.0, inhibited about 90 per cent of the oxidation. Antimycin A up to 10 γ per ml., Versene at $10^{-3} \text{ M}$, pH 6.0, or 8-hydroxyquinoline at $10^{-3} \text{ M}$ did not show any effect.

**Specificity**—Among the sugars tested, only glucose and galactose were appreciably oxidized. The rate of mannose oxidation was very slow, whereas maltose, lactose, fructose, sucrose, cellobiose, raffinose, glucose-6-phosphate, 8-gluconolactone, and calcium gluconate were not attacked. The oxidation of galactose also stopped at 1 atom of oxygen consumption per molecule. Since non-particulate cell-free extracts are unable to oxidize this sugar (16), the galactose oxidation enzyme apparently occurs only in the particulate fraction. The primary product, as with glucose, was a lac-
GLUCOSE OXIDATION AND CYTOCHROMES

tone according to the acylhydroxamic acid test. The apparently higher than theoretical yield of lactone was probably due to the use of gluconolactone as standard in the colorimetry.

Cytochrome Spectra of A. suboxydans

Available spectrophotometers such as the Beckman, Unicam, or Uvispek models were not satisfactory for the examination of absorption spectra of whole cells or particulate matter owing to high light scattering properties of the suspensions and their low cytochrome content. The optically clear extracts of the deoxycholate-treated preparations did not show this complication and thus could be studied without ambiguity.

Solubilized extracts after reduction with dithionate or glucose exhibited three peaks, viz. 426 to 428, 528 to 532, and 558 to 560 m\(\mu\). A difference spectrum is shown in Fig. 3. When the spectra were observed on an enlarged scale of the spectrophotometer, one shoulder each of the \(\alpha\)- and \(\beta\)-band positions was observed. However, no further differentiation was noted at the \(\gamma\)-band, even at a very slow scanning rate (100 minutes).

Dithionate reduction was instantaneous, but at pH 6.0 the reduced

![Graph](https://via.placeholder.com/150)
spectra formed from the glucose reaction were easily observed only in the presence of cyanide. At pH 8.0, the reduced spectra from glucose did not reach a climax until after about 15 minutes. Addition of a strong reducing agent such as dithionite, however, did not further intensify the absorption. This finding was significant in view of the observation that the optimal pH for the glucose oxidation was 5.5. Thus, at acid pH values the reduced cytochrome was rapidly oxidized by molecular oxygen. Although at pH 8.0 the electron transfer from glucose to cytochrome was much slower than

at pH 6.0, the autoxidation of the reduced cytochrome did not take place, and the reduced cytochrome could be demonstrated. All these observations were in line with the fact that the oxidations by molecular oxygen in this organism showed optimal pH values of 5 to 6. It will also be recalled that the optimal pH for growth of this organism with rapid aeration is 5 to 6.

The rate of cytochrome reduction by reduced diphosphopyridine nucleotide (DPNH) and reduced triphosphopyridine nucleotide (TPNH) was much slower than that of glucose at either pH 6.0 or 8.0. The reduction was followed by the appearance of cytochrome peaks or by the disappearance of pyridine nucleotides. This observation was apparently due to the fact that some intermediate carrier in the electron transfer chain between

---

**Fig. 4.** Difference spectra of pyridine hemochromogen from "solubilized" particulate fractions of *A. suboxydans*. The Beckman settings were the same as those in Fig. 3. Pyridine hemochromogen was made according to Vernon and Kamen (17), with protein concentration of 6 mg. per ml.
the pyridine nucleotides and cytochrome was deficient in the solubilized extracts. On the other hand, glucose oxidation may not require this intermediate. This reasoning is also in line with the enzymatic results which showed only glucose, galactose, and mannose to be oxidizable.

When the cytochrome was reduced in strong alkaline solution to produce cyanohemochromogens, as in a previously described method (17), the spectra were not appreciably different from those of cytochromes reduced only by dithionate. However, the Soret peak of pyridine hemochromogen shifted to a lower wave length as shown in Fig. 4.

By using a different method, Smith reported absorption peaks in intact \textit{A. suboxydans} cells at 422, 525, and 554 \textmu m (18). This difference of about 4 to 6 \textmu m between her results and those reported here might be due to interference by other substances in intact cells. That it is not due to deoxycholates in the solubilized preparation is indicated, since dialysis removed practically all the deoxycholate, and added deoxycholate at 1 percent concentration did not change the position of the absorption peaks.

As shown in Fig. 5, soluble cell-free extracts (non-particulate) of this organism showed the Soret peak at 422 \textmu m. The absence of \(\alpha\) and \(\beta\) peaks in cell-free extracts was probably due to the lower extinction coefficients of these bands and to the fact that most of the cytochrome was associated

![Graph](http://www.jbc.org/)

**Fig. 5.** Difference spectra of non-particulate soluble fraction of \textit{A. suboxydans}

The Beckman settings were the same as those in Fig. 3. The protein concentration was about 15 mg. per ml. in 0.02 M phosphate buffer, pH 8.0. The reduced sample was prepared by addition of about 2 mg. of Na$_2$S$_2$O$_4$. 
with the particulate enzymes. The 422 mp maximum was not changed by addition of deoxycholate. Unpublished observations of Keilin and Smith\(^1\) have revealed that, at liquid air temperatures with a low dispersion, direct vision Hartridge spectroscope, three components were observed in the \(\alpha\)-band. Thus the component with a 422 mp maximum observed in the present study in the non-particulate fraction might not be identical with the 426 to 428 mp band associated with particulate fractions. Moreover, the non-particulate peak shifted to 415 ± 1 mp in the oxidized form. This shifting was not observed in the deoxycholate-treated preparations.

The results described in this paper, as well as the observations of Keilin and Smith, suggest that the heme proteins in this organism are of multiple character, as in animal tissues, with as many as three components in the cytochrome group.

**Cytochrome Spectra of \textit{A. pasteurianum}**

The foregoing "solubilization" method could be also applied to the particulate fraction from \textit{A. pasteurianum}. From difference spectra of the solubilized particles from this organism, maxima were observed at 430, 440 to 445, 528 to 532, 558 to 560, and 590 to 595 mp. Smith (18) reported the maxima at 428, 443, 523, 554, and 588 from her studies with intact cells. Here again, about 2 to 6 mp differences were noticed between the data from the solubilized extract and the intact cells. The pyridine hemochromogen showed absorption in the Soret region at 418 to 421 instead of 430 mp.

The initial work on cytochrome in \textit{A. suboxydans} was started in Dr. Keilin's laboratory at the Molteno Institute in 1955 by one of us (T. E. K.). Professor Keilin's valuable ideas and instructions are gratefully acknowledged. Stimulating discussions and suggestions from Dr. Lucile Smith were also helpful in the progress of this work. The authors appreciate the technical assistance of Miss Mary K. Devlin.

**SUMMARY**

The particulate enzyme fraction of \textit{Acetobacter suboxydans} has been rendered soluble by treatment with deoxycholate. The preparations so obtained contain glucose oxidase, which catalyzes the formation of gluconolactone without dependence upon exogenous cofactors or inorganic ions.

The reduced cytochrome of the solubilized particulate preparation shows absorption maxima at 426 to 428, 528 to 530, and 558 mp. The Soret absorption peak shifted to 518 mp in the pyridine hemochromogen.

\(^*\) Personal communication.
Addendum—In cooperation with Professor S. Y. Chen of the Department of Physics, University of Oregon, the absorption peaks described in Figs. 3 and 5 were confirmed by spectrographs obtained from a Bausch and Lomb large Littrow quartz spectrocope with use of iron arc comparison spectra for wave length setting. No niacin was found in the soluble extract of the particulate fraction after acid digestion. (The microbiological assay was performed by Wisconsin Alumni Research Foundation.)

BIBLIOGRAPHY

GLUCOSE OXIDATION AND CYTOCHROMES IN SOLUBILIZED PARTICULATE FRACTIONS OF ACETOBACTER SUBOXYDANS
Tsuo E. King and Vernon H. Cheldelin


Access the most updated version of this article at http://www.jbc.org/content/224/1/579.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/224/1/579.citation.full.html#ref-list-1