Glucose dehydrogenase of *Bacterium anitratum*: an Enzyme with a Novel Prosthetic Group*

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Glucose dehydrogenase of *Bacterium anitratum* catalyzes the reduction by glucose and other aldoses of dyes such as 2,6-dichlorophenol indophenol and phenazine methosulfate without the intervention of pyridine or flavin nucleotides (2, 3). The primary hydrogen acceptor is a group that is linked to the protein and revealed by a broad absorption band in the near ultraviolet in the oxidized state, a band which upon addition of glucose is replaced by a sharper band with maximum at 337 nm (4). In order to throw further light on the nature of this prosthetic group, it was necessary to improve the isolation procedure for the enzyme. The present paper describes the modified procedure and presents observations on the properties of the prosthetic group, in situ and in isolation.

EXPERIMENTAL PROCEDURE

Strains and Conditions of Culture—The strain used was the one originally isolated in this laboratory (2). Several other strains were tested, including strain 7844 of the National Collection of Type Cultures, London. These strains all produced glucose dehydrogenase, but not in yields surpassing that of the original strain.

Several modifications in the growth medium have been introduced since the first report. The yield of enzyme is improved considerably by using tap water for the medium instead of distilled water. We have not yet been able to replace this effect with a trace metal supplement to the distilled water. An ammonium salt has been introduced as the source of nitrogen instead of casamino acids, as this does not affect the enzyme production. The glucose originally included is now omitted since it was found to inhibit rather than to stimulate synthesis of glucose dehydrogenase.

The new medium used for the growth of the organism contains per liter of water 15 g of sodium succinate, 6H2O, 4 g of NH4Cl, 2 g of K2HPO4, and 0.06 g of MgSO4·7H2O.

The cells were grown at 25°C in a 20-liter laboratory fermentor or in a 170-liter pilot plant fermentor (Stainless & Steel Products Company) under vigorous aeration. The growth rate in the large scale fermentor corresponded to a generation time of 100 minutes. In the laboratory fermentor, the growth was frequently considerably slower, for reasons that have not been established. During these slower growths there was an increased differential rate of synthesis of the enzyme.

Enzyme Assay—The determination of the enzyme activity was carried out under the conditions described before (2). A unit of enzyme is defined as the amount that gives an initial absorbance decrease at 25°C of 1 per minute with 10-mm light path in 3 ml of assay mixture. This mixture contains 4.0 × 10⁻³ M 2,6-dichlorophenolindophenol, 0.02 M 6-glucose, and 0.05 M phosphate, pH 6.0. A Beckman model DB spectrophotometer equipped with a Beckman recorder was employed in the determinations. The initial velocity was obtained from transmittance curves rather than from absorbance curves since the former have an inflection point and an approximately linear region between T 0.35 and 0.50. Indophenol, 4 × 10⁻⁴ M, has a T value of 0.25. For a first order reaction the slope of the tangent at T 0.25 was calculated to be 0.93 times the more easily defined maximal slope of the inflection point at T 0.375. The indophenol reduction is governed by the Kₘ for indophenol of 1.6 × 10⁻⁴ M (5), and thus deviates initially somewhat from a first order reaction. The correction factor for obtaining the slope at T 0.25 from the maximal slope was here calculated to be 0.00. A typical transmittance and absorbance tracing for the enzyme reaction is shown in Fig. 1, together with some points of a theoretical transmittance curve for a first order reaction with the same time constant as the later part of the enzymatic reaction. The conversion factor for obtaining dA/dt from dT/dt is (-1/T) logₑ, which for T 0.25 is -1.73.

Physical Measurements—Absorption spectra were recorded automatically in the apparatus described above, except for Fig. 4. A pinhole arrangement allowed measurements in semimicrocuvettes in 0.2 ml of solution. For semiaerobic measurements this cuvette was closed with a rubber lid through which two No. 17 hypodermic needles were inserted. These served as inlet and outlet for highly purified nitrogen gas, which was passed slowly through the cuvette above the solution.

Electron spin resonance spectra were recorded with a Varian V-4500 spectrometer for the range of 2700 to 3700 gauss, through the courtesy of Dr. Bjørn Pedersen, Institute for Industrial Research, Oslo. Analytical ultracentrifuge data were obtained with a Beckman model E centrifuge through the courtesy of Dr. Pål Bjornstad, Institute for Clinical Biochemistry, University of Oslo.

Protein Determination—Protein was determined by the biuret method (6). Crystalline, dehydrated bovine serum albumin was used to verify the conversion factor of 0.285 absorbance units per mg of protein in 1 ml of reaction mixture measured at 540 nm. Protein was also determined from ultraviolet absorption data, with the expression P equal to ΔA₅₀ × 1.55 - ΔA₆₅ × 0.76 (7). It was necessary to apply a correction to these values, changing with the stage of the purification, since the tyrosine-
FIG. 1. Recorder tracings for the reduction of 2,6-dichlorophenolindophenol. Curve T, transmittance; Curve A, absorbance. Points are shown for the calculated transmittance curve for a reaction following the expression $T = 10^{-0.162t}$ where $t$ is reaction time in minutes.

tryptophan content of proteins varies considerably. A solution of bovine serum albumin is thus 20% underestimated by the expression, while the highly purified glucose dehydrogenase is 80% overestimated as compared to the biuret value.

Materials—The solution of α-D-glucose was made from anhydrous dextrose, the solution of β-D-glucose from the crystalline product of Sigma Chemical Company, and the solution of D-glucono-δ-lactone from gluconolactone of Hoffmann-La Roche and Company. These solutions were made in cold water less than 5 minutes before use. All other chemicals were purchased from commercial sources.

RESULTS

Preparation and Properties of Holoenzyme

Isolation of Enzyme—The purification procedure routinely used is summarized in Table I which contains data from one isolation. All steps were carried out at 2-6°C. In detail the procedure was as follows. Frozen cell paste, 137 g was thawed and diluted with 130 ml of 0.1 M phosphate, pH 6. The suspension was passed once through a French pressure cell (American Instruments Company, Inc.) under 9 to 12 tons of pressure and diluted with 45 ml of the above buffer and 200 ml of water. Whole cells and large fragments were sedimented by centrifugation twice, 5 minutes each, at 20,000 × g. The sediment was washed in 80 ml of 0.01 M phosphate, pH 6, and the second supernatant fraction was added to the first. This constitutes the crude extract. Preparation of the extract in this way rather than by grinding with Ballotini beads as previously used (2) was found to give more stable enzyme preparations.

The crude extract was centrifuged for 30 minutes at 20,000 × g to remove smaller fragments, and then made 1% with respect to protamine sulfate. After 30 minutes stirring, the precipitate formed was removed by centrifugation. Solid ammonium sulfate was added in the steps 0 to 45, 45 to 55, 55 to 58, and 58 to 70% saturation, while pH was held at 6.1 to 6.3. The first two steps removed a large proportion of the enzyme units in the form of particle-bound glucose dehydrogenase (2), whereas the soluble enzyme precipitated only when the saturation exceeded 58%. The yellow precipitate formed between 58 and 70% saturation was dissolved in 0.005 M phosphate, pH 7, and dialyzed with internal stirring against the same buffer for 5 hours. The material that became insoluble during the dialysis was removed. At this point the preparation was usually frozen and stored at −20°C, often for several weeks, with only minor losses.

The enzyme is a rather basic protein, a property which is exploited in the last two purification steps. Simple passage through a 2.5 g DEAE-cellulose column (0.7 meq per g) equilibrated with 0.005 M phosphate, pH 7, increased the specific activity of the preparation of Table I 5-fold. The DEAE-cellulose effluent was adjusted to pH 6, and passed through a 1 g CM-cellulose column (0.8 meq per g) equilibrated with 0.01 M phosphate of pH 6. This stepwise elution originally used was replaced by a linear gradient, and the elution profile was as shown in Fig. 2. The fractions around the activity peak were combined and filtered through a bed of 3.3 g of Sephadex G-25, medium grade, equilibrated with 0.01 M phosphate, pH 6. This was followed by adsorption on a 30-mg CM-cellulose bed and elution from this with 0.6 ml of 0.1 M phosphate, pH 6. If 50% of the activity of the crude extract is regarded as soluble enzyme, the yield was 28% and the degree of purification 750-fold. An-
other 25% of the original units were obtained from side fractions of the CM-cellulose column in 500-fold purification.

Purity of Enzyme—Comparison of the 280-mu profile and the activity curve of Fig. 2 indicates glucose dehydrogenase to be the dominating component in the area near the peak. The specific activity for the combined fractions was 9% lower than that of the peak fraction. The specific activity of the pure enzyme is thus equal to or somewhat larger than 1.10 X 2850 ± 3150 units per mg. This estimate agrees fairly well with the appearance in the analytical ultracentrifuge of a preparation of specific activity of 2600 (Fig. 3). The slower sedimenting component is seen to be approximately 20 to 30% of the total. The difficulty encountered in attempting to separate the dehydrogenase from cytochrome b with the stepwise elution procedure (2) is understandable from Fig. 2. In order to obtain a preparation completely free from cytochrome b it is necessary to sacrifice some of the early, rather active dehydrogenase fractions.

Spectral Properties of Intact Enzyme—The absorption spectrum of the enzyme concentrate of Table I is shown in Fig. 4. The oxidized state is characterized by a broad band in the region of 320 to 390 mu with a peak at 347 mu and by a ratio, A$_{347}$/A$_{280}$, of 1.65. On addition of glucose a sharper band appears in the near ultraviolet, as already reported (4), with a maximum at 337 mu. At the same time the absorption below 300 mu is reduced, maximally at about 290 mu, so that the ratio, A$_{290}$/A$_{260}$, is now 1.90. The 337-mu band also appears with dithionite or borohydride as reducing agent. These spectral features are much like those of muscle triosephosphate dehydrogenase (8), which contains 2 moles of bound NAD per mole of enzyme. As documented in an earlier paper (3), NAD or other derivatives of niacin cannot be responsible for these absorptions, however. Further support for this conclusion is found in the magnitude of the extinction coefficients involved, obtained by titration of the enzyme with glucose.

Titrination of Enzyme with Glucose—Addition of 11.2 mumoles\(^1\) of D-glucose to 200 mu of the preparation of Fig. 4 resulted in complete reduction of the prosthetic group, with an absorbance change at 337 mu of 0.430. When only 2.24 mumoles were added to the same amount of enzyme under nearly anaerobic conditions, the reduction observed was partial, the increase in absorbance now being 0.280. From this the molarity of the prosthetic group was calculated to be (2.24 X 0.430)/(0.2 X 0.280) = 1.72 X 10^{-5} M. This value and the absorbance data of Fig. 4 gave the following molar extinction coefficients: $\epsilon_{337}$ (oxidized) = 15,600; $\epsilon_{337}$ (reduced) = 38,900; $\epsilon_{337}$ (difference) = 24,400; $\epsilon_{280}$ (difference) = 15,500.

Two possible objections could be raised against these calculations. One stems from the earlier observation that, of the D-glucose anomers, the $\beta$ form is preferentially attacked by the enzyme (5). If the partial reduction of the prosthetic group were caused by the $\beta$ form alone, the extinction coefficients could be 50% higher since the rate of mutarotation is insignificant compared to the rate of reduction of the enzyme. The other objection concerns the equilibrium of the reaction. If the partial reduction of the enzyme ceases while there are significant amounts of glucose left, the extinction coefficients would again be higher than those computed above.

To check these possibilities, the titration procedure was carried out with $\alpha$- and $\beta$-D-glucose separately, with and without excess D-glucono-6-lactone present. The tracings obtained with the recorder are shown in Fig. 5. The absorbance change ob-

\(1\) The deviation from whole micromoles in this section stems from a 12% calibration correction on the 1-ml micropipette used.
FIG. 5. Titration of glucose dehydrogenase with α- and β-D-glucose. Absorbance was recorded at 337 nm. The cuvette contained, in 200 µl, 0.53 mg of an enzyme preparation with a specific activity of 1800 units per mg. A slow stream of N₂ passed through the cuvette before and during the experiment. The cuvette was flushed briefly after 1 µl of glucose was placed on the cuvette wall, and then the glucose was shaken in. During the slow autoxidation of the reduced enzyme, 1 µl of glucose together with 1 µl of gluconolactone (B) or without gluconolactone (A) were placed on the wall and subsequently mixed with the enzyme. The tracings run from right to left. The absorbance scale was A X 100.

In the experiment of Fig. 5B the slow autoxidation of the partially reduced enzyme was allowed to proceed until an expected 2.1 mmoles were in the oxidized form. Glucose, 2.24 mmoles, was then shaken in together with 22.4 mmoles gluconolactone. The glucose added was barely sufficient to accomplish a complete reduction, as seen from the tracing which within 1 minute reveals reoxidation of the enzyme. It is thus evident that with initially more enzyme than glucose in the mixture, the glucose will become nearly completely oxidized. The reduction of the enzyme may have been incomplete by 0.01 absorbance units without this being detected in the tracing of A versus time in Fig. 5B. This corresponds to 0.08 m mole of unoxidized glucose, when the small correction arising from the incompleteness of the oxidation is neglected. The minimal value for the equilibrium constant (E₄₅₀/(glucose)) is therefore approximately 25.0/0.08 = 312.5. If 2.2 mmoles of glucose are allowed to reduce 3.5 mmoles of enzyme with this equilibrium constant there will be only 0.0027 m mole of glucose left unoxidized. The assumptions underlying the calculation of the extinction coefficients thus appear sound.

Molecular Weight of Enzyme—On the assumption that a specific activity of 3,150 units per mg, as arrived at above, is characteristic of the pure enzyme, the equivalent concentration obtained by titration of the enzyme preparation can be used to calculate the weight of enzyme protein associated with 1 mole of the prosthetic group. A tabulation of four such calculations for three different enzyme preparations is shown in Table II. The average value was 86,000.

The molecular weight could be equal to or a multiple of the equivalent weight. The sedimentation velocity in the analytical ultracentrifuge here leads to a probable decision. In two separate centrifugations, one with a preparation of specific activity of 1,350, the other with specific activity of 2,600 (Fig. 3), the $s_{20,w}$ were found to be 6.03 and 6.25, respectively. The minimal molecular weight compatible with a given sedimentation coeffi-
TABLE II
Equivalent weight and turnover number for glucose dehydrogenase

The measurements were carried out as described in the legend for Fig. 5.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Equivalent weight</th>
<th>Turnover No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg mmoles</td>
<td>mg</td>
</tr>
<tr>
<td>40</td>
<td>0.275</td>
<td>0.432</td>
</tr>
<tr>
<td>41</td>
<td>0.280</td>
<td>0.430</td>
</tr>
<tr>
<td>42a</td>
<td>0.280</td>
<td>0.440</td>
</tr>
<tr>
<td>42b</td>
<td>0.285</td>
<td>0.438</td>
</tr>
</tbody>
</table>

Inserting $\phi_{\omega} = 6.2$ in this formula as well as the viscosity and density of water, and assuming the partial specific volume of the protein to be $0.75 \text{ cm}^3 \text{ per g}$, one obtains $M = 80,500$. This calculation, as well as inspection of tables of known molecular weights and sedimentation constants shows it to be most likely that the value of 86,000 represents both the equivalent weight and the molecular weight for glucose dehydrogenase.

**Turnover Number**—The equivalent concentration for various enzyme preparations obtained through the glucose titration can also be used to compute the catalytic activity per mole of the prosthetic group. The velocity obtained by extrapolating substrate and acceptor concentrations to infinity is 5.25 times the velocity under standard conditions (5). With $\epsilon = 14,900$ for indophenol at pH 6 (5), one obtains the activity in micromoles per minute as the number of enzyme units times 1.06. With this factor and the data of Columns 3, 4, and 5 of Table II, the turnover numbers of Column 9 were computed. The average value is $320,000 \text{ min}^{-1}$.

From this turnover number, the minimal initial velocity of reduction of the enzyme in the titration experiments may be computed (minimal, since the turnover may be limited by the reoxidation of the enzyme). With a $K_m$ for glucose of $5.3 \times 10^{-4} \text{ M}$ (5), $10^{-4} \text{ M}$ glucose will give a rate of reduction that is $\frac{1}{\epsilon} \frac{V_{max}}{V_{s}}$, or one turnover of the enzyme in 0.1 second. It is thus understandable that the recorder did not register the process of enzyme reduction itself, as is apparent from Fig. 5.

If this turnover at infinite acceptor concentration is not limited by the reoxidation reaction, the rate constant for the reaction between reduced enzyme and acceptor may be obtained from the Michaelis constant for the acceptor and the turnover number as follows (5)

$$ k_4 = k_3 \phi_{\omega} \phi_{\tau} = \frac{TN}{K_A} = 3.33 \times 10^7 \text{ sec}^{-1} \text{ M}^{-1} $$

**Nature of Prosthetic Group**

**Absence of Cyclic Disulfide and Copper at Active Site**—In searching for a possible oxidation-reduction group other than pyridine nucleotides that would absorb at 340 $\mu\text{m}$ in the reduced state, it was noted that lipoic acid has a weak absorption band in this position (10), and that it could be oxidized under mild conditions to the sulfoxide, which is also biologically active. The possible involvement of a disulfide of this nature was tested for by preincubating and assaying the glucose-reduced enzyme in the presence of $0.001 \text{ m}$ arsenite. This had no effect on the activity, and the result was the same when the enzyme was preincubated with mercaptoethanol or dimercaptopropanol before adding arsenite. Other enzymes with a disulfide prosthetic group, among them dehydroalanyl dehydrogenase (11) and a number of aldehyde dehydrogenases (12) were inhibited under similar conditions.

Certain copper-proteins, such as hemocyanin and ceruloplasmin, have fairly strong absorption bands at 330 to 350 $\mu\text{m}$, apparently caused by cuprous-cupric pairs, in analogy with the chromophoric ferrous-ferric center in Prussian blue (13, 14). To test for the involvement of copper in the present enzyme, the enzyme was preincubated and assayed in the presence of $0.015 \text{ m}$ solutions of EDTA or o-phenanthroline, with no effect. Dialysis for 18 hours against $0.005 \text{ m}$ EDTA-0.005 $\text{ m}$ KCN likewise did not affect the activity of the enzyme. When enzyme that had been partially inactivated by acid (see below) was supplemented with $2 \times 10^{-4} \text{ M}$ CuCl$_2$, an inhibition rather than activation took place. As a final test for functional copper, a $2 \times 10^{-5} \text{ M}$ enzyme solution was investigated for the presence of electron spin resonance signals in the oxidized and the reduced state. Again there was no indication of involvement of copper nor of manganese or iron.

**Dependence of Function of Enzyme on Dissociable Factor**—It had previously been noted that incubation of the enzyme at room temperature for 1 hour at pH 3.2 reduced the activity to 10% of the original, without NAD or NADP being able to reactivate the enzyme (4). We have now observed that a partial reactivation does take place on addition of a factor contained in boiled juice or a perchloric acid extract of the enzyme. Enzyme preparations, largely inactivated but capable of reactivation, free from dissociated cofactor have been obtained in three ways: (a) by precipitation with ammonium sulfate at pH 2.5, (b) by gel filtration at pH 2 to 2.5, and (c) by freezing and thawing neutral solutions in the presence of high salt concentration, followed by gel filtration. The most reliable of these methods has been gel filtration under acid conditions.

The process of reactivation is slow enough that its progress may be studied. In Fig. 6A is shown the reactivation of an apoenzyme preparation that had a background activity of 0.3 units per ml. After 40 minutes the activity reached 100-fold higher. The temperature dependence is seen to be that of an average chemical reaction. In Fig. 6B is shown the dependence of the rate of reactivation on the concentration of the factor. It is inherent in this type of experiment that the accuracy is not very high. It is apparent, however, that the initial rate is roughly proportional to the concentration of the reactivating factor, as is the extent of the reaction after 10 minutes, except for the highest level of factor, where the amount of apoenzyme becomes limiting. With this concentration of factor, an increase in activity of the apoenzyme preparation from 175 to 950 units per ml was observed.

Experiments such as the one recorded in Fig. 6B give information as to the number of sites capable of reactivation per ml or mg of an apoenzyme preparation, if one assumes that the regenerated enzyme has the same turnover number as the original enzyme. This seems probable, as degrees of reactivation close to 100% have been observed. With a specific activity of 3,150
failed. The apoenzyme preparations on the other hand also failed to show such absorption changes, suggesting that the prosthetic group might after all be dissociated, and that the dissociated form differed in chemical and physical properties from the bound form. The most promising approach to an elucidation of the nature of the prosthetic group appeared to be a further purification and characterization of the dissociable factor.

In concentrating the factor, advantage was taken of the early observation that it is strongly bound by anion exchangers such as Dowex 1, but not by Dowex 50 (1). The diethylaminoethyl derivative of the Sephadex dextran gel was found most convenient for this work. The protein remaining in solution after boiling or perchloric acid treatment is excluded by the gel, and the nonvolatile acid treatment of the original enzyme preparation is washed out by the eluent before the factor. When neutral ammonium formate is used for the elution, the factor appears slowly as the concentration reaches 1.6 M, and rapidly with 4 M ammonium formate. The factor was separated from the concentrated formate solution either by freeze-drying the fraction or by adsorption of the factor on charcoal, elution with ammoniacal ethanol, and evaporation of this solvent.

Several interesting features appeared when this procedure was carried out on concentrated enzyme preparations. Absorption curves from different stages of such a purification are reproduced in Fig. 7. Curve 1 shows the typical 320- to 390-mu band of the intact, oxidized dehydrogenase, with the maximum at 347 mu. Such a band is also found in the boiled and clarified enzyme extract (Curve 2), the maximum is, however, located at a somewhat shorter wave length. This is seen more clearly in Curve 3 units per mg for pure dehydrogenase and 86,000 as the equivalent weight, a concentration of 1 unit per ml corresponds to 3.7 X 10^{-9} M. The concentration of sites capable of reactivation in the apo-enzyme of Fig. 6B thus was 2.9 X 10^{-4} M.

From the converse experiment, where the maximal reactivating capacity of a factor preparation is reached with excess apoenzyme, an evaluation of the equivalent concentration of the factor can similarly be made. This requires, however, an apoenzyme preparation with a lower background activity than is usually obtained. With such apoenzyme, reactivating capacities of more than 80% of the original enzyme activity have been observed in fresh factor preparations.

The lower limit for the concentration of factor in the preparation used in Fig. 6B is seen to be one-tenth of the apoenzyme concentration. From this and the initial slopes of the reactivations, the rate is computed to be \(2.7 \times 10^4\) sec^{-1} M^{-1}. This is 1000-fold lower than the rate constant for the reaction between acceptor and reduced enzyme calculated above.

Identity of Dissociable Factor to Chromophoric Group—The main question left undecided after the discovery of and preliminary experiments (1) with the dissociable factor, was the relationship of this factor to the group responsible for the 347-mu absorption in the oxidized state of the enzyme and the 337-mu band in the reduced. After partial dissociation of the factor, 337-mu absorption and specific activity declined in the same proportion. It could not be ruled out, however, that the dissociable factor was a separate entity from the chromophoric group, required for the reduction of the latter since attempts at showing the expected changes in the absorption spectrum of the factor preparations on addition of borohydride or dithionite had failed. The apoenzyme preparations on the other hand also

![Fig. 6. Reactivation of apoenzyme with boiled juice. A, 350 \(\mu\)l of an enzyme preparation of 600 units per ml (specific activity, 500 units per mg) was brought to pH 2.5 with HCl and filtered in the cold through a 0.33-g Sephadex C-25 column equilibrated with 0.006 N HCl. The protein fraction was immediately neutralized, and denatured material removed. The specific activity was now 18 units per mg, which decreased somewhat during subsequent storage and use. The factor was prepared by keeping a preparation of 480 units per ml for 10 minutes in a boiling water bath. The residual activity was 1 unit per ml. The reactivation was carried out in 200-\(\mu\)l tubes by mixing 10 \(\mu\)l of each component at time zero, and transferring the mixture into an assay mixture at the times indicated. One set of tubes was kept at 4\(^\circ\), the other was set at 25\(^\circ\). The values have been corrected for the background activity of the factor preparation. B, apoenzyme was prepared from a preparation of 3200 units per ml. After acid gel filtration the activity was 175 units per ml, the buffer was 0.01 M phosphate, pH 6. An enzyme preparation in the same buffer of 900 units per ml was used to make the boiled juice. Residual activity was 2 units per ml. Apoenzyme, 1 \(\mu\)l, was preincubated with 2, 5, and 10 \(\mu\)l of factor preparation, and phosphate buffer added to 11-\(\mu\)l total.](http://www.jbc.org/)

![Fig. 7. Purification of the dissociable factor on DEAE-Sephadex. Of an enzyme preparation of original activity of 5100 unit per ml (Curve 1) 600 \(\mu\)l were kept in a boiling water bath for 10 minutes. The supernatant fluid after centrifugation (Curve 2) was filtered through a 5-mm high bed containing 50 mg of dry weight DEAE-Sephadex A-25, fine grade, previously equilibrated with 0.02 M ammonium formate of pH 6.5. The last 200 \(\mu\)l of the effluent contained maximal concentration of excluded protein, and the absorption of this fraction (not shown) was subtracted from Curve 2 to obtain Curve 3. The column was developed with 1000 \(\mu\)l of 0.5 M neutral ammonium formate followed by two portions of 200 \(\mu\)l of 1.5 M formate, and finally 150, 200, 170, and 200 \(\mu\)l of 4 M formate. The last fraction with 1.6 M eluent had an absorbance at 320 mu of 0.040, the following four fractions had an absorbance of 0.078, 0.275, 0.180, and 0.035, respectively. Curve 4 is the spectrum of the peak fraction recorded with formate in the reference cuvette.](http://www.jbc.org/)
which is the difference between Curve 2 and the absorption of the high molecular material of the extract that was excluded by the gel. This curve also shows that the low molecular portion of the boiled juice has characteristic absorption bands in the region of 240 to 300 μm. Some material with absorption at 260 μm appeared with formate concentrations below 1.6 M, unaccompanied, however, by the higher band and by factor activity. The peak fraction of cofactor activity had the absorption spectrum of Curve 1, similar to that of the corrected boiled juice spectrum. These observations give strong support to the hypothesis that the active principle of the boiled juice and the spectrally demonstrable prosthetic group of the intact enzyme are identical. The finding of bands in the region of 240 to 300 μm is in agreement with expectations for the isolated prosthetic group because of the minimum in the difference spectrum for the oxidized-reduced enzyme at 260 μm (Fig. 4).

A final desirable piece of evidence for the identity of the prosthetic group and the cofactor would be the demonstration of the characteristic spectral changes seen in the intact enzyme on reduction. This has indeed been possible with these more concentrated preparations. Fig. 8 shows the oxidized and the borohydride-reduced form of a boiled enzyme preparation after correction for remaining protein. The absorption changes are of the expected magnitude and sign (cf. Fig. 4). There is, however, as was the case for the oxidized enzyme, a change in the position of the maximum when comparison is made with the intact enzyme. These changes may be a sign of a special interaction between apoenzyme and the prosthetic group, and do not invalidate the conclusion that the dissociable cofactor and the prosthetic group are identical. This interpretation is strengthened by the observation that for an enzyme preparation where the prosthetic group had become partly dissociated during cold storage, there was a partial shift in the maximum of the oxidized form.

Some Physical and Chemical Properties of Isolated Prosthetic Group—The prosthetic group, as observed immediately after elution with ammonium formate, is characterized by a broad absorption band with a maximum at 330 μm, a shoulder at about 280 μm, and a second maximum at 248 μm (Fig. 7). On the assumption that the prosthetic group is liberated completely and intact, the concentration in the boiled juice of Fig. 8 is calculated from the activity to be $1.37 \times 10^{-3}$ M. This gives a molar extinction coefficient at 330 μm of 13,000 for the neutral, aqueous solution. The ratio of absorbances at 248 and 330 μm for various preparations and purification stages was found to vary between 2.0 and 2.6. In ammoniacal, aqueous ethanol (Fig. 9A, Curve 1) a more pronounced peak at 330 μm is observed, and the shoulder now is at 270 μm. In 95% ethanol containing 0.25 M HCl the 248-μm band disappears, while the 330-μm maximum is replaced by a diffuse maximum at 310 μm and a sharper maximum at 365 μm (Fig. 9A, Curve 2).

The reduced form of the prosthetic group has a maximum at 302, a shoulder at 340, and a minimum at 270 μm, while the largest reduction changes take place at 305 and 250 μm, respectively (Fig. 8). These basic features were observed in partly rearranged preparations (see below) such as that of Fig. 9B, Curve 1, in formate eluates, and directly in the boiled juice. The position of the maximum was the same at pH 7 and pH 8. Based on the molar extinction coefficient of 13,600 for the 330-μm band and the observations of Fig. 8, an extinction coefficient of 37,000 is obtained for the 302-μm band of the reduced group.

During isolation, the prosthetic group often changed into forms with somewhat different spectral features. Curve 4 of Fig. 7, Curve 1 of Fig. 9A, and Curve 1 of Fig. 9B represent successive stages of the same preparation. Evaporation of the ammoniacal ethanol may lead to replacement of the 248 μm peak by a shoulder if not carried out rapidly. Often this change had already taken place at the charcoal eluate stage. At the same time the peak in the spectrum of aqueous solutions at 320 μm disappears, and shoulders at 320 and 370 μm appear instead. A certain separation of rearranged and intact material took place when the residue after evaporation of the ammoniacal ethanol...
was first extracted with 95% ethanol. In one experiment, 80% of the 330-mu absorbing material and half the factor activity went into solution in ethanol. The residue dissolved in water had the absorption spectrum shown in Curve 2 of Fig. 9B and a factor activity per unit of absorbance at 330 mu equal to that of fresh boiled juice.

Because of the apparent acidic properties of the prosthetic group revealed by the strong binding to anion exchangers, it was of obvious interest to study the effect of pH changes on the spectra. In order to do this on as unarranged material as possible, perechioric extracts or boiled juice were used as such. When pH was changed from 6.9 to 3, no significant changes took place. Below pH 3 there was a gradual transposition of the 330-mu maximum towards longer wavelength, as well as other modifications. The two extreme forms are shown in Fig. 10A. From a plot of the displacement of the maximum versus pH, the pH of the dissociation involved was found to be approximately 1.7. An isosbestic point for the dissociation was found at 325 mu. Similar spectral changes take place in alcohol solution (Fig. 9A).

Observations with the material of Curve 1 of Fig. 9B, indicated another dissociation in the neighborhood of neutrality. This could not be duplicated in fresh boiled juice. However, after the boiled juice had been briefly exposed to pH 9 to 10, the previously biphasic pH 6.6 curve was changed into the triphasic curve of Fig. 10B, with new maxima at 320 and 370 mu. A single, long wavelength maximum could be re-established, either by lowering pH to below 1, in which case it was located at 300 mu, or by increasing pH to 8, restoring the maximum at 325 mu (Fig. 10B). The low pH dissociation now had an isosbestic point at about 340 mu, the second dissociation isosbestic points at 270 and 355 mu. The pH of the latter group was approximately 7.2. From the pH response of the rearranged material of Fig. 9, as well as the position of the shoulders, the hydrolysis resulting in the unmasking of the second acid group is part of the change that takes place in ammoniacal ethanol. The limited alkaline rearrangement of Fig. 10 did not lead to any decrease in the ability of the material to reactivate the apoenzyme.

The strongly acidic character of the prosthetic group is also reflected in its solubility properties. HCl must be added to absolute ethanol in order to bring the dissociated form of the prosthetic group into solution. The undissociated form, while soluble in absolute ethanol, was practically insoluble in cyclohexane and carbon disulfide.

Two further observations shed some light on the chemical nature of the group. When filtering a prosthetic group preparation through Sephadex G-25, a rather strong sorption effect was observed. Such sorption to the Sephadex dextran gel has been reported to take place with aromatic and heterocyclic compounds (15). If the compound were a nitrogen-containing heterocycle, it would be expected in most instances to carry a positive charge at pH values below 2. To test for this possibility, Dowex 50-WX2 in the hydrogen form was added to a prosthetic group preparation in a spectrophotometer cuvette. The expected shift in the spectral maximum took place. There was, however, no binding to the cation exchanger, even after lowering pH to 1.5.

**Discussion**

Glucose dehydrogenase of *B. anitratum* has in the present work been purified to what appears to be near homogeneity. In addition to the chromatographic and ultracentrifugal evidence presented above, which gives a specific activity in the neighborhood of 3,200 units per mg as the probable specific activity of the pure enzyme, it should be mentioned that an increase in this value would correspondingly decrease the equivalent weight. The value of 86,000 for the equivalent weight is, however, already so close to the computed minimal molecular weight, 80,500, that a further decrease would only appear possible if the partial specific volume is appreciably smaller than the commonly found or assumed value for proteins of 0.75 cm$^3$ per g.

The determination of the equivalent weight, which, in light of the sedimentation constant found, most likely is also the molecular weight, became possible through a titration of the prosthetic group of the enzyme with glucose. From the equivalent weight the turnover number of the enzyme could be computed and, the average value found was 320,000 min$^-1$. Glucose dehydrogenase of *B. anitratum* thus belongs among the most active of dehydrogenases, its turnover number, for instance, being 30-fold higher than that of the flavoprotein glucose oxidase (16).

The most noteworthy feature of the present enzyme is, however, not its high catalytic activity, but the nature of its prosthetic group. It has so far, because of scarcity of material, only been possible to study this by spectral observations of the intact enzyme or the liberated prosthetic group, and by enzymatic assay of the reactivation of apoenzyme by prosthetic group preparations. For these studies quantities of a few micrograms have been sufficient, while 100- to 1000-fold larger amounts are required for chemical studies that could lead to a complete identification. Several distinct chemical and physical characteristics of the group have nevertheless been uncovered by these limited means.

The free prosthetic group has an absorption maximum at 330 mu, a shoulder at 280 mu, and a second maximum at 249 mu. On reduction, a band appears at 302 mu, with a shoulder at 340

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**Diagram**: Fig. 10. pH dependence for absorption spectrum of the prosthetic group. A, 1 ml of an enzyme preparation of 5000 units per ml was treated in the cold with 200 mu of 37% HClO$_4$. The supernatant fluid, with a pH of 0.5 (Curve 1), was treated with 240 ml of 37% HClO$_4$. The supernatant after precipitation of KCIO$_4$ had a pH of 6.9 (Curve 2). The absorption values were corrected to the same volume, and the absorption of the material subsequently excluded on a 30-mg DicEAE-Sephadex column subtracted, as in Fig. 7. B, boiled juice was prepared as above from a preparation of 2800 units per ml, and the absorption corrected for residual protein. The initial absorption spectrum recorded (pH 6.6) was similar to Curve 2 of A and to the pH 8.0 curve of B. To 300 mu of the boiled juice in 0.1 M phosphate buffer was added 5 N HCl and KOH to give different pH values between 6 and 9. *Curve 1*, pH 8.0, was recorded after brief overhanging of the pH to between 9 and 10. The pH was then lowered to 6.6 with the absorption spectrum shown in Curve 2.
The absorption in the region of 240 to 280 μm at the same time declines. The free prosthetic group thus has spectral properties agreeing with observations made with the intact enzyme. The maxima are, however, shifted to shorter wave lengths, the change being 17 μm for the oxidized form and 35 μm for the reduced form. These shifts may be an expression of a rather strong interaction between the prosthetic group and the protein, such as have been observed for liver alcohol dehydrogenase and NAD (17), the old yellow enzyme and flavin mononucleotide (18), and in other instances. The shift is exceptionally large for the reduced form. A shoulder remains, however, for the free prosthetic group at the position of the maximum of the bound form, and the difference spectrum for the intact enzyme conversely indicates a shoulder at the position of the maximum for the free group (Fig. 4).

Equally as important for the characterization of the compound as the position of the absorption bands are, of course, their extinction values. These have been determined through titration with glucose. The long wave length band of the oxidized prosthetic group is rather strong, log ε being 4.13. Even stronger is the absorption of the reduced group, log ε here being close to 4.6 both for the free and the attached group. This is 0-fold higher than the molar absorbitivity for reduced pyridine nucleotides at 340 μm. The 248 μm band of the oxidized form has a log ε of about 4.5.

These spectral features, together with the finding of an uncharged acid group of pK 1.7 and an additional, easily hydrolyzed group of pK 7.2 greatly narrows the field of possible candidates for the unknown oxidation-reduction group of the enzyme, without, however, in any way deciding the issue. One type of compound which offers itself as an attractive possibility is a suitably substituted 1,4-naphthaquinone. 1,4-Naphthaquinone has absorption maxima at 330 and 250 μm, coupled with increased absorption at about 330 μm in the reduced state, and decreased absorption between 260 and 280 μm. Furthermore, certain naphthaquinones are known for their participation in electron transport, at least in vitro (19). They have suitable oxidation-reduction potentials for this task, E′0 (pH 7) ranging between +0.036 and −0.260 volt for various derivatives (20). A glucose dehydrogenase with a naphthaquinone as a prosthetic group would thus become completely reduced by an equivalent amount of glucose, as was indeed observed for the present enzyme, since the glucose-glucolactone couple has an E′0 (pH 7) of −0.32 volt (21). The main difficulty with the naphthaquinone hypothesis is that the 330 μm band, especially, of the unsubstituted compound is rather weak, log ε being 3.44 (22). It does not appear impossible, however, that suitable substitutions with hydroxyl, carbonyl, and carboxyl could both increase the intensity of this band, and at the same time give the compound the required water solubility and acidity. Anthraquinone, which contains the same chromophore, C₆H₄—CO-R, thus has a log ε for this band of 3.75, and 2,6-dihydroxyanthraquinone has a log ε of 3.9 for the same band (22).

The prosthetic group of the soluble and the particulate glucose dehydrogenases of R. anitratum may well have a wider occurrence. A particulate glucose dehydrogenase from *Rhodopseudomonas spheroides* was observed to require an unknown factor present in the boiled soluble fraction for the reduction of phenazine methosulfate or ferricyanide (23, 24). This factor was adsorbed by anion exchange resins and charcoal, as is the B. anitratum factor. The *Rhodopseudomonas* enzyme, furthermore, could be reactivated by a boiled extract of *B. anitratum* (2). Glucose-oxidizing particles of *Pseudomonas fluorescens* and *Acetobacter suboxydans* were shown to have several traits in common with the *B. anitratum* particles (25). These, and the long series of other particle-bound glucose dehydrogenases of *Acetobacter* and *Pseudomonas* species are thus the most promising material in a further search for the new prosthetic group. It is possible that one of these other organisms would be the richer source which is desirable in order to produce the group in quantities sufficient for chemical analysis.

**SUMMARY**

Glucose dehydrogenase of *Bacterium anitratum* has been purified to a state close to homogeneity. Its equivalent weight was found to be 86,000 ± 4%, based on titration of the prosthetic group of the enzyme with glucose. The ε₉₀,₀ value of 6.2 suggests this to be the molecular weight as well. The turnover number of the enzyme is 320,000 min⁻¹.

The prosthetic group is revealed in the absorption spectrum of the dehydrogenase as a broad band at 347 μm (log ε, 4.18). On reduction of the enzyme with glucose, a sharper band at 357 μm appears (log ε, 4.59). When the enzyme is boiled, a factor that will reactivate enzyme preparations inactivated by gel filtration at pH 2.5 is liberated. This factor has been concentrated and purified by chromatography on small columns of DEAE-Sephadex. The spectral properties of the product strongly suggest that the dissociable factor and the prosthetic group are identical. The maximum of the oxidized form, as well as the maximum observed upon reduction with sodium borohydride are, however, shifted to somewhat shorter wave lengths, to 330 and 302 μm, respectively. The extinction coefficients at the maxima were similar to those observed for the intact enzyme, log ε being 4.13 and 4.57, respectively. The liberated prosthetic group in its oxidized form is further characterized by a maximum at 245 μm (log ε, 4.5), and a shoulder at 270 to 280 μm. A decrease in absorption takes place in the region of 240 to 270 μm upon reduction, a phenomenon also observed for the glucose-reduced intact enzyme.

Spectral observations of the free prosthetic group at different pH values revealed an acid dissociation constant with a pK of 1.7. Under mildly alkaline conditions, the prosthetic group readily underwent a hydrolysis resulting in the appearance of a second dissociation, with a pK of 7.2. The latter form of the prosthetic group showed undiminished capacity to reactivate apoenzyme.

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**REFERENCES**
