PURIFICATION AND PROPERTIES OF GLUCOSE DEHYDROGENASE AND CYTOCHROME *b* FROM *BACTERIUM ANITRATUM**

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SUMMARY

A soluble glucose dehydrogenase with a turnover number of 28,000 moles of glucose/min per 10^5 g of protein has been purified from *Bacterium anitratum*. The enzyme does not posess a flavin or heme prosthetic group. It catalyzes the reduction of 2,6-dichlorophenolindophenol and phenazine methosulphate well, but ferricyanide and methylene blue only very slowly. Reduction of DPN, TPN, FMN, FAD, cytochrome *c*, tetrazolium, or oxygen could not be detected.

The dehydrogenase was accompanied during all purification steps except the last by a *b*-type cytochrome with absorption maxima at 419 and 530 mμ in the oxidized state, 428, 532, and 562 mμ in the reduced. It was purified to the stage of $2.9 \cdot 10^5$ g protein per mole of protoporphyrin. In the presence of the dehydrogenase and possible unidentified linking factors, the hemoprotein was reduced by glucose.

Evidence is presented and the possibility discussed that the two proteins are soluble precursors or degradation products of the particulate glucose oxidizing system from the same organism.

INTRODUCTION

Oxidation of glucose to gluconic acid is carried out by many bacteria, notably by *Acetobacter*, *Pseudomonas*, *Aerobacter*, and *Azotobacter* species[2-6]. In these organisms glucose oxidation has been found linked to structural elements, in some instances shown to carry cytochromes reducible by glucose[2,3]. No evidence has here been found for the intervention of DPN or TPN in the primary dehydrogenation. A few instances are, however, known where bacteria or their spores contain a soluble glucose dehydrogenase resembling the mammalian liver enzyme in being DPN-linked[7,8]. The present communication describes a soluble glucose dehydrogenase which is independent of externally added DPN or TPN for its action. It is purified from a bacterial homogenate, where a large part of the dehydrogenase activity is associated with particles in the manner usually found.

Abbreviations: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; DIP, 2,6-dichlorophenolindophenol.

* A brief note on the early phase of this work has appeared elsewhere[7].

METHODS

Microorganism

The strain of *Bacterium anitratum** used was isolated in this laboratory and kindly identified by Dr. H. LAUTROP, Statens Seruminstitut, Copenhagen. It was grown under vigorous aeration and agitation in a 25 l container at 37°, either in batch or continuous culture. The medium contained 4.7 g Difco Casamino Acids, 4.7 g glucose, 5.5 g Na_2HPO_4, 1.7 g K_2SO_4, 0.8 g $MgSO_4 \cdot 7H_2O$, 0.8 mg $FeSO_4 \cdot 7H_2O$, and distilled water to 1 l. During the growth pH was kept between 6.9 and 7.4 by addition of ammonium hydroxide. The cells were harvested at a density of 1.0–1.5 g dry wt./l in a super centrifuge, and washed, when required, by resuspension in several liters of distilled water, followed by a second centrifugation. The cell paste was then dried from the frozen state and stored at —20°. It was usually found, for reasons yet to be investigated, that the cells grown in batch process gave a higher yield of soluble enzyme per gram of cells than the cells from continuous culture.

Preparation of cell-free extract

The cells were disintegrated by grinding with Ballotini glass beads, No. 12, in a MSE homogenizer cooled with ice-water. 5 g of cells and 20–25 ml of water were added to 40 ml of wet glass beads, and the homogenizer run at close to full speed for 2 × 8 min. As the cells were broken, the mass turned from a thick to a thin paste. This paste was then diluted with 20–30 ml water, and centrifuged for 5 min at 24,000 × *g* in the cold. The sediment was suspended in 30 ml 0.1 *M* phosphate buffer, pH 6.0, and recentrifuged. The two supernatants were combined and centrifuged for 90 min at 24,000 × *g*. A well packed layer of cells and heavy fragments, a suspended layer of smaller fragments near the bottom, and a clear supernatant, showing Tyndall effect, were obtained. The clear supernatant represents the starting material for the enzyme studies. 40–50 ml with 600–700 mg of protein was obtained from 5 g of cells.

Analytical methods

Protein was measured with the biuret method[12], with the addition of 20 μl 10% desoxycholate per 3 ml of reaction mixture when lipoprotein complexes were present. The mixture then had to be centrifuged before reading of the O.D. In purer preparations, the protein concentration was calculated from the absorptions at 260–280 mμ.

Glucose was determined by the anthrone method[13], and gluconolactone determined as hydroxamate with authentic D(+)-gluconolactone as standard, according to HESTRIN[14].

Aerobic oxidation was followed in the Warburg apparatus at 30° with air as the gas phase, in a medium of 0.05 *M* phosphate, pH 6.0. Dehydrogenase activity was measured spectrophotometrically in a Beckman DU at 25° by reduction of DIP in 3 ml total volume in 1 cm cuvettes with an initial O.D. at 600 mμ of 0.6. The buffer

* *Bacterium anitratum* is a commonly found apathogenic organism whose position in the taxonomic system has not yet been finally agreed upon[9,10]. It has many similarities to species within the family *Achromobacteraceae*, particularly *Alcaligenes viscolactis* and *Achromobacter butyri*. BRISOU AND PREVOT[11] have suggested a new arrangement and definition of *Achromobacteraceae*, in which among others these two species and *Bacterium anitratum* fall in a common new genus denoted *Acinetobacter*. The present genus *Alcaligenes* is proposed eliminated.

was as above, and glucose was added to an initial concentration of 0.02 M. The reaction was started by the stirring in of the enzyme preparation. Enzyme activities were calculated based on the time needed for an initial O.D.-decrease of 5–10 %. 1 unit of enzyme is defined as the amount giving an O.D.-reduction of 1/min under these standard conditions, and specific activity is equal to units/mg of protein. Reaction rates in terms of μmoles DIP reduced were computed based on an extinction coefficient of $21.0 \cdot 10^6$ cm$^2 \times$ mole^{-1} at pH 7.0 (see ref.[15]), which at pH 6.0 corresponds to a value of $14.9 \cdot 10^6$ (experimentally determined).

Other electron acceptors were tested under the same substrate, volume, buffer, and pH conditions. Cytochrome c and ferricyanide reductions were measured at 550 and 418 mμ respectively, using extinction coefficients of $19.1 \cdot 10^6$ and $1.02 \cdot 10^6$ respectively. Reduction of methylene blue, triphenyl tetrazolium chloride, FAD and FMN were studied in Thunberg tubes *in vacuo*.

The presence of flavin was investigated in the following manner: 125 μg of highly purified enzyme in 250 μl phosphate buffer, pH 6.0, was denatured by brief heating to 100° and treated with 50 μg Difco Trypsin, added in 20 μl 0.5 M Na_2HPO_4, for 1 h at 37°. The sample was again heat-denaturated and precipitated with 40 μl 50 % trichloroacetic acid. The absorption spectra were recorded at the various stages in this procedure, following clarification by centrifugation. The final supernatant was adjusted to pH 2.7 with ammonia, and the fluorescence spectrum recorded in the micro-cuvette of a Farrand spectrophotofluorimeter, the excitation beam having a wavelength of 365 mμ.

Pyridinohemochromogens were prepared by making the hemoprotein solution 0.1 N with respect to NaOH, and adding 1/3 volume of pyridine. They were reduced with $Na_2S_2O_4$. As the extinction coefficient for the α-band of protohemin hemochromogen[16] $34.8 \cdot 10^6$ was used.

Absorption spectra were recorded in volumes of 0.20–0.25 ml in semi-micro cuvettes, the Beckman DU being equipped with an appropriate diaphragm.

MATERIALS

Ballotini glass beads were obtained from H. Mickle, Gomshall (Surrey); D-gluconolactone from Hoffmann-La Roche and Co; N,N-diethylaminoethylcellulose (DEAE-cellulose) from Eastman Kodak Company; carboxymethylcellulose (CM-cellulose) from Serva Entwicklungslabor; triphenyltetrazolium chloride, crystalline bovine serum albumin, and FAD (80 % pure) from Nutritional Biochemicals Corporation; horse heart cytochrome c, reduced DPN (90 % pure), FMN, TPN (95 % pure), p-chloromercuribenzoate, and phenazine methosulphate from Sigma Chemical Company; DPN from Pabst Laboratories; trypsin 1:250 from Difco Laboratories; atebrin from Bayer, Leverkusen; methylene blue from G. T. Gurr, London; 2,6-dichlorophenol-indophenol from Hopkin and Williams Ltd.

EXPERIMENTAL AND RESULTS

Purification

The purification was initiated by precipitation with protamin sulphate, which was added in quantities sufficient to reduce the 260 mμ O.D. of the extract from 180 to around 20. This usually was 300 mg for a 5 g cell batch. Inactive protein and a

portion of the enzyme activity were also precipitated, giving a supernatant of specific activity close to that of the extract. This first step was necessary for a successful subsequent ammonium sulphate fractionation. This was carried out at pH 6, in steps given in Table I. Some purification was achieved in the 61–76 % saturation fraction. After dialysis for 4 h against 0.005 *M* phosphate, pH 7.0, this fraction was applied to an equilibrated DEAE-cellulose column of 1 g DEAE-cellulose/50 mg of protein, and

TABLE I

MAIN PURIFICATION

Purification step	*ml*	*mg protein*	*Units*	*Units/mg*
Extract	238	2,900	1,050	0.36
Protamin sulphate supernatant	250	2,300	780	0.35
Ammonium sulphate: 0–45 %	16	240	100	0.41
45–55 %	36	1,140	300	0.29
55–61 %	4.3	160	40	0.24
61–76 %	3.7	115	100	0.91
Supernatant	220	460	0	0
DEAE-cellulose eluate	15	5.5	102	18
Calcium phosphate gel eluate	1.9	2.0	82	41
Ammonium sulphate: 50–55 %	38	530	125	0.24
140,000s240*	31	350	28	0.08
140,000p240	9	93	54	0.58
Semi-sedimented layer	2	60	16	0.27

* Ultra centrifuge supernatant and precipitate fractions, with relative centrifugal force and centrifugation time (min) indicated.

elution started with the dialysis buffer. The dehydrogenase activity was under these circumstances not bound by the column, while much of the inactive protein remained attached. Concentration and further purification was achieved by adsorption on calcium phosphate gel, 2 mg added/mg of protein. Most of the activity was adsorbed, and eluted again with 0.1 *M* phosphate of pH 6, containing 10 % ammonium sulphate. All steps were carried out at a temperature of 0–5°. The protocol for a purification up to this stage, starting with 25 g of cells from a continuous culture, is summarized in the upper part of Table I.

For further purification, a small scale column fractionation on carboxymethyl cellulose was set up. 3–6 mg protein was dialyzed against 0.01 *M* phosphate buffer of pH 6.0 and adsorbed on a column of 100 mg carboxymethyl cellulose that had previously been equilibrated with the same buffer. Elution was carried out with phosphate buffers in the following steps of molarity and pH: 0.01–6.00, 0.02–6.30, 0.03–6.60, 0.04–6.70, 0.05–6.85, 0.06–7.00, and fractions of 0.6 to 0.9 ml collected. The progress of the elution was followed in terms of enzymic activity, protein concentration (280 mμ absorption), and cytochrome content (difference in absorption between 419 and 380 mμ). The dehydrogenase activity was slowly eluted at pH 6.70, and rapidly by the following buffer. In one such fractionation, to be further discussed below, 125 units of enzyme, specific activity 36, were applied to the column. 80 units of specific activity 390 were recovered in the first fractions of the pH 6.85 buffer. The following fractions also contained considerably enriched material. The protein eluted was very dilute, around 0.1 mg/ml. It was concentrated as follows: after 2 h dialysis

against 0.01 *M* phosphate of pH 6.0, suitably pooled fractions were led through a bed of 5–10 mg carboxymethyl cellulose, equilibrated as above. 0.3 ml buffer of a pH and molarity somewhat in excess of what was necessary for initial elution was used to reelute the fractions.

Inspection of Table I shows a high loss of enzyme units in the 0–45, and particularly in the 45–55 % ammonium sulphate fraction. Refractionation of the 45–55 fraction did not bring more activity into the 61–76 range. A part of the material precipitating between 50 and 55 % was then subjected to ultra centrifugation at 140,000 × g* in the preparative Spinco centrifuge for 4 h. The lower part of Table I demonstrates that a large part of the activity of this fraction was sedimentable under these conditions. The degree of purification was twofold.

The purification process thus presents us with two different forms of the enzyme. The major part of the glucose dehydrogenase units appears to be linked to small sedimentable particles, and this portion is purified to a small degree only. Another portion of the extract activity is not sedimentable under the above conditions; it behaves like a soluble enzyme in the purification process and is considerably purified. The relative amount obtained of the soluble form varied from one batch of cells to another. Studies on the small particle fraction, purified as above, have been carried out alongside the investigation of the properties of the soluble dehydrogenase, on the hypothesis that the latter might be a degradation product or a precursor of the former, and that they therefore might show some similar traits.

The stability of the enzyme preparations at all stages of purity is fairly good. When kept in the frozen state at $\div$ 20°, the decline in activity was on the order of 5–15 % in a month for the soluble enzyme. Occasionally there have been unexplained larger losses at some point of purification or in storage.

Hydrogen acceptors

The original extract oxidizes glucose with oxygen as well as with DIP as hydrogen acceptor, the DIP rate usually being 2–4 fold higher than the rate with oxygen. After fractionation, the ability to react with oxygen is retained in the 45–55 % ammonium sulphate fraction and in the particles isolated from this, whereas it is lost in the 61–76 fraction, and all further purification steps. The high speed precipitate referred to in Table I carried out oxidation of glucose at a rate of 150 μl O_2/mg protein/h, while the equivalent rate with DIP was half this figure.

In addition to DIP and oxygen, TPN, DPN, FAD, FMN, phenazine methosulphate, triphenyltetrazolium chloride, methylene blue, cytochrome *c*, and ferricyanide were tested. The results are given in Table II. The original extract or dialyzed fractions were not stimulated in their DIP reduction by addition of pyridine nucleotides, neither were these reduced when added in the absence of DIP. Cytochrome *c* was not measureably reduced by glucose in the presence of either the dehydrogenase or the particle, unless small amounts of DIP or phenazine methosulphate were present as carrier. Ferricyanide was reduced very slowly by the purified dehydrogenase, and somewhat faster by the particle. Again, DIP was a very effective bridge, and with DIP added, the reduction of ferricyanide proceeded linearly at a rate determined by the DIP concentration, until the ferricyanide remaining was of the same order of

* Centrifugal force at tube bottom.

magnitude as the DIP added. No reaction with tetrazolium could be detected with either of the two forms. Methylene blue was reduced too slowly to be an effective bridge to oxygen for the soluble enzyme. Phenazine methosulphate, however, coupled the dehydrogenase to oxygen in manometric experiments, at a rate close to the rate obtained with DIP.

TABLE II

RELATIVE RATES WITH DIFFERENT HYDROGEN ACCEPTORS

Acceptor	*Concn.** ($M \times 10^4$)	*Relative rates*	
		Sol. enz.	*Part. enz.*
DIP	4	100	100
Oxygen	(air)	< 1	50–200
Phenazine methosulphate	35	100	
Methylene blue	6	2	< 2
Ferricyanide	50	0.3	3
FAD, FMN	3	0	0
DPN, TPN	14	0	0
Cytochrome *c*	8	0	0
Triphenyltetrazolium chloride	500	0	0

* These figures represent convenient rather than saturating concentrations. The dependence of the rate on the concentration of the main acceptor DIP is discussed in the accompanying paper[19].

Nature of the product

Resting cells oxidized glucose rapidly to the level of 1 atom oxygen consumed/molecule glucose, and from then on at a reduced rate. In this first period CO_2-evolution was slight. Cell-free extracts and particle preparations oxidized glucose only one step under the present test conditions. This all pointed towards gluconic acid as the probable candidate for the product, particularly since gluconic acid or gluconolactone has been shown to be the product in other particulate glucose oxidation systems[2,4,6].

A manometric experiment was set up with soluble dehydrogenase from the gel eluate level and phenazine methosulphate as link to oxygen, as described in Table III. Two parallel vessels were used, which were removed at different times. The reaction was stopped with trichloroacetic acid, and glucose and gluconolactone determined. It is evident that hydroxamate-forming material was produced in conjunction with

TABLE III

PRODUCT FORMATION

The Warburg vessels contained 150 μmoles phosphate buffer, 37 μg dehydrogenase of specific activity 70, 2.5 mg bovine serum albumin, 20 μmoles glucose (added at zero time), 0.3 mg phenazine methosulphate, and water to 3 ml at a pH of 6.0. The reaction was stopped by addition of 0.5 ml 10% trichloroacetic acid, and aliquots taken after centrifugation for determination of glucose and gluconolactone. It was necessary to correct for the ability of glucose itself to form some color in the hydroxamic acid test, as well as for the color of decomposed phenazine methosulphate.

Time (min)	*Glucose oxidized (μmoles)*	*Gluconolactone formed (μmoles)*	*Oxygen consumed (μmoles)*
25	11.3	7.1	6.9
60	14.0	8.3	10.0

oxygen uptake and glucose disappearance, and it is assumed that this hydroxamate represents gluconolactone. There is not full correspondence between glucose oxidized and lactone formed. Loss of gluconolactone by spontaneous hydrolysis must, however, be expected to occur during the reaction and handling before the assay. The oxygen uptake is between 1 and 2 atoms per molecule glucose, indicating partial decomposition of the hydrogen peroxide formed during the reoxidation of phenazine.

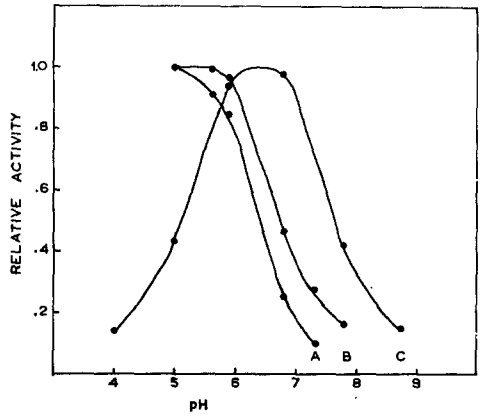

Fig. 1. pH dependence. The following buffers were used: pH 4.1 and 5.0 (acetate); pH 5.9, 6.8 and 7.3 (phosphate); pH 7.8 and 8.7 (tris(hydroxymethyl)aminomethane). Curve A, particulate enzyme with DIP; B, soluble enzyme with DIP; C, particulate enzyme with oxygen. The assays were otherwise carried out under standard conditions. The DIP reduction rates were corrected for decline in extinction with pH according to an empirical extinction curve with pK about 5.8.

pH-dependence

Fig. 1 shows the dependence on pH of the dehydrogenase reaction with DIP for the soluble and the particulate enzyme, and also of the aerobic reaction of the latter. There is a pronounced shift in the optimum when oxygen replaces DIP, indicating a still higher pH optimum for some reaction along the chain to oxygen, or a lower pH optimum for the dehydrogenation with DIP than with the natural primary acceptor.

TABLE IV

INHIBITION BY ATEBRIN

The measurements were carried out in the DIP-system under standard conditions, without preincubation of the enzymes with atebrin.

Atebrin concn. ($M \times 10^5$)	*Per cent inhibition*	
	Sol. enz.	*Part. enz.*
0.5	19	43
1.0	24	50
2.0	35	
4.4	52	64
8.8	68	
13.2	77	82

Inhibition studies

The soluble and particulate enzymes were inhibited by atebrin in the concentrations given in Table IV. The particle is slightly more sensitive to the inhibitor than the soluble dehydrogenase, but concentrations of 10^{-3} *M* or more are in both cases required to give 50% inhibition. *p*-Chloromercuribenzoate in a concentration of $3.6 \cdot 10^{-3}$ *M* did not interfere with the activity of the dehydrogenase, even after prolonged action. HCN was likewise found without effect on the dehydrogenase reaction in a concentration of 0.01 *M*. At this concentration, the aerobic oxidation with the particle was inhibited 20%. 0.001 *M* was, however, without effect on the oxygen uptake.

The involvement of cytochromes

Work by others on glucose oxidizing particles from microorganisms has demonstrated the reducibility of cytochromes of these particles by glucose. Such is also the case with the particles obtained from this organism. Fig. 2 gives difference spectra for the fraction of small particles, showing maxima at 427, 530 and 560 mμ. The particles,

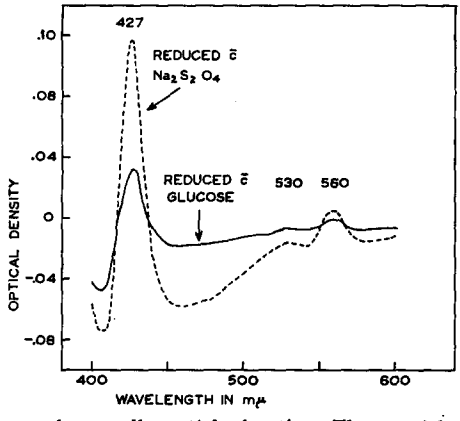

Fig. 2. Difference spectrum for small particle fraction. The particles were sedimented from a 45–55% ammonium sulphate fraction at 140,000 × *g* for 4 h. The protein concentration was 8.4 mg/ml, specific activity 0.5. Glucose and dithionite were added to a phosphate buffer suspension of the particles, and the absorption read against a cuvette with the same particle concentration.

as one would expect, also carry other enzymes. DPNH dehydrogenase and oxidase activities were demonstrated, and found somewhat lower than the corresponding glucose activities, but of the same order of magnitude. The particles also posessed succinic dehydrogenase.

These other dehydrogenase activities are absent from the purified dehydrogenase. Fig. 3 gives the spectral characteristics of the preparation which was the product of the particular purification reproduced in Table I. Before reduction, the preparations at this stage have maxima at 419 and 530 mμ. In some early preparations there was also a shoulder at 440–460 mμ, as is the case in Fig. 3. Upon addition of glucose to the cuvette, there is a more or less pronounced shift of the SORET band towards higher wavelengths and an appearance of bands around 530 and 560 mμ. These latter peaks are intensified by addition of dithionite, forming maxima at 532 and 562 mμ, while the SORET band shifts to 428 mμ. Preparations have been encountered where glucose alone

causes this maximal reduction. For preparations with lower glucose–cytochrome reduction, one could instead of dithionite add traces of phenazin methosulphate, which, upon reduction by the dehydrogenase present, rapidly reduced the heme. DIP also acted as carrier in this fashion, but not as effectively as phenazine methosulphate.

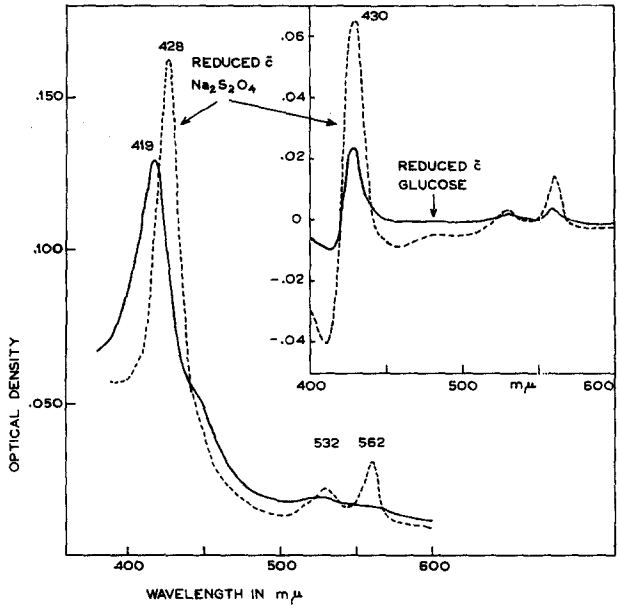

Fig. 3. Absorption spectrum of the dehydrogenase preparation at the gel eluate stage. The protein concentration was 1.6 mg/ml, specific activity 41.

The dehydrogenase is thus accompanied through the main purification process by a cytochrome of the *b*-types, and this cytochrome also appeared to have a functional relationship to the dehydrogenase reaction. It was natural here to first think of the enzyme as an analogue to yeast lactic dehydrogenase[1] or *Propionibacterium pentosaceum* succinic dehydrogenase[18]. There were suggestions of the presence of functional flavin (the shoulder at 440–460 mμ, the inhibition by atebrin). Purer preparations were, however, needed to decide this question of the possible role of flavin. The role of the heme was also somewhat obscure, not only in the variable glucose-reducibility, but also in the fact that the gel eluate fraction from different cell batches gave ratios of enzyme activity to heme content varying in a tenfold range, the highest specific activities corresponding to the lowest heme concentrations.

The further purification on carboxymethyl cellulose described above threw surprising light on some of these questions. Practically all the heme was eluted at pH values below 6.85, most of it appearing with the 0.03 *M* pH 6.60 buffer. The enzymic activity in these fractions was, however, negligible. The dehydrogenase appeared, as mentioned, with 0.05 *M* pH 6.85 buffer. Depending on the purity and heme content of the material applied to the column, the dehydrogenase fractions were either found very low in or essentially free from heme. Fig. 4 gives the spectral characteristics after concentration of pooled fractions with high heme/protein and activity/protein ratios respectively.

The material of Fig. 4, curve A, represents a 7-fold purification of the cytochrome *b* in the preparation applied to the column, a preparation similar in heme/protein/activity ratios to the preparation of Fig. 3. Based on an α-band extinction coefficient of $36 \cdot 10^6$ cm^2 × mole^{-1}, there is one mole of heme/290,000 g of protein. This coeffi-

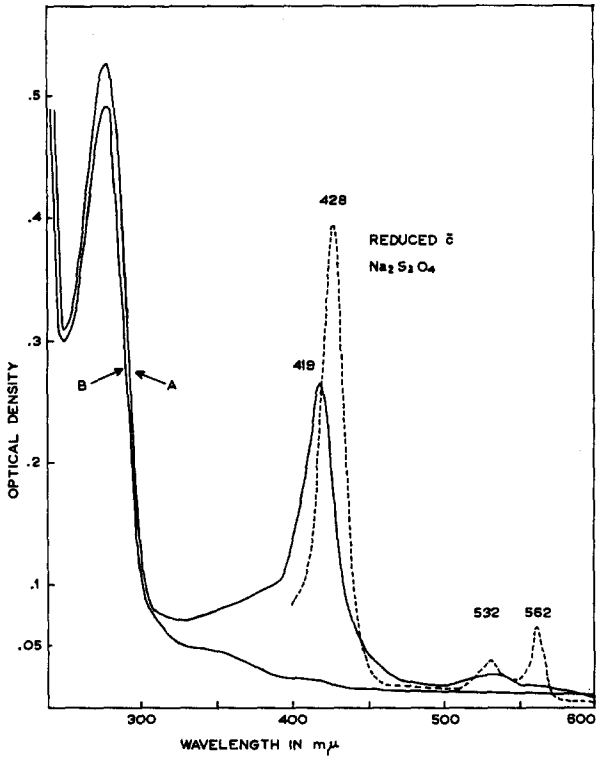

Fig. 4. Absorption spectrum of dehydrogenase and cytochrome *b* after carboxymethyl cellulose. A represents a concentrate of fractions 9–14 (0.03 *M* phosphate pH 6.60), B, fractions 21–23 (0.05 *M* phosphate pH 6.85), with specific activity 390. The actual O.D. obtained for A are here multiplied by 2.5, for the sake of comparison.

cient was found by making the pyridin hemochromogen of a sample of cytochrome *b* similar to that of Fig. 4, and it is nearly the same as that of crystalline yeast lactic dehydrogenase[17]. The spectral maxima above 400 mμ are at the same wavelengths as noted for the preceding purification stage, and the pyridin hemochromogen showed maxima in the reduced state at 419, 525, and 557 mμ, confirming the assignment of the hemoprotein to the *b*-class of cytochromes, having protoheme as prosthetic group.

The material of Fig. 4, curve B, represents an 11-fold purification with respect to the dehydrogenase activity of the preparation applied to the column. Here there is only a small suggestion of heme absorption. The dehydrogenase spectrum further shows no indication of any flavin being present, and determination of the fluorescence of the preparation after digestion with trypsin likewise showed no sign of the characteristic flavin band.

The degree of purity of the final dehydrogenase preparations is not known. Too little material has so far been available to make the necessary homogeneity tests. The

activities are, however, high. The preparation of Fig. 4 represents a 1,300-fold purification above the original extract, and it had a catalytic activity of 80 μmoles DIP reduced/min/mg under standard conditions. For excess glucose and DIP, the rate should ideally be 5-fold higher[19]. Because of mutual inhibition at high glucose and DIP concentrations, only 70% of this may be attained. This means that the maximal turnover-number for the preparation at 25° and pH 6.0 was 28,000 moles/min/100,000 g of protein.

DISCUSSION

An association between the dehydrogenase activity and the hemoprotein accompanying it through the main purification was anticipated, partly because of the observed reductions of the heme by glucose, partly from analogy considerations. Already the rather variable ratio of heme to activity for different cell batches showed, however, that there was at least a certain independence between the two. The final purification step proved this independence to be complete. The two proteins, which have a high isoelectric point and other characteristics in common, were finally separated on carboxymethyl cellulose. But the difference in buffer concentration and acidity for their elution was only 0.02 *M* and 0.25 pH units respectively.

It still appears plausible, however, to look upon the enzyme when operating in the cell, as cytochrome linked, the chain starting with cytochrome *b*. There are two main reasons for this: the dehydrogenase is able to reduce the cytochrome accompanying it, and the difference spectrum of the dehydrogenase-carrying particles upon addition of glucose and dithionite is closely akin to that of the dehydrogenase–cytochrome *b* mixture. The displacement of the maxima to slightly lower wavelengths could be due to some cytochrome *c*-like hemes also becoming reduced.

The first of these phenomena was, as already noted, rather variable, extending from full reduction to almost nil, independent of the dehydrogenase activity. Such variation in cytochrome reducibility has been observed by others, even with dehydrogenases where the heme still is a part of the enzyme[20]. Preliminary observations indicate that the level of cytochrome *b* reduction in the present work were expressions of steady states, where the reduction rate was equal to the slow autooxidation taking place. Factors decreasing the latter or increasing the former should then give a higher degree of observed heme reduction. The efficient transfer of electrons from the substrate to cytochrome *b* could possibly *in vivo* involve a cellular equivalent to the artificial bridge afforded by phenazine methosulphate in our experiments. This natural carrier might to a large degree be lost during the purification. Flavin does apparently not play this role, as there is no reduction by the dehydrogenase of either FAD or FMN. An efficient *in vivo* transfer may, however, alternatively be brought about by a close proximity of dehydrogenase and cytochrome *b* in the cell structure.

The argument from the observations on the spectral properties of the particles rests on the assumption that the particle is an expression of how the dehydrogenase is fitted into a larger structure in the cell. The similarity in acceptor specificity, in pH dependence, and in sensitivity towards inhibitors, together with the fact that both forms lead to a one-step oxidation of glucose, supports this view. The substrate specificity data given in the accompanying paper[19] also support this contention, if one is willing to grant that the incorporation of the dehydrogenase into, or separation

from, the particles may modify the architecture of the substrate site to a certain degree.

The enzyme and cytochrome-carrying particles discussed are probably to be considered as artifacts produced in the grinding. There exist in the homogenate many sizes of particles carrying the ability to oxidize glucose, some of which are sedimented in the clarification centrifugation, some in the protamin sulphate and 0–45 % ammonium sulphate fractions, and the smallest particles finally in the 45–55 % fraction. In accordance with observations on ghost fractions in other organisms[21,22], these particles probably represent smaller and larger fragments of the cytoplasmic membrane of the bacterium.

Microbial cytochromes of the *b*-group are usually classified into three classes, cytochrome *b*, cytochrome b_1 and cytochrome b_2. PAPPENHEIMER[23] differentiates according to the position of the α-band, so that cytochromes with maxima at 562–565, 560, and 556 mμ should be designated with *b*, b_1, and b_2 respectively. According to this convention, the cytochrome here purified should be classified as a cytochrome *b* without subscript.

Few microbial *b*-type cytochromes have been purified in the soluble state. VERNON[24] has isolated soluble cytochrome b_1 from *Micrococcus lysodeikticus* and *Pseudomonas denitrificans*, showing maxima at 426, 528, and 559 mμ. A soluble form of succinic dehydrogenase from *Propionibacterium pentosaceum*[18] has also been somewhat purified, showing maxima around 430, 530, and 560 mμ. Cytochrome b_2 is best represented, with the essentially homogeneous or crystalline lactic dehydrogenase preparations from yeast[17,20]. These have equivalent weights of about 100,000, as compared to 290,000 for the present preparation, which probably still is inhomogeneous.

The *Bacterium anitratum* glucose dehydrogenase functions without addition of niacinamid coenzymes, and does not reduce these. If the enzyme, as postulated, stands at the entry of the cytochrome system, it is perhaps even more noteworthy that flavin is not involved. The inhibition by relatively high concentrations of atebrin observed for the present enzyme is without relation to flavin function. Instead, it could be shown that atebrin competes with the acceptor DIP for a site on the enzyme[19].

As already mentioned, it can not be ruled out that another unidentified compound acts as a carrier *in vivo*, reducing cytochrome *b* after having itself become reduced, and a flavoprotein might conceivably play this role. But whether DIP in the present assay is to be regarded as a substitute for cytochrome *b*, or for an intervening carrier, there remains the question whether the dehydrogenase "itself" becomes reduced in a particular group, or whether there is a direct transfer of the hydrogen from substrate to acceptor. As is discussed elsewhere[19], the kinetic data with glucose and DIP are in line with the concept of a reducible group tightly bound to the enzyme taking part in the reaction, and do not show the characteristics of the usual direct reduction-oxidation reactions. This might possibly be fortuitous, and further chemical evidence is required to settle the question of mechanism.

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REFERENCES

1 J. G. HAUGE, *Acta Chem. Scand.*, 13 (1959) 2124.
2 T. E. KING AND V. H. CHELDELIN, *J. Biol. Chem.*, 224 (1957) 579.
3 W. A. WOOD AND R. F. SCHWERDT, *J. Biol. Chem.*, 201 (1953) 501.
4 J. H. DOWLING AND H. B. LEVINE, *J. Bacteriol.*, 72 (1956) 555.
5 E. A. DALBY AND A. C. BLACKWOOD, *Can. J. Microbiol.*, 1 (1955) 733.
6 A. F. BRODIE AND F. LIPMANN, *J. Biol. Chem.*, 212 (1955) 677.
7 T. E. KING AND V. H. CHELDELIN, *Biochem. J.*, 68 (1958) 31P.
8 H. HALVORSON AND B. CHURCH, *3rd Intern. Congr. Biochem. Brussels, 1955*, p. 90.
9 I. G. SCHAUB AND F. D. HAUBER, *J. Bacteriol.*, 56 (1948) 379.
10 M. S. BROOKE, *Acta Pathol. Microbiol. Scand.*, 28 (1951) 338.
11 J. BRISOU AND A. R. PREVOT, *Ann. inst. Past.*, 86 (1954) 722.
12 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
13 D. L. MORRIS, *Science*, 107 (1948) 254.
14 S. HESTRIN, *J. Biol. Chem.*, 180 (1949) 249.
15 E. P. STEYN-PARVÉ AND H. BEINERT, *J. Biol. Chem.*, 233 (1958) 843.
16 K. G. PAUL, H. THEORELL AND Å. ÅKESON, *Acta Chem. Scand.*, 7 (1953) 1284.
17 C. A. APPLEBY AND R. K. MORTON, *Nature*, 173 (1954) 749.
18 F. J. S. LARA, *Biochim. Biophys. Acta*, 33 (1959) 565.
19 J. G. HAUGE, *Biochim. Biophys. Acta*, 45 (1960) 263.
20 A. P. NYGAARD, *Biochim. Biophys. Acta*, 33 (1959) 517.
21 P. MITCHELL AND J. MOYLE, *Biochem. J.*, 64 (1956) 19P.
22 R. STORCH AND J. T. WACHSMAN, *J. Bacteriol.*, 73 (1957) 784.
23 A. M. PAPPENHEIMER JR., in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. II, New York, 1955, p. 744.
24 L. P. VERNON, *J. Biol. Chem.*, 222 (1956) 1035.