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GLUCOSE DEHYDROGENASE OF *BACILLUS MEGATERIUM* KM. COUPLING OF THE CYTOPLASMIC ENZYME WITH MEMBRANE-BOUND CYTOCHROMES

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SUMMARY

1. The oxidation of glucose by O_2 catalyzed by lysed-cell preparations of *Bacillus megaterium* KM involves the entire complement of respiratory pigments present in the cell membrane; difference spectra indicate complete reduction of the cytochromes on adding a combination of glucose *plus* NAD^+.

2. The initial step of glucose oxidation occurs in the soluble fraction of the bacterial cell and requires NAD^+ or $NADP^+$ as coenzyme. Pyridine nucleotide reduction with either coenzyme is observed in the presence of dialyzed soluble fraction only when substrate is added. Spectral studies of concentrated solutions of the dialyzed soluble fraction indicate total reduction of a flavin component on the addition of only glucose.

3. Lysed protoplast preparations show a negligible respiration rate compared to the rate catalyzed by intact cells. Supplementation with glucose *plus* NAD^+ increases the rate of O_2 uptake to a significant fraction of the respiration of intact cells. The data support the conclusion that in this organism, free glucose is oxidized directly *via* a soluble NAD-linked glucose dehydrogenase (which may involve the participation of a functional endogenous flavin component) coupled to the electron-transport chain of cytochromes.

INTRODUCTION

Many of the substrates that are utilized by aerobic bacteria are oxidized by a complex reaction sequence that involves initially a reaction catalyzed by a soluble NAD-linked dehydrogenase present in the cytoplasm, and secondly, a reoxidation of the product, NADH, by O_2 *via* the array of cytochrome components present in the bacterial cell membrane. Despite the basic importance of this coupling between the soluble dehydrogenases and the insoluble lipoprotein membrane components, there are very few relevant quantitative data available. It is generally assumed that the dehydrogenase activity in the presence of a given substrate measured in a cell-free extract is an indication of the oxidative capacity of the intact cell with respect to that substrate, though the preparation of an extract necessarily dilutes the soluble cell

materials. The soluble step is considered rate-limiting because the individual dehydrogenase activities of soluble fractions obtained from cell extracts are lower than the rate of oxidation of NADH by isolated membrane fractions[1], and because in one instance it has been possible to confirm this by rate measurements in intact cells[2]. There has been little systematic attempt to resolve the difficulties involved in relating the measured *in vitro* activities of fractions obtained from ruptured cells to the observed O_2 uptake of intact cells[3]. The limited data available indicate large discrepancies between the rates *in vivo* and *in vitro*[4–7], suggesting that the model of a soluble dehydrogenation reaction limited only by the inherent catalytic properties of the enzyme, followed by rapid diffusion of free NADH to the cell membrane and reoxidation there, is too simple.

The present study describes a stable system in which the interaction of soluble and membrane respiratory enzymes can be studied. The soluble fraction from osmotically lysed protoplasts of the obligate aerobe, *Bacillus megaterium*, strain KM, contains an active pyridine nucleotide-linked glucose dehydrogenase; the membrane fraction contains a relatively simple respiratory chain which is completely and rapidly reduced by NADH[8]. With the exception of glucose 6-phosphate, which shows an absolute requirement for $NADP^+$ as coenzyme, and malate, which is oxidized by a membrane-bound enzyme that exhibits no requirement for or stimulation by pyridine nucleotide, no other substrate has been found which gives rise to significant rates of O_2 uptake in comparison to the sequence of reactions that oxidize glucose. There is, therefore, relatively little interference from other oxidation reactions of products that may arise from metabolism of added glucose.

METHODS

Bacillus megaterium, strain KM, was grown aerobically in 2% trypticase soy broth on a New Brunswick rotary shaker. Cultures were grown at 30° and routinely harvested in the early stationary phase of growth.

For studies using intact cells, the cultures were washed with distilled water and resuspended in a volume of 0.05 M phosphate buffer (pH 7.0) equal to 1/25 the volume of the original culture medium. For measurements of respiration, 0.1 ml of this concentrated whole cell suspension was added to 3 ml of fresh, autoclaved 2% trypticase soy broth at 25°, and O_2 uptake measured with a Clark oxygen electrode.

Protoplasts were prepared from whole cells by suspending washed cell pellets in 4/25th of the original culture volume of 0.5 M sucrose–0.05 M phosphate (pH 7.0) and incubating at room temperature with Sigma Grade I lysozyme (EC 3.2.1.17) at a final concentration of 0.25 mg/ml until conversion from intact cells to protoplasts was complete, as determined by observation with a phase-contrast microscope. After the incubation, usually 45 min–1 h, the intact protoplasts were collected by centrifugation at 16 000 × *g* for 30 min. Protoplasts were lysed by resuspending the pellet in 1/25 the original culture volume of 0.05 M phosphate buffer (pH 7.0). Worthington crystalline deoxyribonuclease (EC 3.1.4.5) was added at a final concentration of 0.02 mg per ml, and the mixture stirred at room temperature for approx. 15 min. For measurements of respiration, 0.1 ml of this protoplast lysate was used per 3 ml assay volume.

Soluble and particulate fractions were obtained from the lysate by centrifugation at 33 000 × *g* for 1 h in a refrigerated centrifuge. After decanting the clear, pale

yellow supernatant fraction, the sedimented residue consisted of a yellow particle fraction above a white fraction. In resuspending the residue in a volume of 0.05 M phosphate buffer equal to the volume of lysate used, only the yellow layer was used; as much as possible of the white material was left in the tube.

A Cary Model 14 recording spectrophotometer supplied with a strong light source and a 0–0.1 absorbance slide wire was used for difference-spectra determinations.

Protein concentrations of all fractions were determined using a modified biuret method[9], which involves pretreatment with alkali before the colorimetric assay is carried out. For whole cells, however, this treatment was not sufficient to clarify the samples; microscopic observation indicated that insoluble cell-wall material remained. The material was solubilized by an initial incubation of the samples of intact cells with an aliquot of lysozyme (final concentration of 1 mg/ml) for approx. 1 h; a control tube containing the same concentration of lysozyme was incubated simultaneously. Following the lysozyme treatment, all samples were assayed colorimetrically. Absorbance due to lysozyme was subtracted from that of all the samples of intact cells; it was found to provide only a very small fraction of the total absorbance in all cases.

D-Glucose 6-phosphate (disodium salt), β-NAD (Grade III), and NADP (Sigma Grade), were purchased from Sigma Chemical; trypticase soy broth was obtained from the Baltimore Biological Laboratory.

RESULTS

Respiratory activities of intact cells and subcellular fractions

In order to assess the contribution of a single oxidative reaction sequence *in vitro* to the overall respiratory activity of intact cells, it was necessary to determine the rate of oxidation of intact cells of *B. megaterium* supplied with excess substrate. In Table I are listed the respiration rates of five separate preparations of whole cell suspensions measured upon adding the washed cells to aerated fresh culture medium, in this case 2 % trypticase soy broth. The average of the rates obtained was approx. 2 nmoles of O_2 taken up per sec per mg protein, which was assumed to be a measure of the maximum respiratory capacity of the intact cells at the time of harvest, that is, in the early stationary phase of growth. Although intact cells exhibited significant rates of O_2 uptake even in the absence of added substrate, it was found that under these conditions, the endogenous rates of respiration as measured with an oxygen electrode were neither linear over the period of measurement nor consistent for a given preparation, as they were when culture medium was present during the assay.

WEIBULL[4] showed that the cells of *B. megaterium* may be lysed osmotically if they are first converted to protoplasts by lysozyme treatment. Lysed protoplast preparations have been prepared and found to have a much lower capacity for O_2 uptake than do the intact cells from which they have been derived. In Table I, the observed respiration rates of four lysed preparations are given. With no additions of substrate to the assay mixture, *i.e.*, in the presence of only the cellular materials originally present, the O_2 uptake was negligible; in the presence of 2 % trypticase soy broth, the rate varied with the preparation, but accounted for only about 1/10th that observed with the corresponding amount of intact cells. During lysis of intact

TABLE I

RATE OF O_2 UPTAKE BY INTACT CELLS AND BY OSMOTICALLY LYSED PROTOPLASTS OF *B. megaterium*

Rates determined at 25° using a Clark oxygen electrode; assay volume, 3 ml. Intact cells washed and resuspended in sterile 2% trypticase soy broth with 0.05 M phosphate (pH 7.0). Lysed protoplasts were diluted with the same buffered 2% trypticase soy broth or with 0.05 M phosphate buffer (pH 7.0). Amounts of additional components: NAD^+, 3 μmoles, and glucose, 30 μmoles.

	Rate of O_2 uptake (nmoles O_2/sec per mg protein)					
	In 2% trypticase soy broth		*In 0.05 M phosphate buffer*			
	No additions	*+ NAD^+*	*No additions*	*+ glucose*	*+ NAD^+*	*+ NAD^+ + glucose*
Intact cells	2.0					
	2.3					
	2.6					
	1.8					
	2.1					
Lysed protoplasts	0.1	1.2	0	0.06	0.4	1.1
	0.2	0.7	0	0.04	0.2	0.5
	0.3	0.9	0	0.04	0.1	0.6
	0.3	1.3	0	0.07	0.2	1.2

protoplasts, soluble materials such as substrates, cofactors, and soluble enzymes are all diluted. On the other hand, the concentrations of the insoluble membrane-bound respiratory pigments presumably are not changed with respect to one another, though their relative concentration with respect to the soluble materials with which they interact is different. All of the factors responsible for the marked decrease in respiration on lysis are not known, but the respiration can be partially restored by adding substrate to the osmotically lysed cellular material. In Table I, the effects of these additions are listed. Some enhancement of the rate is observed on adding only substrate; in buffer there is a low but measurable rate observed when glucose is added as contrasted to a negligible rate with no additions, and similarly, adding only trypticase soy broth (which contains glucose and nitrogen-containing substrates in addition) permits a low but measurable rate with lysed preparations. Addition of NAD^+ to protoplasts lysed in buffer provided a much greater increase in respiration rate than addition of substrate, and adding both glucose and NAD^+ gave a much higher rate than either substance alone or than the sum of the two separately. Lysate in the presence of trypticase soy broth also showed a marked increase on adding NAD^+. This latter system showed a slightly higher rate in all experiments than the same preparation to which glucose had been added as substrate. The data indicate that supplements of glucose (or glucose-containing culture medium) *plus* NAD^+ to lysed cellular material will restore the respiration rate to a significant fraction, 25–55% of the rate of corresponding intact cells.

If measurements of O_2 uptake are carried out using higher levels of a lysed protoplast preparation than were used above for comparison with intact cells, the stimulatory effect of adding NAD^+ is more evident. In Fig. 1A an oxygen-electrode trace is presented: Curve a indicates a measurable rate of O_2 uptake on adding only NAD^+ at time t_1; in contrast, Curve b indicates a very slow rate when only glucose is

added at time t_1. The data suggest that since the addition of NAD^+ is sufficient to observe O_2 uptake, both substrates and the enzymes which oxidize those substrates, using NAD^+ as cofactor, are present in sufficient quantity in the lysed preparation to give a measureable respiration rate. Also, since subsequent addition of glucose gives an additional increase, then the enzyme system which oxidizes glucose, using NAD^+ must also be active enough to catalyze a significant rate of respiration.

It was found that the endogenous NAD-linked substrate or substrates could be removed from the lysed protoplast preparation by dialysis. Fig. 1B represents the oxygen-electrode trace of the same preparation pictured in Fig. 1A after the preparation had been dialyzed. Results indicate negligible reaction with either NAD^+ or glucose added separately but a significant reaction when both were present. No further attempt was made to identify the metabolites that comprised the pool of dialyzable endogenous substrates.

The lysed protoplast preparations which permit O_2 uptake in the presence of glucose and NAD^+ can readily be separated into soluble and particulate components by centrifugation. Neither the soluble nor the particulate system is sufficient by itself to catalyze any O_2 uptake in the presence of glucose and NAD^+, though when the two fractions are present together at the concentrations tested separately, O_2 uptake is observed. Dialysis of the isolated soluble fraction is as effective in removing endogenous substrate as dialysis of the unfractionated lysed protoplast material, indicating that the endogenous substrate is not bound to the insoluble fraction of membrane fragments.

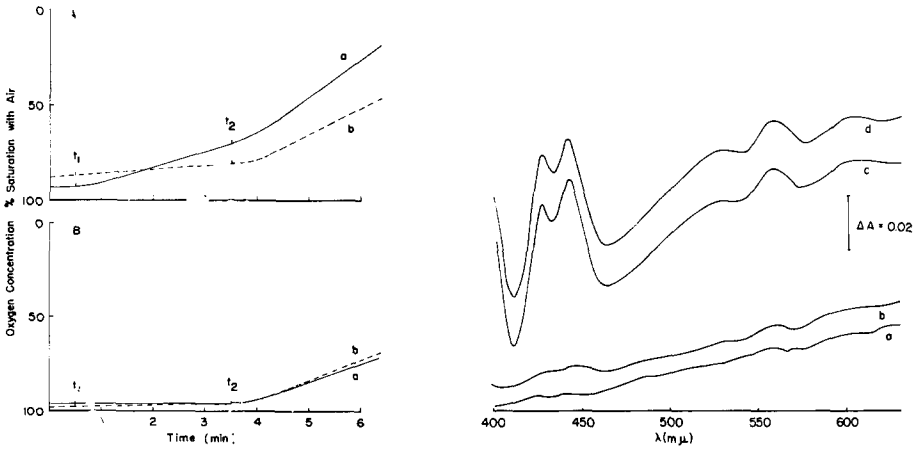

Fig. 1. The effect of adding NAD^+ and glucose to lysates of *B. megaterium* protoplasts. A comparison of the O_2 uptake catalyzed by dialyzed and by untreated preparations. Oxygen-electrode trace at 25°; assay volume, 3 ml. A. Undialyzed lysate containing 3.1 mg protein. B. Lysate dialyzed against 0.05 M phosphate buffer (pH 7.0) containing 3.0 mg protein. For both plots: Curve a, addition of 3 μmoles NAD^+ at t_1 and 30 μmoles of glucose at t_2; Curve b, reverse order of addition: 30 μmoles glucose added at t_1 and 3 μmoles NAD^+ added at t_2.

Fig. 2. Reduction of cytochromes by glucose *plus* NAD^+. Substrate reduced *minus* oxidized difference spectra of reconstituted lysate after dialysis of soluble fraction. Total protein concentration: 10.1 mg in the 3-ml sample volume. Additions were made to the sample cuvette sequentially as follows: Curve a, no additions; Curve b, 3 μmoles NAD^+ (+ 10 min); Curve c, 60 μmoles glucose (+ 20 min); Curve d, solid $Na_2S_2O_4$. Cuvettes were protected from light during the incubation periods between scans on the Cary spectrophotometer.

Reduced minus oxidized difference spectra of lysed protoplasts; cytochrome reduction by glucose plus NAD$^+$.

Though the membrane fraction of the bacteria has been shown above to be essential for O_2 uptake on addition of glucose *plus* NAD$^+$, this is only indirect evidence that the cytochromes located in the membrane are involved in the oxidation. Difference spectra of a mixture of the soluble and membrane fractions, however, indicated directly that the cytochromes present are completely reduced by the addition of both glucose and NAD$^+$. After preparations of lysed protoplasts were separated into their soluble and membrane fractions, the soluble fraction was dialyzed against 0.05 M phosphate buffer (pH 7.0) to remove endogenous substrate. The two fractions were then recombined to give a reconstituted lysate in which the relative concentrations of the two fractions were the same as in the original lysate. Difference spectra are given in Fig. 2; Curve b on addition of only NAD$^+$ shows no change from Curve a, the base line. However, glucose subsequently added to the system (Curve c) gives visible peaks at 602 and 557 mμ and Soret peaks at 443 and 427 mμ, a spectrum characteristic of the reduced *minus* oxidized difference spectra of membrane fragments isolated from this organism, as shown earlier in this laboratory[8]. Addition of $Na_2S_2O_4$ (Curve d) caused no further change in the reduced *minus* oxidized difference spectrum. It was concluded that soluble and membrane fractions at the relative concentrations present in the lysate contain an enzyme system active enough to reduce all the cytochromes completely when glucose and NAD$^+$ are added. Stated in another way, the data indicate the participation of all membrane-bound cytochromes in the oxidation of glucose by O_2.

Properties of the soluble reaction in the oxidation of glucose

By analogy with other cytochrome-linked systems[10] and in agreement with the data above, the role of the soluble fraction appears to be that of catalyzing an enzymatic redox reaction between glucose and NAD$^+$, producing NADH. The rate of appearance of NADH as measured by the increase in absorbance at 340 mμ is dependent upon the concentration of dialyzed supernatant fraction, as indicated in Fig. 3, Curve A. Each point represents the initial rate of reduction observed on adding glucose to a diluted aliquot of the soluble fraction which has been incubated with NAD$^+$ for 5 min. The initial rate of reduction increases linearly with the level of soluble fraction over the range of concentrations used. Substitution of NADP$^+$ for NAD$^+$ gave the results given in Curve B of Fig. 3; the rates of reduction are lower than the rates with NAD$^+$ for each concentration of enzyme used (at a concentration of 1 mg protein per 3 ml assay volume, the NADP$^+$ rate is 0.7 of that with NAD$^+$), but the initial rate of reduction of NADP$^+$ by glucose is also linearly dependent upon the amount of enzyme present.

Glucose 6-phosphate can be utilized as substrate in place of glucose if NADP$^+$ is used as coenzyme. With this combination of substrate and coenzyme the rate of reaction was almost identical to that with free glucose *plus* NADP$^+$ and only slightly lower than the rate with glucose *plus* NAD$^+$. The rates of increase in absorbance at 340 mμ per 15 sec per mg protein were: NADP$^+$ *plus* glucose 6-phosphate, 0.040, NADP$^+$ *plus* glucose, 0.037, and NAD$^+$ *plus* glucose 0.044 in one preparation; the corresponding values were 0.050, 0.056, and 0.078 for a second preparation. The dialyzed soluble preparations used as the enzyme source in the above studies apparently

do not contain significant levels of endogenous substrate that can react with added NAD^+ or $NADP^+$. In neither case is there any significant increase in absorbance at 340 mμ when the aliquot of enzyme is incubated for several minutes with either coenzyme. There is no detectable lag period after adding substrate; absorbance begins to increase linearly immediately upon the addition of substrate, regardless of which coenzyme, NAD^+ or $NADP^+$, which substrate, glucose or glucose 6-phosphate, or which level of enzyme is used.

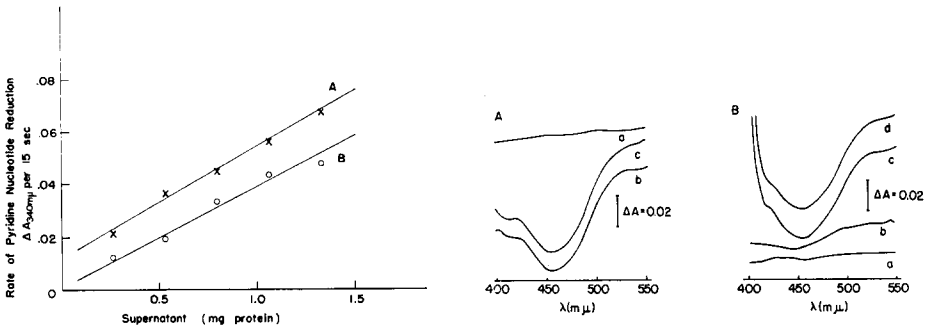

Fig. 3. Dependence of pyridine nucleotide reduction on concentration of soluble fraction. Rates determined at 25°, assay volume, 3 ml. Aliquots of enzyme diluted with 0.05 M phosphate buffer (pH 7.0). 3 μmoles of either NAD^+ or $NADP^+$ and 30 μmoles glucose present in each assay. Curve A, NAD^+ + glucose; Curve B, $NADP^+$ + glucose.

Fig. 4. Spectral changes of soluble fraction on adding glucose and NAD^+. Difference spectra; both cuvettes initially contained only the dialyzed soluble fraction. Sequential additions as described made to sample cuvette only. Samples were protected from light between scans on the Cary spectrophotometer. Samples A and B each contained 13 mg protein per ml. A. Curve a, no additions; Curve b, 60 μmoles glucose (+ 5 min); Curve c, solid $Na_2S_2O_4$. B. Curve a, no additions; Curve b, 3 μmoles NAD^+ (+ 30 min); Curve c, 60 μmoles glucose (+ 15 min); Curve d, solid $Na_2S_2O_4$.

Measurements of O_2 uptake catalyzed by dialyzed soluble fraction *plus* particle fraction are in agreement with the observations above on the specificity of the soluble reaction with respect to glucose 6-phosphate and $NADP^+$, determined spectrophotometrically. These studies have been extended to other related substrates and indicate a very low activity toward maltose and complete lack of activity with glucose 6-phosphate, gluconate, glucuronate, glucose 1-phosphate, galactose, fructose, and *N*-acetylglucosamine, all in the presence of NAD^+.

Spectral studies of the soluble fraction; difference spectra obtained on the addition of glucose and NAD^+.

Substrate-induced spectral changes in the membrane-bound cytochromes present in lysed protoplast preparations have already been described. Using more highly concentrated solutions of the soluble fraction from which the insoluble materials have been removed, spectral changes can also be shown to result from the addition of substrate. Fig. 4A gives the difference spectrum obtained by adding glucose to a dialyzed soluble fraction. The result, Curve b, is a single broad trough with the maximum absorbance change from the untreated sample at 450–460 mμ. Subsequent addition of $Na_2S_2O_4$ shows no further reduction (Curve c is very similar to Curve b)

and indicates that addition of glucose is sufficient to reduce completely the material in the soluble fraction with the spectral properties observed.

If NAD^+ is added to a similar dialyzed soluble fraction, no change is observed as is indicated in Fig. 4B, Curve b. Glucose added after the NAD^+ (Curve c) gives the same spectral changes as glucose alone gave above. The spectrum is unchanged by the subsequent addition of $Na_2S_2O_4$ (Curve d). (In obtaining the difference spectra presented in Fig. 4, the baseline was moved between successive runs in order to prevent overlap of the tracings.) It is concluded that regardless of the presence of added NAD^+, the addition of glucose results in complete reduction of soluble material having the spectral characteristics of a flavin.

DISCUSSION

The extent to which measurements *in vitro* of redox reactions catalyzed by subcellular fractions reflect respiration as it occurs in intact cells is open to question. When it is possible to make measurements of the intact system, these may provide a standard for comparison of the values *in vitro*. WEIBULL[4] has presented data relevant to the work in this paper in his report concerning the isolation of protoplasts from *B. megaterium*. The results of his manometric studies include Q_{O_2} values for intact cells of 68 and 120 in the absence and in the presence of added glucose, and Q_{O_2} values for completely lysed cells of 3 and 27, also in the absence and presence of exogenous glucose. Therefore lysis at WEIBULL's concentration of 5.5 mg bacterial dry weight per 2 ml reaction volume caused a decrease of about 95% in the respiration rate for unsupplemented cells and about 78% for the lysed cells supplemented with glucose as substrate.

The measurements of O_2 uptake by *B. megaterium* whole cells and cell lysates presented here were obtained with an oxygen electrode and made use of a more dilute suspension of cellular materials than WEIBULL's. (The average concentration of the lysed preparation listed in Table I was 1.5 mg protein per 3 ml reaction mixture). With no supplement of substrate, the rate was negligible; with glucose it was measureable but low, only about 2 % of the rate of intact cells.

The marked increase in respiration rate obtained by adding NAD^+ to the lysates has been observed in other cell-free bacterial systems which require both soluble and particulate fractions for O_2 uptake. JONES AND REDFEARN[6] demonstrated a 20-fold increase in the rate of O_2 uptake on the addition of NAD^+ to a cell-free extract of *Azotobacter vinelandii* oxidizing the substrate, β-hydroxybutyrate. A simple possible explanation is that NAD^+ is normally present in relatively low concentrations in a free state in the cytoplasm of the cell and that on lysis of the cell membrane, it is diluted from its original concentration within the cell by a factor dependent upon the volume of solution used for lysis. The dilutions used lower the NAD^+ concentration to a level where it is a limiting factor in the dehydrogenase reaction *in vitro*.

The same reasoning can be applied to the soluble dehydrogenases present in the cytoplasm, provided that they are not originally bound to membranes or associated with other specific cellular constituents that affect their activity. If the enzymatic activity toward a substrate is present in a dilute lysate, and the NADH formed by that enzyme can diffuse to the suspended membrane particles and rapidly undergo

oxidation by O_2 *via* the cytochromes there, the assumption is that *in vivo* the enzyme has the same role.

Calculation of the rate of formation of NADH catalyzed by isolated supernatant fraction (from data in Fig. 3) indicates that the rate of formation of reduced coenzyme is approximately the same on a molar basis as the rate of O_2 uptake catalyzed by lysed protoplasts supplemented with NAD^+ and either glucose or trypticase soy broth. Also, the rate of O_2 uptake of intact cells is only 2.5 times (Table I) that catalyzed by the supplemented lysate preparations. The data are interpreted as evidence for a stable and very active soluble glucose dehydrogenase which when diluted by lysis of intact cells (at least 30-fold *plus* another undetermined factor representing the volume of lysate originally occupied by cells, in the oxygen electrode measurements reported in Table I) is still capable of coupling with the cytochrome chain in the membrane particles to give O_2 uptake at almost half the rate of intact cells.

Many substrates were tried with lysed cell preparations at dilutions where easily measureable oxidation rates were obtained with glucose and NAD^+. No other substrate that involved both soluble and particulate cell fractions, except glucose 6-phosphate in the presence of $NADP^+$ as described in the RESULTS section, was able to support significant rates of O_2 uptake at these concentrations of cellular materials; and none except malate was oxidized by isolated membrane fragments at significant rates compared to the NADH-oxidase activity of the particulate fraction.

As mentioned earlier, $NADP^+$ appears to be able to substitute for NAD^+ in glucose oxidation, giving somewhat lower rates of pyridine-nucleotide reduction and O_2 uptake than NAD^+. And with $NADP^+$ as coenzyme, glucose 6-phosphate will substitute for glucose in both assays as well. However, the combination of glucose 6-phosphate and NAD^+ gives no activity in either assay. This specificity, together with the observation that the dialyzed soluble fraction with no added energy source will oxidize either glucose or glucose 6-phosphate in the presence of coenzyme with no preliminary lag period leads to the interpretation that two active enzymes are present in the soluble fraction, one specific for glucose and one enzyme which will use glucose 6-phosphate and requires $NADP^+$. The reduced pyridine nucleotide produced by both reactions can react with membrane particles to provide measurable rates of O_2 uptake.

The data presented here indicate that in *B. megaterium* the oxidation of glucose need not involve either of the well established pathways, the Embden–Meyerhof or the hexose monophosphate shunt, but may be oxidized without prior phosphorylation by transfer of hydrogen atoms to NAD^+. The oxidation product of glucose, gluconic acid, will not support oxidation in the presence of NAD^+ and the same level of enzyme that readily oxidizes glucose. The data are in agreement with a brief report of the presence of glucose dehydrogenase activity in cell-free extracts obtained by sonic oscillation of *B. megaterium*[11]. In this study, measurements of activity could be made only after NADH-oxidase activity had been removed by treatment with protamine sulfate and $(NH_4)_2SO_4$. Two additional purification steps gave a fraction which indicated an increase in absorbance at 340 mμ on adding glucose and NAD^+, and a decrease on subsequent addition of the product, γ-gluconolactone. The purified enzyme could use either NAD^+ or $NADP^+$, but glucose could not be replaced by any of the sugars tested, although glucose 6-phosphate and maltose were not reported, so a comparison with those activities in the present system cannot be made. The rate of

reduction of the most highly purified fraction was approx. 8.5 times as active as the dialyzed soluble fraction reported in this paper. In another study of glucose metabolism by Bacilli, DEDONDER presented evidence for the reduction of $NADP^+$ by glucose 6-phosphate catalyzed by a cell-free extract of *B. megaterium*; a quantitative comparison of this data with the present work is not possible from the information given[12].

STORCK AND WACHSMAN[13] have made a survey of several enzyme activities in fractions obtained from *B. megaterium*. Their manometric studies indicate that the four most active substrates for O_2 uptake by lysates in the presence of an array of coenzymes and cofactors, including NAD^+ and $NADP^+$, are glucose, citrate, fumarate, and malate. The four substrates give similar rates of O_2 uptake at 36°, which are a little lower than the lowest rates presented in Table I for lysate at 25° supplemented with only glucose and NAD^+. The rates they give are corrected for endogenous rates of O_2 uptake, but the extent of the correction is not given. Of the four most active substrates listed, only two of them, glucose and citrate, require both soluble and particulate fractions for activity; the other two show the highest specific activity in the particle fraction. The difference between their work and the present study, namely our failure to detect activity with citrate equal to glucose activity, may be due to the 10-fold greater dilution of cellular materials in our measurements using the oxygen electrode to measure activity. Or it may reflect the fact that citrate oxidation is not direct and may require a longer incubation time as is inherent in manometric measurements before O_2 uptake can be observed.

The present data clearly indicate that a dialyzed lysed protoplast preparation shows complete reduction of cytochrome components when both glucose and NAD^+ are added; that is, the soluble enzyme system is active enough to keep the pigments reduced even without special precautions against diffusion of O_2 from the air into the sample cuvette. It seems reasonable to assume from this that *in vivo*, where the cytoplasmic enzyme, which produces NADH from glucose and NAD^+, and the insoluble membrane fragments which reoxidize the NADH by O_2 are in much closer proximity, the glucose oxidizing system is at least as active in this respect. Perhaps the generalization that the dehydrogenase is always the rate-limiting step in an oxidation sequence which involves a soluble dehydrogenation reaction coupled to a chain of electron-transport components may not hold for all systems.

This system differs from the particulate cytochrome-linked glucose oxidation system of Pseudomonas species, *Bacterium anitratum* and *Acetobacter suboxydans*[14,15], in which exogenous NAD^+ is not required for reduction of cytochromes. In all of these systems difference spectra obtained by addition of glucose indicate reduction of components giving α-peaks at 555–560 mμ and γ-peaks at 425–428 mμ, characteristic of *b*-type cytochromes. *B. megaterium* used in the present study contains pigments with similar spectral properties but also possesses components with properties of *a*-type cytochromes[8]. Both types of pigments show complete reduction by glucose *plus* NAD^+ in the present study.

The spectral evidence for reduction of a soluble flavin component by substrate does not in itself indicate the role, if any, of this component in respiration of intact cells of *B. megaterium*. The observations are consistent with, but do not prove, the hypothesis that the nondialyzable material is a relatively nonspecific acceptor of reducing equivalents from substrates that support measurable O_2 uptake in a lysed

protoplast preparation, NADH, glucose, glucose 6-phosphate, and malate all give reduction of the same soluble material but at different rates. Presumably the reduced *minus* oxidized difference spectra represent steady states of reduction by substrate and oxidation by O_2; spectra of the reduced flavin-like component indicate that with all of the above substrates, the steady-state level is almost completely reduced. It is concluded that the material is not readily autooxidizable by O_2.

Possibly *in vivo* the unidentified material may act as a reservoir for reducing power under conditions of excess substrate and anaerobiosis; and under aerobic conditions it may become reoxidized by reversal of its formation or by transfer of reducing equivalents to membrane-bound pigments. Calculations from difference spectra indicate about 3 times the amount of flavin present (on a molar basis) as cytochromes of the *a*-type, using published extinction coefficients for flavin[16] and for mammalian cytochrome oxidase[17]. To obtain the difference spectra reported here, it is necessary to use much more highly concentrated soluble fractions than is required to carry out measurements of respiratory activity, either spectrophotometrically, or with the oxygen electrode. Therefore, to test for participation of the substrate-reducible material in respiration, further experiments will have to be carried out with more sensitive techniques for assaying the flavin-like material under conditions where respiration can also be measured.

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