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VITAMIN K-MEDIATED ELECTRON TRANSFER IN *BACILLUS SUBTILIS*

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Abstract

Downey, Ronald J. (University of Notre Dame, Notre Dame, Ind.). Vitamin K-mediated electron transfer in *Bacillus subtilis*. J. Bacteriol. **88**:904-911. 1964.—Electron transfer enzymes were obtained from log-phase cells of *Bacillus subtilis* after aerobic and anaerobic cultivation. The cytochrome content was found to be related to oxygen tension, there being little, if any, cytochrome operative in anaerobic cells. Vitamin K levels in the two cell types did not vary as markedly. A soluble diaphorase-type flavoprotein was obtained from both types of cells which reacted with vitamin K_2, K_3, and certain dyes but not bovine cytochrome *c*. Almost 90% of this diaphorase activity was leached from intact protoplasts without the use of solvating agents or sonic oscillation. Electron transport particles capable of coupled phosphorylation were inhibited by light (360 mμ) or 2,3-dimercaptopropanol (BAL), whereas these had no effect on the diaphorase activity. Phosphorylation in a BAL-inhibited system was restored after addition of the soluble diaphorase from either aerobic or anaerobic cells. The results suggested that soluble flavoprotein components are linked to vitamin K in both fermentative and phosphorylative pathways, and that this segment is indispensable to aerobic and anaerobic respiration in the bacillus.

The oxidation of reduced nicotinamide adenine dinucleotide (NADH) by extracts of bacillus has been demonstrated to occur in both particulate and soluble systems (Doi and Halvorson, 1961; Downey, 1962). Quinone-mediated oxidation of NADH via electron transfer particles (ETP) from *Bacillus stearothermophilus* was demonstrated to bear a commensal relationship to the reaction catalyzed by a soluble flavoprotein component from the same cell (Downey, 1964*a*). The particle-catalyzed oxidation was sensitive to light (360 mμ) or extraction with organic solvents, or both, and could be restored by the addition of certain quinones. Phosphorylation in this system was observed only when coupled to the vitamin K-dependent oxidation of malate. Current studies with the electron transfer system of *B. subtilis* have disclosed that the naphthoquinone which is essential to electron transfer is a vitamin K_2C_{35} and is apparently synthesized to a similar extent by anaerobically and aerobically grown cells (Downey, 1964*b*).

In bacillus, vitamin K has thus far been demonstrated to function only in the respiratory sequence essential to aerobic electron transfer. It was of interest to investigate the role of vitamin K in relation to the flavoprotein respiration which is prevalent when bacilli are grown anaerobically on a complex medium. Evidence suggesting that vitamin K serves as a functional link between a soluble diaphorase-type enzyme and a phosphorylating ETP is presented.

Materials and Methods

Organism and growth medium. *B. subtilis* ATCC 957 was cultured on a complex medium containing 1% Tryptone (Difco); 1% yeast extract; 1% glucose; 0.4% $MgSO_4 \cdot 7H_2O$; 0.02% NaCl; 0.2% $FeSO_4 \cdot 7H_2O$; 0.02% $MnSO_4 \cdot 4H_2O$; and 0.4 ml of HCl per liter. The salts were sterilized as a separate solution and added aseptically to the base medium. Special Pyrex carboys, with a 1.5-in. port near the bottom, were fitted with a sintered-glass sparge head, a vent, and a thermometer. The medium was aerated with filtered air preheated to 37 C. For anaerobic growth, the vessels were filled to the neck with warm sterile medium and tightly stoppered. Upon inoculation of 250 ml of a 10-hr log-phase culture of *B. subtilis*, anaerobiosis was established quickly. All cultivations were conducted at 35 C.

Spores were obtained by growing the organism on a nutrient agar (Trypticase; BBL) surface for 2 weeks at 40 C. Spores were washed from the agar and examined microscopically for the presence of vegetative cells.

Measurement of oxygen tension. The concentration of oxygen in the cultivation medium was

monitored amperometrically with a Clark electrode (Beckman model 160 physiological gas analyzer). The macroelectrode was exposed to ultraviolet light for 3 hr prior to insertion into the low port in the growth vessel. The fluid mass was rotated with a large magnetic stirring device in both anaerobic and aerobic cultivation to assure a continuous renewal of the surface at the electrode.

Enzyme assay. The oxidation of NADH was determined in a manner described previously (Downey, 1962). Diaphorase activity was measured in the same manner, except the reaction was allowed to proceed anaerobically, and 6 μmoles of menadione or 2,6-dichlorophenol indophenol were added to serve as electron acceptor. Phosphorylation, respiratory rates, and flavin analyses were determined as described previously (Downey, Georgi, and Militzer, 1962; Downey, 1962).

Cytochromes. A somewhat crude estimate of cytochromes in various cell-free extracts was achieved by the difference in absorption of the reduced minus the oxidized hemeprotein at 550 mμ for cytochrome *c* and 600 mμ for cytochrome a_3. The heme was reduced with a few crystals of sodium hydrosulfite. The differences were determined in supernatant fluids (20,000 $\times$ *g* for 30 min) of sonically disrupted cells, and were expressed per 100 mg of Folin protein in the fraction.

ETP from B. subtilis. The cells were grown at 35 C for 14 hr and washed three times in cold distilled water. The final sediment was dispersed in 0.1 M NaCl, and the pH of the suspension was adjusted to 7.0 with tris(hydroxymethyl)aminomethane (tris) buffer. The turbid suspension was subjected to sonic oscillation (Downey, 1962), and the mixture was centrifuged at 20,000 $\times$ *g* for 30 min. The resulting supernatant fluid was then centrifuged at 60,000 $\times$ *g* for 60 min. The sediment was suspended in 5 ml of a mixture containing 0.1 M KCl, 0.01 M tris-Cl, and 0.02 M $MgCl_2$, and was centrifuged again at 60,000 $\times$ *g* for 60 min. The sediment was suspended as before, and the fraction was referred to as the ETP_I. The supernatant fluid of the first sedimentation at 60,000 $\times$ *g* was centrifuged at 140,000 $\times$ *g* for 90 min. The supernatant fluid was referred to as the Sol_I fraction, and contained NADH oxidase, diaphorase, and a heat-stable soluble factor, which is essential for optimal phosphorylation. The sediment was resuspended and washed as above. The washed particles (ETP_{II}) oxidized succinate but were incapable of coupled phosphorylation.

Isolation and indentification of vitamin K_2. The physiologically active vitamin K_2 homologue was isolated from vegetative cells of *B. subtilis* by saponification and extraction into petroleum ether (Gale et al., 1962). The golden-yellow residue resulting after vacuum drying was taken into a small volume of petroleum ether and chromatographed on a column (2 by 20 cm) of magnesium alumno silicate. After elution with 2% ether in petroleum ether, the fractions were air-dried and resuspended in a small volume of absolute ethanol. Upon overnight storage at 6 C, yellow crystals formed.

The ultraviolet absorption spectrum of a suspension of crystals in isooctane exhibited maxima at 243.5, 247.5, 259, 268, and a broad peak at 325 mμ. Such a spectrum agreed quite closely with that of synthetic vitamin $K_2(C_{35})$. Such spectral peaks were typical of vitamins of the K_2 series. When chromatographed with other vitamin K isoprenologues on silicone-impregnated paper (Whatman no. 3), the quinone from the bacillus displayed an R_F equal to that of vitamin $K_2(C_{35})$.

Iron analysis. Heme and nonheme iron in the ETP and diaphorase were determined in the manner of Doeg and Ziegler (1962).

Protein. The method of Lowry et al. (1951) was used for protein.

Vitamin K isoprenologues. Vitamin $K_2(C_{35})$ was a gift of O. Isler, Hoffman-La Roche, Inc., Basle, Switzerland; vitamin $K_2(C_{30})$ and vitamin $K_2(C_{45})$ were a gift of A. F. Brodie, University of Southern California Medical School. Other chemicals were obtained as previously reported (Downey, 1962).

RESULTS

The extent to which the cytochromes *c* and a_3 were synthesized by vegetative cells of the bacillus depended upon the oxygen tension in the cultivation medium. In experiments where oxygen tension was varied in the broth cultures, the comparative levels of the above hemeproteins were appreciably affected (Table 1). Aeration rates exceeding 2.5 liters per min resulted in maximal levels of cytochrome per unit dry weight of vegetative cells. Since higher rates of aeration retarded growth on complex medium, the oxygen tension approaching that necessary for maximal

aerobic growth on complex medium was taken as the base line of aerobiosis during each cultivation. The respiratory rates of washed cells from each log-phase culture grown at a lessened oxygen tension declined in a nonlinear fashion. No oxygen consumption was detected during 20 min of glucose dissimilation by washed anaerobic cells.

The vitamin $K_2(C_{35})$ concentration in the various cell types (anaerobic vs. aerobic) was not as markedly affected by aeration. In no case was it observed to be less than 75% of the concentration in normal aerobic cells (Table 1). Spores of *B. subtilis* grown in the manner described contained no detectable quantities of vitamin K or cytochromes.

High-speed supernatant fluids (140,000 × *g* for 90 min) of sonically disrupted cells exhibited NADH oxidase activity which was not sensitive to light (360 mμ) and not stimulated by vitamin K_1 or K_2. This oxidase activity was removed by passage on diethylaminoethyl (DEAE) cellulose (Peterson and Sober, 1956). A diaphorase-type enzyme prevalent in the eluate catalyzed rapid oxidation of NADH with vitamin K_2, K_3 (menadione), or 2,6-dichlorophenol indophenol as electron acceptor. The vitamin K level in the above supernatant fluids (<0.001 mμmoles/mg of protein) was not sufficient to support the oxidative rates which were obtained with or without added menadione as acceptor. The diaphorase activity was isolated from both aerobic and anaerobic cells. It was not sensitive to light (360 mμ) or

TABLE 1. *Influence of oxygen tension on oxidative components of Bacillus subtilis*

Oxygen tension during growth period (mm of Hg)	$K_2(C_{35})$[a]	Cytochrome *c*[b]	Cytochrome a_3[c]	Qo_2[d]
160	0.81	20.0	1.7	135.0
80	0.76	14.6	1.1	46.3
40	0.60	3.1	0.1	2.2
0	0.64	0.1	0.1	0

[a] Expressed as micromoles per gram (dry weight) of cells.

[b] A_{550}-A_{574} per 100 mg of Folin protein in fraction upon reduction with $Na_2S_2O_4$.

[c] A_{600}-A_{610} per 100 mg of Folin protein in fraction upon reduction with $Na_2S_2O_4$.

[d] Glucose oxidation by vegetative cells; expressed as microliters of O_2 per milligram of protein per hour.

TABLE 2. *Iron and flavin content of particulate and soluble oxidative components from mid log-phase cells of Bacillus subtilis*

Fraction	Iron in fraction*			Flavin in fraction*
	Heme Fe	Nonheme Fe	Total Fe	
ETP_I	3.0	5.4	8.4	1.31
ETP_{II}	1.2	4.0	5.2	0.72
Diaphorase (+0)†	0	26.1	26.1	0.25
Diaphorase (−0)‡	0	24.2	24.2	0.19

* Expressed as millimicromoles per milligram of Folin protein.

† Aerobic cultivation; oxygen tension, 160 mm of Hg.

‡ Anaerobic cultivation; oxygen tension, 0 mm of Hg.

acetone-ethanol extraction. No evidence of vitamin K was found in the soluble fraction from *B. subtilis*. The diaphorase preparation did not react with reduced nicotinamide adenine dinucleotide phosphate (NADPH) as substrate or with cytochrome *c* as electron acceptor. Its reaction with vitamin K prompted examination of the iron content, since many oxidases, reductases, and diaphorases bear metal components along with the flavin moiety.

Analyses of the iron content of phosphorylating (ETP_I) and nonphosphorylating (ETP_{II}) particles revealed differences in heme iron on a weight basis (Table 2). Whereas no heme iron was evident in the diaphorase, appreciable quantities of nonheme iron were detected. The ratio of iron to flavin in the ETP was approximately 4:1.

The vitamin K_2 isolated from the bacillus was tested along with other electron acceptors to determine the relative specificity of the diaphorase for these compounds (Table 3). The greatest oxidative rates were observed in the presence of vitamin $K_2(C_{35})$. A comparatively high rate of NADH oxidation was observed with the enzyme from anaerobic cells and with menadione or 2,6-dichlorophenol indophenol as acceptor.

The question arose as to whether the diaphorase represented a normally soluble system or merely a modified flavoprotein fragment of the particulate complex which was released during sonic oscillation. Although flavoprotein bypass reactions are prevalent in facultative anaerobes, the reaction with vitamin K has not yet been elucidated.

Vitamin K seems to be bound to the particulate fraction from which it can be extracted with organic solvents.

Approximately 90% of the diaphorase activity, in which vitamin K_2 and K_3 serve as electron acceptors, was leached from protoplasts by washing. Protoplast suspensions were washed three times in 4 volumes of a mixture containing 0.1% KCl, 0.01 M ethylenediaminetetraacetic acid (EDTA), and 0.67 M phosphate buffer (pH 7.0). Few, if any, active ETP were obtained by centrifugation (120,000 × *g*; 60 min) of these washings.

When the washed protoplasts were osmotically disrupted by sudden stirring in 4.0 volumes of distilled water and centrifuged, as above, very little diaphorase activity was detected in the supernatant fluid. If the washed protoplast fraction was subjected to prescribed periods of sonic oscillation, the magnitude of NADH oxidation catalyzed by the supernatant fluid was a function of the time under sonic treatment (Fig. 1).

Oxidation of NADH by the soluble diaphorase was not sensitive to respiratory inhibitors, some of which proved effective against the activity of the particulate fraction (Table 4). A smaller concentration of amytal than that shown in the table partially inhibited the diaphorase; however, it also stimulated NADH oxidation by the ETP. The oxidation of exogenous NADH by the ETP_I was inhibited by 2,3-dimercaptopropanol (BAL), whereas the oxidation of malate was not. No

TABLE 3. *Effect of various electron acceptors on the oxidation of NADH by the diaphorase from Bacillus subtilis**

Addition	Concn	NADH oxidation†	
		Aerobic	Anaerobic
	µmoles		
None		2	0
Q_6	2.3	0	0
Q_{10}	2.0	4	1
$K_{1(20)}$	1.8	18	20
$K_{2(30)}$	1.4	40	16
$K_{2(35)}$	1.4	93	84
$K_{2(45)}$	1.4	14	12
K_3	2.5	9	153
2,6-Dichlorophenol indophenol	3.0	13	189
TTC	3.0	4	10

* Each cuvette contained 1.0 ml of 0.1 M phosphate buffer (pH 7.0), 0.05 ml of enzyme, 0.45 mg of protein, quinone or dye as stated, in 0.2 ml of absolute ethanol and water to a volume of 2.8 ml. After bubbling with N_2 for 3 min, the reaction was started by addition of 0.72 µmoles of NADH and was followed anaerobically at 37 C. The cuvettes were not gassed or evacuated for the aerobic reaction.

† Expressed as micromoles per minute per milligram of protein.

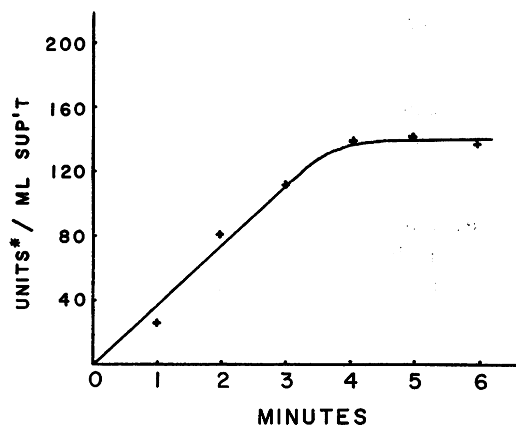

FIG. 1. *Rate release of bound NADH oxidase upon sonic oscillation of washed protoplasts from Bacillus subtilis. Units = millimicromoles of NADH oxidized per minute. Each cuvette contained 1.0 ml of 0.1 M phosphate buffer (pH 7.0), 1.0 ml of electron transfer particles (20 mg of protein), and water to a volume of 2.5 ml. The reaction was started by addition of 0.72 µmole of NADH. The ΔA_{340} was recorded for 3 min at 35 C. The control cuvette lacked substrate.*

TABLE 4. *Effects of inhibitors on NADH oxidation by a soluble and particulate fraction from Bacillus subtilis*

Inhibitor	Concn	NADH-O_2* ETP_I	NADH-vitamin K_2* Sol_I
	M		
None		121	153
NaCN	3.0×10^{-3}	120	149
NaN_3	1.5×10^{-2}	37	150
Antimycin A	2.5×10^{-3}	120	153
BAL†	1.5×10^{-3}	0	144
Amytal Na	2.0×10^{-2}	2	141
p-CMB‡	2.0×10^{-2}	48	126

* Expressed as millimicromoles per minute per milligram of protein.

† 2,3-Dimercaptopropanol.

‡ *p*-Chloromercuribenzoate.

phosphorylation was observed in the presence of BAL with malate as substrate.

The inhibition with BAL was not reversed by washing in buffer, dialysis, or addition of oxidants such as potassium ferricyanide or menadione. The concentration of ETP was varied in proportion to the concentration of the diaphorase to test the affinity, if any, of the latter for BAL. If the diaphorase restored activity by exchanging with the ETP for BAL, then pretreating the diaphorase with an excess of BAL, prior to mixing with the ETP, should retard restoration of the activity which is unique to the ETP.

Phosphorylation was used as an indicator of the integrity of the ETP to assess the extent of restoration or support rendered by the added diaphorase. Phosphorylation was also used to test the possible circumvention of the quinone locus upon addition of the diaphorase. An ETP suspension was irradiated (Brodie and Ballantine, 1960) to establish vitamin K dependency prior to treatment with BAL. The photo-lability of coupled

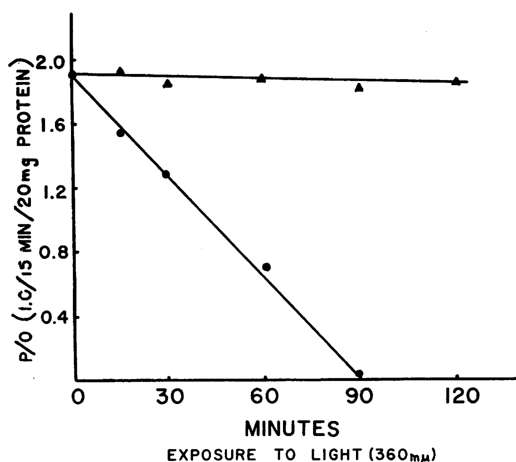

FIG. 2. *Effect of light (360 mμ) on coupled phosphorylation in the electron transfer particles. The reaction vessel contained 0.5 ml of particles (ETP_I), 20 mg of protein, 0.5 ml of Sol_I, 15.6 mg of protein, 15 μmoles of inorganic phosphate, 15 μmoles of $MgCl_2$, 1.0 mg of yeast hexokinase, and water to a volume of 2.0 ml. The sidearm contained 75 μmoles of iodoacetate, 30 μmoles of mannose, 10 μmoles of adenosine diphosphate, 10 μmoles of NAD^+, and 50 μmoles of sodium malate. Oxygen uptake was terminated after 15 min at 35 C with 10% trichloroacetic acid, and inorganic phosphate was determined. Symbols:* ▲, *nonirradiated control;* ●, *irradiated.*

TABLE 5. *Restoration of phosphorylation in 2,3-dimercaptopropanol-inhibited particles by the soluble diaphorase from Bacillus subtilis*[a]

Fraction	Phosphorylation[b]	Oxidation[b]	Phosphorylation/oxidation
$Sol_I(+0)$[c]	<0.1	0	0
$Sol_{II}(-0)$[d]	0	0	0
ETP_I	<0.1	1.28	0
$ETP_I + Sol_I$	0.35	1.52	0.23
$ETP_I + Sol_{II}$	0.11	1.40	0.08
$ETP_I + Sol_I + K_2$	2.37	1.26	1.87
$ETP_I + Sol_{II} + K_2$	2.13	1.96	1.09

[a] The reaction vessel contained additions as in Fig. 2 plus 1.44 μmoles of vitamin $K_2(C_{35})$ where indicated.

[b] Expressed as microatoms consumed per 15 min at 35 C.

[c] Diaphorase from aerobically grown cells.

[d] Diaphorase from anaerobically grown cells.

phosphorylation in the ETP_I is seen in experiments where particles exhibiting a P/O of 1.85 were subjected to light (360 mμ; Fig. 2). Such particles possessed a demonstrated dependence on vitamin K_2, since the latter restored at least 75% of the original phosphorylative capability.

The diaphorase which was treated with BAL prior to addition to the BAL-blocked ETP mixture restored oxidative and phosphorylative activity to the ETP (Table 5). The restoration was effected by a diaphorase from either aerobic or anaerobic cells. If vitamin K was omitted from the irradiated, BAL-blocked ETP preparation, the activity was not restored.

These results suggest that the site of BAL inhibition precedes the site of diaphorase coupling in the respiratory chain. The restoration was also vitamin K-dependent, since no phosphorylation was observed without the quinone. Observations in which a nonphosphorylating system can be coupled to a BAL-inhibited ETP and a phosphorylating entity result might well be an artifact. In view of these data, a schematic diagram of the electron transfer chain of the *B. subtilis* system would seem premature.

In any case, certain vitamin K-linked respiratory components seem to be utilized by both aerobic and anaerobic cells. The relationship of the quinone to the amino acid fermentation occurring anaerobically in the absence of glucose is presently under investigation.

DISCUSSION

The data suggest that under anaerobic conditions *B. subtilis* exhibits a lower respiratory activity and synthesizes little, if any, cytochrome. This contradicts the report of Schaeffer (1952), in which a cytochrome deficiency was noted in anaerobically grown *B. cereus*, whereas no impairment of glucose dissimilation was detected. The same author reported that glucose dissimilation remained as cyanide-sensitive in anaerobic cells as in aerobic cells. Whereas *B. stearothermophilus* was shown to exhibit a cyanide-sensitive respiration (Downey, 1962), the pathway in *B. subtilis* appears to be insensitive to cyanide and sensitive to azide. The same observations were reported earlier with whole-cell preparations of *B. subtilis* (Gary and Bard, 1952).

Although no general statement can be made regarding the level of heme components in relation to oxygen tension in the aerobic sporeformers, it would appear that availability of oxygen directly influences the synthesis of cytochrome. If glucose is made available to anaerobic cultures of *B. subtilis*, a homolactic fermentation ensues, whereas in the absence of oxygen amino acids are fermented with the subsequent production of ammonia and no gas (Downey and Sundstrom, *in preparation*). It is possible that the flavoprotein oxidases function in the bacillus with or without the cytochromes. Given the same medium and inoculum, growth as measured by dry weight is three times greater aerobically than anaerobically. No ETP as such are attainable from anaerobically grown cells. Whether or not cytochrome synthesis in the bacillus is induced by oxygen remains to be shown.

The presence of near aerobic levels of vitamin K in anaerobic cells of bacillus would attest to the importance of this compound in a role perhaps different from the demonstrated flavin to heme transfer in the ETP from aerobic cells. Although naphthoquinone was not evident in the soluble fraction from vegetative cells, its ability to act as electron acceptor for the diaphorase suggested that oxidations initiated in the soluble components of the cell may enter the respiratory chain through the quinone.

Although no evidence for vitamin K in the soluble fraction was found, appreciable levels of NADH oxidase activity were detected. Oxidation of NADH by this fraction was observed to proceed via two systems after resolution on DEAE cellulose. The first enzyme, a NADH oxidase, was not stimulated by vitamin K and reacted with mammalian cytochrome *c*. The second enzyme reacted only with NADH, was stimulated by vitamin K_1, K_2, or certain dyes, and did not react with mammalian cytochrome *c*. Since this enzyme exhibited broad acceptor reactivity, it is referred to in this report as a diaphorase, as opposed to a reductase. It is interesting to note that a similar preparation from *B. stearothermophilus* readily reduced beef heart cytochrome *c*.

The iron to flavin ratio of 4:1 in the ETP of *B. subtilis* appears closely related to that of animal diaphorase (Slater, 1950). The role of iron in the system with vitamin K as acceptor is not clear. It is possible that iron may interact with inorganic iron and iron-riboflavine chelates (Mahler, Fairhurst, and Mackler, 1955; Weber, Lenhoff, and Kaplan, 1954), in which case vitamin K reactivity with the diaphorase would be an effective bypass. However, until a functional link between vitamin K and cytochromes can be attributed to a specific enzyme, the role of quinone in relation to inorganic iron remains an enigma. An enzymatic reduction of the natural cytochrome *c* in the ETP_I from the bacillus has been observed when incubated with malate or succinate. It is possible that exogenous beef heart cytochrome is not available to the particle-bound flavoprotein.

Although the greatest diaphorase activity was observed with vitamin $K_2(C_{35})$ as acceptor, we have recorded assays in which vitamin $K_2(C_{30})$ or vitamin K_1 have performed nearly as well. The comparatively high activity with menadione or 2,6-dichlorophenol indophenol as acceptor is typical for these cell-free systems. Failure to narrow the acceptor reactivity by passage on DEAE cellulose is puzzling. This would seem to be characteristic of a diaphorase, as opposed to a reductase which might catalyze a closely allied reaction.

It is difficult to assess the role of the quinone bypass reactions if indeed they exist in the intact cells. It is, however, interesting that the bulk of diaphorase activity can be leached from protoplasts of *B. subtilis*. Although vitamin K_2 remains a functional acceptor for this soluble diaphorase, it is nevertheless tightly bound to the ETP and is distributed with it upon fractionation of physically disrupted cells or protoplasts. Sonic oscillation of washed protoplasts apparently released

the membrane-bound NADH oxidase activity in the form of discrete particulate material. Repeated attempts to fragment further the ETP with deoxycholate (Crane and Glenn, 1957) and digitonin (Cooper and Lehninger, 1957) failed to yield an active flavoprotein or cytochrome component.

The restorative behavior of the soluble diaphorase, as reported here, would support the notion that normally soluble oxidative components, which may contribute heavily to reoxidation of NADH, possess the ability to interact with particulate catalysts when metabolic circumstances warrant it.

Further investigation with other specific inhibitors may reveal the nature of the quinone-mediated electron transfer in facultative microorganisms. A soluble fraction was shown to be essential for oxidative phosphorylation in cell-free systems from *E. coli* (Kashket and Brodie, 1963). A particulate NADH oxidase isolated from *Mycobacterium tuberculosis* was shown to require a naphthoquinone for activity (Segel and Goldman, 1963). The enzymatic reduction of vitamin $K_2(C_{45})$ by NADH, and the solubilization of the enzyme system responsible for this reduction, was reported by Kusunose and Goldman (1963). In addition to the naturally occurring naphthoquinone, at least one additional factor is necessary for restoration of oxidation of NADH in ETP from *M. phlei* (Weber and Rosso, 1963).

Evidence that vitamin K behaves as a functional intermediate between flavoprotein oxidases and the terminal members of the respiratory chain is accumulating. It may logically be expected that the study of soluble and particulate oxidative phenomena in the cell will be intensified, because the key to coupled phosphorylation in microbial and animal systems will depend on it.

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