MECHANISM OF DIPICOLINIC ACID STIMULATION OF THE SOLUBLE REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE OXIDASE OF SPORES[1]

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Received for publication October 13, 1960

The presence of dipicolinic acid in bacterial spores has aroused much interest in its possible role in dormancy. It has been suggested that dipicolinic acid forms a part of the spore cortex (Mayall and Robinow, 1957), that a dipicolinic acid-calcium-protein complex stabilizes the spore against physical agents (Powell, 1953; Young, 1959), that dipicolinic acid is a factor in heat resistance (Church and Halvorson, 1959) and X-ray irradiation resistance (Woese, 1959), and that it is an activator of spore enzymes during germination by a chelate mechanism (Harrell, Doi, and Halvorson, 1957).

The observation of Harrell (1958) that dipicolinic acid stimulated glucose and DPNH[2] oxidation in spore extracts threefold suggested that dipicolinic acid played a role in regulating spore metabolism. In preliminary studies, Halvorson, Doi, and Church (1958) found that it stimulated DPNH cytochrome *c* reductase and diaphorase of spores and vegetative cells. A more thorough examination of the electron transport system of spores (Doi and Halvorson, 1961) has made it possible to find a specific site of action of dipicolinic acid in relation to its observed stimulation of oxygen uptake in crude extracts. The present results demonstrate that it stimulates the soluble DPNH oxidase of spores and suggest that its mode of action is via an electron accepting mechanism.

MATERIALS AND METHODS

The spores of *Bacillus cereus* strain T (formerly called *Bacillus cereus* var. *terminalis*) were used throughout this study. The methods for preparing and heat activating spores have been previously reported by Church, Halvorson, and Halvorson (1954) and Church and Halvorson (1957). Spore extracts were prepared by the colloid mill method of O'Connor, Doi, and Halvorson (1960).

Oxygen uptake was measured by conventional Warburg manometric techniques. Protein was measured by the method of Lowry et al. (1951) using serum albumin as the standard. Soluble DPNH oxidase activity was measured spectrophotometrically at 340 mμ at 25 C. The reaction mixture contained 100 μmoles potassium phosphate (pH 7.4), 0.1 μmole of DPNH, enzyme, plus additions as indicated in a total volume of 1.0 ml. The reference cuvette held the same materials except only 0.05 μmole of DPNH and no enzyme.

Diketopimelic acid was synthesized from the commercial sodium salt of diethyloxaloacetate (Eastman Organic Chemicals) by the method of Cope and Fournier (1957). The melting point of the crystalline compound was 127 to 130 C (the literature melting point was 127.8 to 129.6 C (Cope and Fournier, 1957)). FMN and DPNH were products of Sigma Chemical Company. Atabrine was a product of Winthrop-Sterling Company.

The purification procedure for the soluble DPNH oxidase is presented in Table 1. The crude fraction was freed of glass beads and debris by centrifugation at 10,000 $\times$ *g* for 30 min. To this crude fraction was added streptomycin to a final concentration of 1.5%. The preparation was centrifuged at 144,000 $\times$ *g* to remove the precipitated nucleic acids and other particulate matter. An ammonium sulfate fractionation at 0.4 to 0.7 saturation contained most of the oxidase activity. After dialysis against 0.001 M phosphate buffer, pH 7.4, the yellow enzyme preparation was placed on diethylaminoethyl column I (1.8 by 35 cm). Fractions (5 ml each) 150 to 175 of this column were concentrated by

[1] This research was supported in part by an Institutional research grant from the American Cancer Society and a grant-in-aid from the Wisconsin Alumni Research Foundation.

[2] The following abbreviations will be used: DPNH, reduced diphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; FMN, flavin mononucleotide; FAD, flavin adenine nucleotide; EDTA, ethylenediaminetetraacetate; and tris, tris(hydroxymethyl)aminomethane.

TABLE 1

Purification of the soluble DPNH oxidase of spores

Fractions	Volume	Total Units*	Total Protein	Specific Activity
	ml		*mg*	*units/mg protein*
Crude	233	403	2560	0.16
Streptomycin-(Spinco)	225	471	2020	0.23
Ammonium sulfate, 0.4–0.7	26	352	755	0.47
Diethylaminoethyl I	4.8	139	67.2	2.07
Diethylaminoethyl II	2	45.5	8.7	5.23

* One unit equals 1 μmole of DPNH oxidized per min. The assay system contained 0.2 μmole DPNH, 0.1 μmole FMN, 100 μmoles phosphate buffer (pH 7.3), and enzyme in 1.0 ml. The reference cuvette held 0.1 μmole DPNH, 0.1 μmole FMN, 100 μmoles phosphate buffer, and water in 1.0 ml. Change in optical density at 340 mμ was followed at room temperature.

placing the fractions in a dialysis tubing and dialyzing against a volume of saturated ammonium sulfate that would give a final saturation of 0.7. The precipitate in the dialysis tubing was resuspended and dialyzed against phosphate buffer. This preparation was then placed on diethylaminoethyl column II (1 by 25 cm). Fractions (5 ml each) 58 to 64 were dialyzed against saturated ammonium sulfate as mentioned above. This final preparation had a specific activity of 5.2. The highest specific activity obtained was 7.2. The enzyme had absorption peaks at 455, 405, and 374 mμ. The highest activity was at pH 7.4 and the enzyme was quite stable at this pH at 0 to 4 C. Some of the other properties of this enzyme have been reported (Doi and Halvorson, 1961).

RESULTS

Dipicolinic acid stimulation of the soluble oxidase. During the study of the electron transport system of spores (Doi and Halvorson, 1961), it was found that spores contained a particulate and a soluble DPNH oxidizing system and that dipicolinic acid stimulated only the soluble DPNH oxidase. FMN addition was necessary for full activity of the soluble oxidase, but dipicolinic acid was able to partially replace

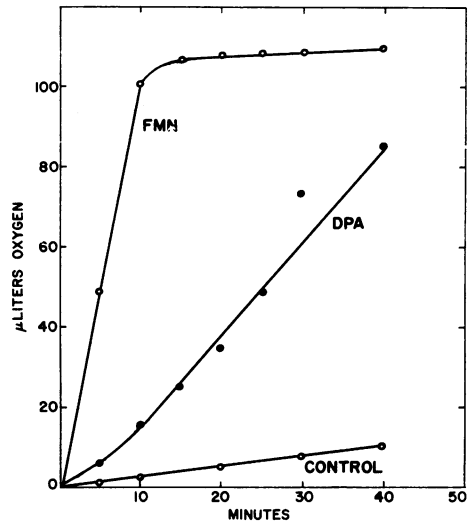

Fig. 1. FMN and dipicolinic acid (*DPA*) stimulation of the soluble DPNH oxidase of spores of *Bacillus cereus*. For dipicolinic acid stimulation, the Warburg cup contained: DPNH, 10 μmoles; dipicolinic acid, 50 μmoles; phosphate buffer (pH 7.4), 200 μmoles; enzyme, 0.64 mg; and water for a total volume of 1.8 ml. The control contained the same except for enzyme. For FMN stimulation, the Warburg cup contained: DPNH, 10 μmoles; FMN, 0.2 μmole; phosphate buffer (pH 7.3), 200 μmoles; enzyme, 0.26 mg; and water for a total volume of 1.8 ml. The control contained the same materials except for enzyme. The enzyme control contained: DPNH, 10 μmoles; phosphate buffer (pH 7.4), 200 μmoles; enzyme, 0.64 mg; plus water to 1.8 ml. The reaction temperature was 30 C. The inner well contained 0.2 ml of 20% KOH.

FMN. This is illustrated in Fig. 1. The rate with FMN was 384 μl of oxygen uptake per 10 min per mg protein. The rate with dipicolinic acid was 31 μl of oxygen uptake per 10 per mg protein. Both oxygen uptake studies and spectrophotometric studies after DPNH oxidation at 340 mμ gave identical results.

To show that the decrease in optical density at 340 mμ during spectrophotometric studies was due to DPNH oxidation and not to DPNH degradation, DPN was recovered from the reaction mixture by paper chromatography (Kaplan and Stolzenbach, 1957). After elution of the DPN spot from the paper with 0.01 M phosphate buffer, pH 7.4, the DPN was tested for its substrate activity with alcohol dehydrogenase and for the formation of a DPN-CN complex

TABLE 2

Recovery of DPN from soluble DPNH oxidase assay

Assay	DPNH in Oxidase Assay	DPN Recovered	Recovery
	μmole	*μmole*	%
Alcohol dehydrogenase	0.64	0.57	89
Potassium cyanide	0.64	0.58	91

Alcohol dehydrogenase assay contained 100 μmoles phosphate buffer (pH 7.4), 10 μmoles ethanol, 10 μmoles semicarbazide, 0.22 mg of alcohol dehydrogenase, and 0.6 ml of the paper chromatogram eluate in a total volume of 1.0 ml. Optical density at 340 mμ was followed until the reaction stopped. The increase in optical density from the start of the experiment to the end was used as the change in optical density. The extinction coefficient of 6.2 mM was used for DPNH.

KCN assay contained 100 μmoles phosphate buffer (pH 7.4), 500 μmoles of KCN, 0.6 ml of the paper chromatogram eluate, and water in a total volume of 1.0 ml. The optical density at 325 mμ was read before and 5 min after the addition of KCN. The blank contained the same materials except for the eluate. Correction for eluate absorption was used in determining the optical density for the test cuvette. The extinction coefficient of 6.3 mM was used for the DPN-CN complex.

(Ciotti and Kaplan, 1957). The recovery of DPN by the alcohol dehydrogenase method was 89%, whereas recovery by the use of KCN was 91% (Table 2). The spectrum of the paper eluate had a sharp absorption peak at 260 mμ, indicative of DPN. After reduction with alcohol dehydrogenase, the spectrum showed a new peak at 340 mμ, the absorption maximum for DPNH. These results showed that DPNH was oxidized and not degraded.

Since dipicolinic acid is a good chelating agent (Powell, 1953), it was thought that the dipicolinic acid stimulation was due perhaps to a removal of an inhibitory metal; however previous dialysis of the soluble oxidase against EDTA, tris-thioglycolate, or dipicolinic acid did not diminish stimulation of the enzyme (Table 3). Dipicolinic acid still stimulated the oxidase 2- to 4-fold after dialysis treatment suggesting that a mechanism other than chelation was involved.

Cofactor requirement for the soluble oxidase. Without the addition of FMN to the assay system, enzymic activity was low but significant. To determine the cofactor requirement of the enzyme, the flavin was completely stripped from the enzyme by the acid ammonium sulfate treatment of Warburg and Christian (1938). The deep yellow enzyme preparation was rendered a pure white color after the treatment. Table 4

TABLE 3

Stimulation of soluble DPNH oxidase of spores of Bacillus cereus by dipicolinic acid after dialysis

Dialysis Treatment	Control*	+DPA*	+EDTA*	DPA/Conrtol
None	1.14	2.15	0.93	1.9
TTG; TTG†	0.93	2.40	1.14	2.6
DPA; TTG†	0.64	2.15	0.86	3.3
EDTA; TTG†	0.12	0.50	0.14	4.1

* DPNH μmoles oxidized per min per mg protein × 10^3.

†TTG = 0.05 M tris-thioglycolate buffer (pH 7), DPA = 0.05 M dipicolinate (pH 7), and EDTA = 0.05 M ethylenediaminetetraacetate (pH 7).

The enzyme was dialyzed against the chelating agents for 12 hr.; then dialyzed against TTG buffer for 12 hr to remove the chelating agents.

The reaction mixture contained 0.1 μmole of DPNH, 100 μmoles of phosphate buffer (pH 7.3), 25 μmoles dipicolinic acid or 25 μmoles of EDTA, enzyme, plus water for a total volume of 1.0 ml. Reaction temperature was 25 C.

TABLE 4

Cofactor requirement for the soluble DPNH oxidase after removal of flavin

Cofactor Added	Final Concn	Rate*
None		0
FMN	5×10^{-5} M	26.3
FAD	5×10^{-5} M	18.3
Dipicolinic acid	5×10^{-3} M	0.3
FMN + dipicolinic acid	As above	19.6

* Change in optical density at 340 mμ per min × 10^2.

Sample cuvette contained 0.2 μmole DPNH, 100 μmoles phosphate buffer (pH 7.4), enzyme, plus cofactors as indicated above and water for a total volume of 1.0 ml. Reference cuvette contained 0.1 μmole DPNH, 100 μmoles phosphate buffer (pH 7.4), plus cofactors as indicated above, and water for a total volume of 1.0 ml. Reaction run under air at 30 C.

TABLE 5

Dipicolinic acid-FMN interaction with the soluble DPNH oxidase of spores of Bacillus cereus

Additions	Specific Activity*		Inhibition By Atabrine
	−Atabrine	+Atabrine	
			%
Control	0.33	0.26	22
+ Dipicolinic acid	1.66	0.80	52
+ FMN	5.31	2.39	55
+ dipicolinic acid + FMN	4.25		

* DPNH μmoles oxidized per min per mg protein × 10^2.

Reaction mixture contained 0.1 μmole DPNH, 0.1 ml enzyme, 100 μmoles phosphate buffer (pH 7.4), and 2.5 μmoles dipicolinic acid, 0.1 μmole FMN, or 0.1 μmole atabrine. Reaction temperature was 25 C. Change in optical density at 340 mμ was followed.

gives the results of the reactivation of the enzyme by cofactors. There was no activity without addition of cofactors. FMN addition resulted in the greatest reactivation, whereas FAD gave only 70% of the rate of FMN. Dipicolinic acid stimulated the enzyme only slightly. However, the addition of FMN and dipicolinic acid to the apoenzyme decreased the rate of activity suggesting a competition for the enzyme.

Atabrine inhibition of the soluble DPNH oxidase. Atabrine, a flavin analogue, inhibits both FMN and dipicolinic acid stimulation of the soluble oxidase. In Table 5 it is demonstrated that atabrine inhibits the reactions about 50%. Another interesting observation shown in Table 5 is the interaction between FMN and dipicolinic acid. It is apparent that the rate of DPNH oxidation with both of these compounds is less than the rate with FMN alone. If both compounds are acting on the same site, and if dipicolinic acid can substitute as a cofactor for FMN, then the stimulation should be competitively inhibited by atabrine. That this is the case is shown in Fig. 2. Atabrine competitively inhibits both dipicolinic acid and FMN stimulation. The inhibitor constants for atabrine determined by using dipicolinic acid and FMN are 2.2 × 10^{-6} M and 5.5 × 10^{-6} M, respectively, the difference probably being insignificant. These results suggest that dipicolinic acid is acting on or near

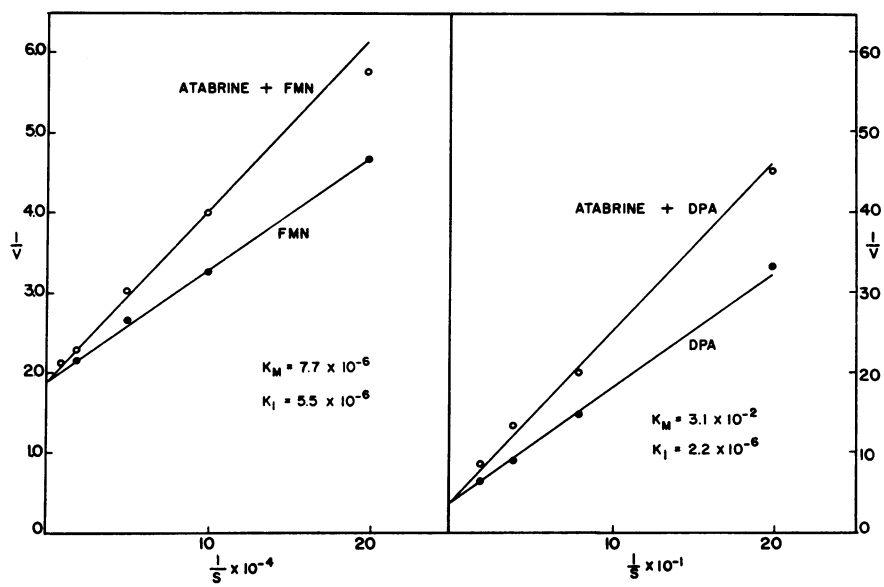

Fig. 2. Competitive inhibition of FMN and dipicolinic acid (*DPA*) by atabrine on the soluble DPNH oxidase. The reaction mixture contained: phosphate buffer (pH 7.4), 100 μmoles; DPNH, 0.1 μmole; enzyme; 6 μg plus varying concentrations of dipicolinic acid and FMN in a total volume of 1.0 ml. The atabrine content for competition with FMN was 0.005 μmole and with dipicolinic acid was 0.005 μmole. The reference cuvette held the same materials except only 0.05 μmole DPNH and no enzyme.

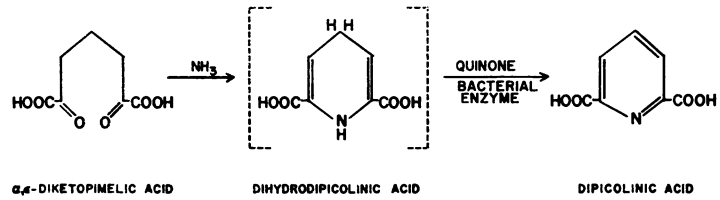

Fig. 3. Synthesis of dipicolinic acid from 2,6-diketopimelic acid (Powell and Strange, 1959)

the site of FMN action and that it may be an electron acceptor.

Dihydrodipicolinic acid. A reduced form of dipicolinic acid has been postulated by Powell and Strange (1959) as illustrated in Fig. 3. They observed that 2,6-diketopimelic acid, ammonia, and an oxidizing system gave oxygen uptake with production of dipicolinic acid. The stoichiometry of the reaction made dihydrodipicolinic acid the logical intermediate.

These results were confirmed by using the soluble DPNH oxidase of spores as the oxidizing system. Dipicolinic acid was isolated from the reaction mixture by ascending paper chromatography using equal volumes of 95% ethanol and 0.1 M acetic acid as solvent. The spectrum of the isolated compound was identical to authentic dipicolinic acid (Powell, 1953). Fig. 4 demonstrates the oxygen uptake with soluble DPNH oxidase as the oxidizing enzyme. Flavin requirement is illustrated by the lack of oxygen uptake when oxidase from which flavin has been removed is used. FMN alone is able to substitute for the enzyme. These results suggest that some reduced compound, probably dihydrodipicolinic acid can either be oxidized by the spore oxidase or a suitable electron acceptor as FMN.

Many attempts to reduce dipicolinic acid and isolate dihydrodipicolinic acid have been unsuccessful. The low solubility of dipicolinic acid in both aqueous and organic solvents caused difficulty in finding the appropriate reducing medium. Attempts to reduce dipicolinic acid with sodium borohydride and aluminum chloride in diglyme at various temperatures (0 to 75 C) by the method of Brown and Subba Rao (1956) resulted only in the formation of a bright orange borohydride complex. Also no reaction occurred when sodium hydrosulfite was added to dipicolinic acid in a sodium carbonate solution under nitrogen at 4 C (Mumm and Beth, 1921). Several modifications of these methods using different solvent systems were carried out without success.

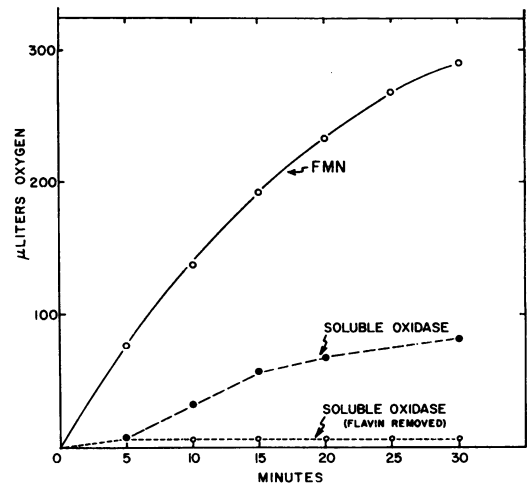

Fig. 4. The flavin requirement for the oxidation of 2,6-diketopimelic acid. The Warburg cups contained: 2,6-diketopimelic acid, 45 μmoles; ammonium chloride, 100 μmoles; phosphate buffer (pH 7.4), 200 μmoles; or FMN, 0.2 μmole; soluble oxidase, 0.9 mg; soluble oxidase with flavin removed, 0.5 mg; plus water for a total volume of 1.8 ml. The reaction was run at 30 C. The inner well contained 0.2 ml of 20% KOH.

Isolation of dihydrodipicolinic by ring closure of 2,6-diketopimelic acid with ammonia has been attempted. When diketopimelic acid, ammonia, and phosphate buffer are incubated at room temperature, the resulting solution has an absorption spectrum with peaks at 270, 280, 240, and 380 mμ. The reaction is complete within 30 min and proceeds most rapidly under alkaline conditions. The 270 mμ peak is characteristic of dipicolinic acid. The other peaks may be due to the reduced form of dipicolinic acid or side products. Paper chromatography of the reaction mixture results in several ultraviolet absorbing spots; however, none of those spots gives rise to a material that can be oxidized by FMN or the enzyme. Several ultraviolet absorbing fractions are obtained when the reaction mixture is eluted

from a Dowex 1-formate column. The compounds in these fractions were not oxidized by the enzyme. If in the reaction mixture of Fig. 4, either the oxidase or FMN is omitted, or if the enzyme from which the flavin has been removed is used, no oxygen uptake occurs; however, dipicolinic acid is still formed in the reaction mixture. Thus it appears that a dismutation of dihydrodipicolinic acid occurs resulting in dipicolinic acid and perhaps tetrahydrodipicolinic acid. If this is the case, one would expect that dihydrodipicolinic acid will be most difficult to isolate. Work is still in progress for the isolation of the reduced dipicolinic acid.

DISCUSSION

The large quantity of dipicolinic acid in bacterial spores has been adequately substantiated (Powell, 1953; Perry and Foster, 1955); however, its role remains obscure. Evidence presented here indicates that dipicolinic acid has a significant effect on the soluble DPNH oxidase of spores. The results demonstrate that it does not act simply by chelation of an inhibitory metal, but acts as a cofactor to the soluble oxidase. The atabrine inhibition studies point to some common site of activity for dipicolinic acid and FMN. When flavin is completely removed from the enzyme preparation, dipicolinic acid will not reactivate the enzyme; however, it will still compete with FMN for the apoenzyme.

The facts that dipicolinic acid will not reactivate the enzyme after flavin removal and that flavin is required for oxidation of dihydrodipicolinic acid suggest that a mechanism more complex than a cofactor role of dipicolinic acid on one enzyme exists. One hypothesis is that two flavin compounds are involved in DPNH oxidation by dipicolinic acid—a DPNH aerodehydrogenase and a flavoprotein which oxidizes reduced dipicolinic acid (Fig. 5). The DPNH aerodehydrogenase acting with added FMN catalyzes the transfer of hydrogen from the substrate to flavin, this in turn being reoxidized by air (Bentley, 1955). With dipicolinic acid, the aerodehydrogenase catalyzes the transfer of hydrogen from DPNH to dipicolinic acid. The reduced dipicolinic acid is oxidized nonspecifically by another flavoprotein. Exogenously added FMN and dipicolinic acid are cofactors and competitors for the DPNH apo-aerodehydrogenase. This hypothetical pathway explains the

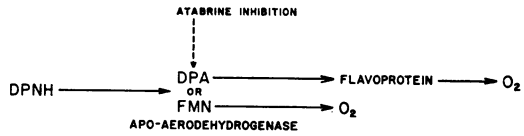

Fig. 5. The pathway of DPNH oxidation with dipicolinic acid (*DPA*) as cofactor.

competition of dipicolinic acid and FMN for a common enzyme site and the flavin requirement for dipicolinic acid oxidation. The requirement for exogenous FMN or dipicolinic acid for aerodehydrogenase may stem from the removal of the loosely bound cofactor during extract preparation. Dipicolinic acid in vivo, would serve as the cofactor for this apoenzyme.

Since the soluble oxidase is the primary pathway of electron transport in spores during dormancy and germination (Doi and Halvorson, 1961), the stimulation by dipicolinic acid of this system can play an important role in dormancy and morphogenesis. Although spore respiration is undetectable (Spencer and Powell, 1952), it is difficult to conceive of a biological system which is metabolically inert. A basal level of energy would appear to be necessary for maintenance of structural and physiological integrity. Although oxygen uptake does not occur, it appears possible that metabolism does take place at a low rate and that dipicolinic acid may be acting as an internal electron sink. This capacity would explain the substrate levels of dipicolinic acid found in spores. To test this hypothesis would require an analysis of dormancy and spontaneous germination in spores with different amounts of internal dipicolinic acid and to see whether dormancy is broken earlier in those spores with less dipicolinic acid. Dipicolinic acid in vivo would accept electrons through the mediation of the soluble oxidase during dormancy; then during germination the reduced form would be oxidized and serve as a cofactor for the oxidase until the particulate vegetative system was synthesized. Further studies on this working hypothesis should produce evidence of its feasibility.

ACKNOWLEDGMENTS

The authors wish to express their appreciation to Lucile Smith of the Dartmouth Medical School and to Jens G. Hauge for their advice and helpful discussions.

SUMMARY

Kinetic and cofactor studies strongly suggest that dipicolinic acid activates the soluble reduced diphosphopyridine nucleotide (DPNH) oxidase of spores by an electron accepting mechanism. The flavin requirement for oxidation of dihydrodipicolinic acid places dipicolinic acid between DPNH and flavin in the electron transport sequence. A method for purification and some properties of the soluble DPNH oxidase of spores are presented. The significance of the dipicolinic acid stimulation of the soluble oxidase is discussed in relation to spore morphogenesis.

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