*DORMANCY OF BACTERIAL ENDOSPORES: REGULATION OF ELECTRON TRANSPORT BY DIPICOLINIC ACID**

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Communicated by Kenneth Raper, October 27, 1958

One of the most common features in the metamorphism of diverse biological systems is the conversion of these systems into a quiescent, dormant state. Although the properties of these dormant states have in general been well described (inert or weak metabolic activity and resistance to external chemical and physical factors), there is relatively little information on the internal regulatory mechanisms involved in either maintaining the dormant state or elevating its conversion to a metabolically active form.

In microbes these stages are well illustrated by the processes of sporulation and germination of bacterial endospores. The mature endospore is a highly refractile body which is resistant to heat and chemical agents and, although it contains several enzymes, is inert metabolically. Activation evokes a latent carbohydrate metabolizing system, a number of individual enzymes, and prepares the system for a triggered germination with loss of heat resistance upon the addition of simple germinating agents.[1] These observations, together with the dependence of over-all germination on metabolic activity,[2] suggest an enzymic regulatory mechanism for dormancy.

A priori one would expect this regulatory mechanism to reflect some fundamental chemical or biochemical difference between the dormant and vegetative states. Two such dramatic differences are the unique presence in spores of DPA[1, 3] and elevated levels of divalent metals.[4, 5] Our attention was directed toward these components as playing a role in dormancy, since (i) DPA synthesis and divalent metal uptake are essential for, and immediately precede, the production of resistant spores;[5, 6] (ii) activation of dormant spores is associated with the partial release into the medium of DPA salts;[7] (iii) germination parallels the complete release of DPA.[7, 8]

The recent demonstration that DPA stimulates the rate of oxygen uptake from glucose oxidation in spore extracts[9] and an analysis of the pathway of glucose metabolism[10] provided a basis for examining the regulatory role of DPA on spore metabolism. The present experiments were designed to classify further the basis of the DPA stimulatory effect.

MATERIALS AND METHODS

Spores of *Bacillus cereus* var. *terminalis* were used throughout this work. The methods employed for the preparation of washed spores, heat activation, and the preparation of spore extracts (1 gm spores/10 ml buffer) have been previously described.[2] To maintain reducing conditions during breakage of the spores, a steady stream of N_2 was included in the chamber of the Raytheon 10-kc. sonic oscillator. Unless otherwise indicated, 0.1 *M* glycylglycine, pH 7.3, was employed as buffer.

Oxygen uptake was measured by standard Warburg methods at 30°. Protein was determined by the method of Lowry *et al;*[11] using serum albumin as a standard. ATP and ADP hydrolysis was measured by following the rate of release of inorganic phosphate by the method of Fiske and SubbaRow.[12]

Diaphorase and cytochrome c reductase activities of the preparation were both measured spectrophotometrically at 30° with a DK2 recording spectrophotometer.[13] Diaphorase activity was followed by the rate of oxidation of DPNH by ferricyanide at 410 mμ and cytochrome c reductase by the rate of reduction of cytochrome c by DPNH at 550 mμ.

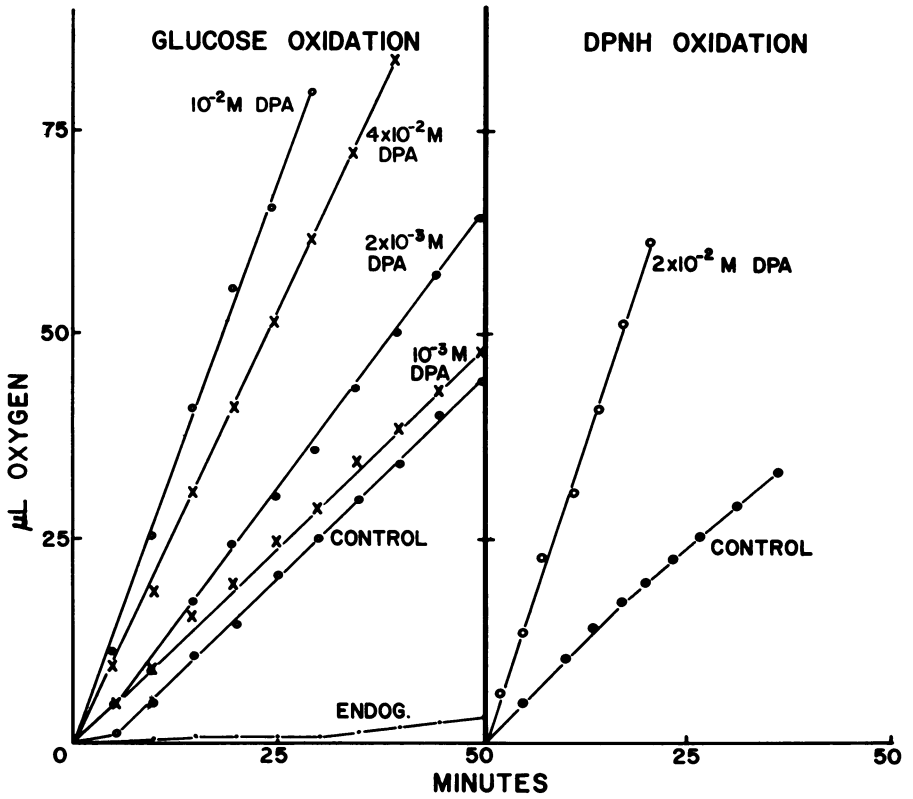

FIG. 1.—Stimulation of glucose oxidation by DPA. The Warburg vessels contained: glycylglycine buffer, pH 7.3, 75 μmoles; enzyme fraction with 4 mg. protein; DPA as indicated and (*a*) glucose, 20 μmoles; DPN, 0.7 μmoles; (*b*) DPNH, 10 μmoles. Final volume 1.8 ml. Center well contained 0.2 ml. of 20 per cent KOH. Incubation temperature, 30°.

In the assay for diaphorase activity the experimental cuvette contained 20 μmoles glycylglycine (pH 7.3), 0.5 μmole ferricyanide, 0.46 μmole of DPNH, enzyme and water to 1.0 ml. The control cuvette was identical except for the absence of enzyme. In the assay of DPNH cytochrome c reductase activity the final concentration of reactants in the 1-ml. cuvettes were as follows: 20 μmoles glycylglycine (pH 7.3), 0.46 μmole DPNH, and 0.05 μmoles of oxidized cytochrome c.

DPA was obtained from the Aldrich Chemical Company. The product was repeatedly (6 times) extracted from acid extracts with ether, and the concentrated preparation was crystallized from water. The resulting product gave the identical

spectra reported for DPA,[8] melted at 239° and stimulated oxygen uptake and DPNH cytochrome c reductase in a manner identical with that of the commercial product. ATP, ADP, AMP, DPN, DPNH, D-amino acid oxidase, and cytochrome c were products of the Sigma Chemical Company. Glycylglycine, EDTA, and 8-hydroxy quinoline were commercial samples.

RESULTS

Effect on DPA on Oxygen Uptake.—Harrell has observed that the addition of DPA to spore extracts leads to a twofold stimulation in the rate of oxygen uptake from the oxidation of glucose.[9] The results of a similar experiment, as seen in Figure 1, a,

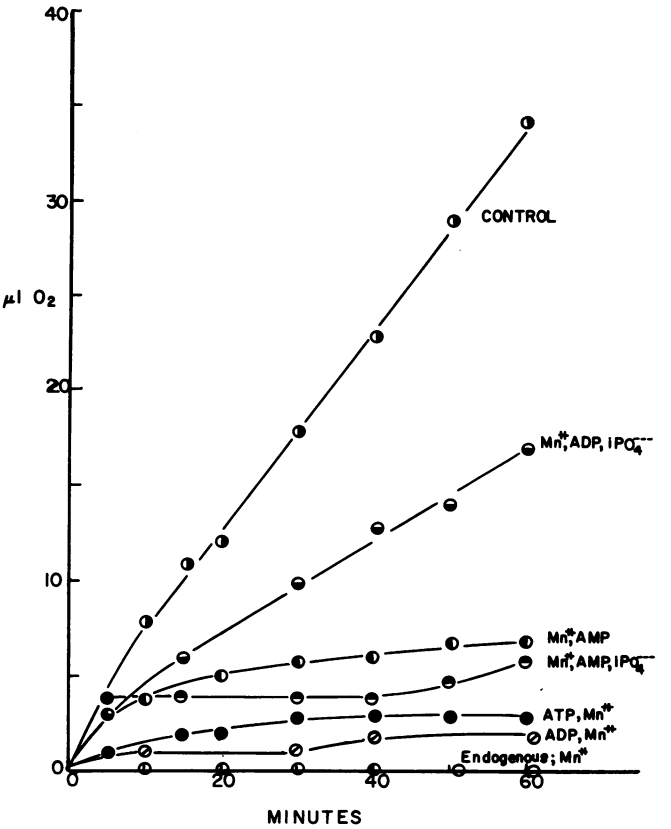

FIG. 2.—Reversal of Mn^{++} inhibition by ADP and inorganic phosphate. Spore extracts were dialyzed 12 hours against *M*/15 phosphate–*M*/150 EDTA buffer, pH 7.3, and 4 hours against *M*/20 glycylglycine buffer, pH 7.3. Warburg cup contents are identical with experiment in Fig. 1, *a*, except for $FeSO_4$, 1 μmole; $MnCl_2$, 100 μmoles; as indicated AMP, ADP, ATP, or $PO_4^{=}$, 5 μmoles.

show a threefold increase in the presence of 0.01 *M* DPA. Higher levels of DPA are slightly inhibitory. DPA is not metabolized by these extracts, nor is it stimulatory for the endogenous respiration.

Glucose oxidation by these extracts is mediated by a DPN-linked glucose dehydrogenase.[10] Since glucose dehydrogenase activity, as measured by the rate of DPNH formation, was found to be unaffected by the addition of DPA, the observed stimulation of O_2 uptake must occur in the electron-transport system. In support of this conclusion, the rate of oxygen uptake from DPNH is stimulated to the same extent by DPA as is glucose oxidation (Fig. 1, *b*).

Effect of Metallic Ions.—DPA forms chelate complexes with a number of divalent metals, including calcium, manganese, zinc, and copper. These findings, as well as

the stimulatory effect of another chelating agent, EDTA,[9] suggest that the observed stimulation may result from the chelation of some endogenous inhibitory metal. To test this possibility further, the sensitivity of the system to divalent ions was examined.

At 5×10^{-3} *M*, Fe^{++}, Fe^{+++}, Mg^{++}, and Ca^{++} enhanced glucose oxidation, whereas Mn^{++}, Ag^{+}, Co^{++}, Zn^{++}, Cu^{++}, Hg^{++}, and Pb^{++} inhibited oxygen uptake. The highest stimulations were observed by the addition of Fe^{++} (80 per cent) and Mg^{++} (42 per cent). Mn^{++} gave variable inhibitions of 60–100 per cent. Calcium (15–20 per cent stimulation at 5×10^{-3} *M*) is markedly inhibitory at concentrations above 2×10^{-2} *M*. Both the stimulatory and the inhibitory effects of metals were more pronounced when the extracts were first dialyzed to remove endogenous DPA. In general, the addition of DPA reversed the inhibition by metals.

The Mn^{++} inhibition was further examined, to characterize the locus of the metal inhibition. Employing a dialyzed spore extract, Mn^{++} completely inhibits glucose oxidation (Fig. 2). When substrate levels of ADP and PO_4^{---} were added to the Mn^{++} inhibited reaction mixture, oxygen uptake was again observed at 50 per cent of the normal rate. Additions of ATP, ADP, AMP, AMP + PO_4^{---} or PO_4^{---} alone were ineffective.

Test of DPA Control on the Supply of ADP.—The results in Figure 2 led us to speculate earlier[9] that in these extracts a coupling between the electron-transport system and oxidative phosphorylation may exist similar to that reported for other systems. The inhibition by Mn^{++} could readily be understood on the basis of a regulation of ATPase, whose activity is required to regenerate ADP from ATP for oxidation phosphorylation. A control of respiration of this type by calcium has been reported in heart preparations.[14]

Examination of the extract revealed the presence of an ATPase, pH optimum 8.1, which liberates PO_4^{---} from ATP. The dialyzed extract displays an optimum activity in the presence of 10^{-3} *M* Ca^{++}. Addition of 10^{-4} *M* Mn^{++} or 10^{-3} *M* Zn^{++} give 75 and 65 per cent of maximal activity. At 10^{-2} *M* concentrations, the enzyme is inhibited 45 per cent by Ca^{++} and Zn^{++} and over 90 per cent by Mn^{++} and Cu^{++}.

To provide a comparative basis for interpreting the respiration data, the subsequent experiments were conducted at pH 7.3 At this pH the enzyme has 53 per cent of its activity at pH 8.1. The extract hydrolyzes ADP and ATP at the same

TABLE 1

THE SUBSTRATE SPECIFICITY AND EFFECT OF DPA ON ATPASE*

Substrate	DPA	Specific Activity†
ATP	.....	0.35
ADP	.....	0.37
AMP	.....	0.023
ATP	3.2×10^{-2} *M*	0.42
ATP	8×10^{-3} *M*	0.46
ATP	1.6×10^{-3} *M*	0.40

* The systems for ATPase assay contained: glycylglycine buffer, pH 7.5, 100 μmoles; ATP, ADP, and AMP, 5 μmoles; DPA; and 2.0 ml. enzyme (16 mg.) in a final volume of 4.0 ml. Incubation for 60 min. at 30°. 2.0-ml. aliquots were taken at 0 and 60 min., deproteinized with 0.2 ml. of 50 per cent TCA, and centrifuged. 1.0 ml. of the supernatant was tested for PO_4^{---} by the Fiske-SubbaRow method. The results are corrected for endogenous PO_4^{---}.

† μM PO_4^{---}/mg protein/hr.

rate, while the rate of hydrolysis of AMP is at least 15 times slower than that of ATP (Table 1).

Based on the hypothesis that DPA regulates respiration by controlling the ADP level via its effect on ATPase, one would predict in crude extracts that (i) ATP hydrolysis would be stimulated two- to threefold by ADP, and ((ii) respiration would be dependent on ADP and PO_4^{---} levels. To test these possibilities, spores were heat-shocked, washed three times to remove endogenous phosphate, and cell-free extracts prepared. ATPase had a maximum stimulation of 30 per cent at 8×10^{-3} *M* DPA (Table 1).

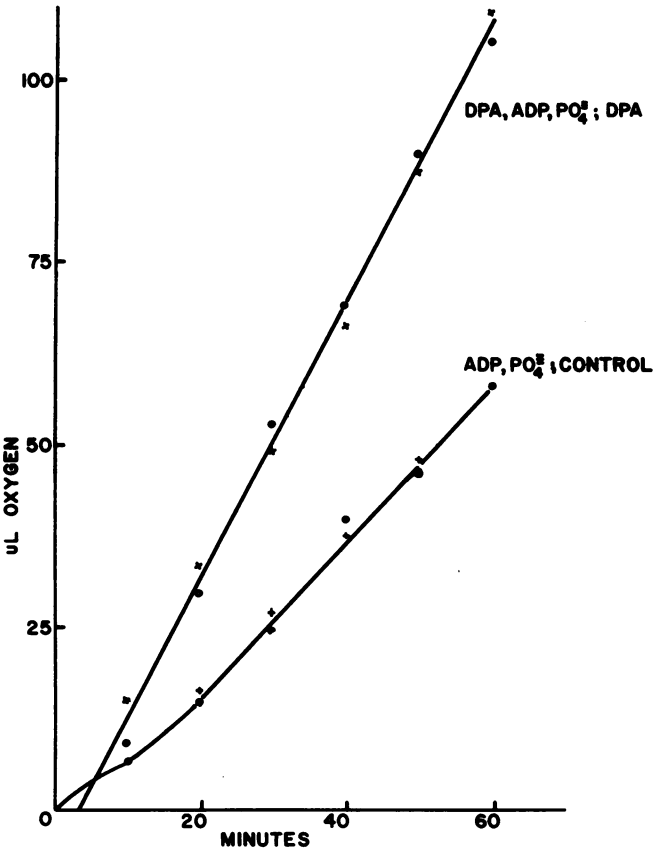

FIG. 3.—Independence of glucose oxidation from ADP. See Fig. 1, *a*, for details. Where indicated, ADP, $PO_4^{=}$, 5 μmoles; DPA, 20 μmoles.

Furthermore, the addition if ADP and PO_4^{---} to the extract did not significantly stimulate oxygen uptake from glucose (Fig. 3). The stimulation observed normally in the presence of DPA was unaffected by the presence of ADP and PO_4^{---}. Similar results were found either when DPNH was employed as substrate or when ATP substituted for ADP.

From these results it is clear that the major effect of DPA on oxygen uptake cannot be attributed to its effect on ATPase. The reason for the reversal of Mn^{++} poisoning by ADP and PO_4^{---} is not known at this time.

Effect of DPA on DPNH Cytochrome c Reductase.—Employing DPNH as substrate, a DPNH dehydrogenase was detected in extracts of heat-shocked spores.

The enzyme, which reacts with cytochrome c, ferricyanide, 2,6-dichlorophenol indophenol, and neotetrazolium as electron acceptors, may be similar to the diaphorase previously reported in spores and vegetative cells of *B. cereus* and *B. megaterium*.[1, 15] The enzyme is heat-labile and soluble. The activity is quantitatively recovered in the supernate fraction after centrifugation for 1 hour at 140,000 $\times$ *g*. in a Spinco preparation centrifuge.

The activity of the DPNH dehydrogenase, as well as over-all glucose oxidizing capacity, is variable in successive spore stocks. At 30° the diaphorase activity of the cell-free extracts, assayed by the ferricyanide method, varies from 3.5 to 5.3 μM DPNH/mg protein/hr, whereas the cytochrome c reductase activity is 0.23–0.50 μM DPNH/mg protein/hr. In these same extracts the variation in the rate of O_2 uptake from glucose and DPN or DPNH was 0.15–0.40 and 0.20–0.51 μM O_2/mg protein/hr, respectively. These rates were in general agreement with the DPNH cytochrome c reductase activities of the various extracts.

The enzymes usually mediating electron transfer from DPNH to cytochrome c have some form of riboflavin as the prosthetic group.[13] Spectroscopic examination of the spore extract, reduced either by dithionite or under anaerobic conditions by DPNH, showed a general absorption in the 450-mμ region where flavins would be expected to absorb. Spencer and Powell[15] have previously reported the presence of both free and combined FAD in spore extracts of *B. subtilis* and *B. megatherium*. Dialysis or ammonium sulfate fractionation of the spore extracts of *B. cereus* var. *terminalis* resulted in a loss of DPNH cytochrome c reductase activity, indicating the possible loss of prosthetic group during the procedures. Accordingly, the ability of flavins to reactivate an extract partially inactivated by dialysis was examined, with the results given in Table 2. The addition of FMN to the extract reactivates both the reductase and over-all respiration. FAD is less effective. Although direct evidence that the DPNH cytochrome c reductase of these spores is a flavoprotein is lacking, the reactivation obtained with added FMN is presumptive evidence that this enzyme is a flavoprotein, as are the other DPNH cytochrome c reductases examined to date. The purification and properties of the enzyme will be reported in a subsequent publication.

TABLE 2

REACTIVATION OF OXIDATIVE CAPACITY* AND DPNH CYTOCHROME C REDUCTASE†

	Rate of O_2 Uptake Per Cent Control	Reductase Per Cent Control
Extract	100	100
Extract + FAD	104	140
Extract + FMN	148	600
Extract + FAD + FMN	153	600

* See Fig. 1, *a*, for details. Where indicated FAD, 25 mμmoles; FMN 39 mμmoles.

† Reductase activity was determined as described under "Materials and Methods" except for the addition of FAD, 25 mμmoles and FMN, 39 mμmoles. The co-factors were added to both the reference and the test cuvettes.

Table 3 shows the effect of DPA addition on the diaphorase and DPNH cytochrome c reductase activities. The dehydrogenase, extracted from both heat-shocked spores and vegetative cells, is stimulated by DPA to approximately the same degree as over-all oxygen uptake with either glucose or DPNH as substrates (Fig. 1, *a*, *b*). When the soluble enzyme is further purified by ammonium sulfate precipitation and fractionation by DEAE cellulose chromatography, the resulting

preparation retains both its DPA-enhanced diaphorase and DPNH cytochrome c reductase activities. The locus of a DPA effect at the DPNH cytochrome c reductase level is further supported by the fact that in extracts of heat-shocked spores, the rate of cytochrome c reduction by glucose and DPN is stimulated threefold by 0.02 *M* DPA.

TABLE 3

STIMULATION OF DPNH OXIDATION BY DPA

SOURCE OF EXTRACT	REDUCTASE*		DIAPHORASE*	
	Control	DPA†	Control	DPA
Heat-shocked spores	0.23	0.90	4.9	13.2
Vegetative cells	0.98	3.32	33.8	96

* μM DPNH/mg protein/hr.
† 0.02 *M*.

Table 4 summarizes experiments designed to determine whether the stimulation of DPNH cytochrome c reductase could be ascribed to the removal of an inhibitory agent by chelation with DPA. A survey of chelating agents on the activity of the soluble enzyme indicates that the stimulatory phenomenon is specific for DPA. One of these agents—8 hydroxyquinoline—has no effect on the system, whereas EDTA inhibits DPNH cytochrome c reductase activity. Dialysis of the extract from heat-shocked spores against glycylglycine buffer leads to a 40–50 per cent reduction of enzymatic activity, probably because of a loss of flavins. A similar loss in activity occurs when the preparation is first dialyzed for 12 hours against DPA, EDTA, or 8 hydroxyquinoline and then dialyzed against buffer to remove the chelating agents. In none of the dialyzed preparations has the DPA effect been abolished. Also, when care was taken to decrease the divalent metal content of extracts of vegetative cells which had either been grown in a medium from which Zn^{++}, Mn^{++}, or Ca^{++} were withheld, disrupted in non-metal containers (freeze and thawing, Nossal disintegrator, or Hughes's press), or assayed with DPA-dialyzed cytochrome c, a threefold increase in reductase was observed in the presence of 0.02 *M* DPA.

TABLE 4

EFFECT OF CHELATING AGENTS ON DPNH CYTOCHROME C REDUCTASE*

Dialysis‡	INCUBATION CONDITIONS†				DPA/ Control
	Control	DPA	Versene	8-OHQ§	
None	2.28	6.67	1.40	2.28	2.9
Buffer	1.61	4.92	0.95	..	3.0
DPA; buffer	1.08	3.79	..	..	3.5
Versene; buffer	1.12	2.73	..	..	2.2
8-OHQ; buffer	1.30	4.21	..	1.30	3.2

* μM DPNH/ml./hr.
† Reductase activity was determined as described in methods except for the addition of 10^{-2} *M* chelating agents where indicated.
‡ Dialyzed at 2° for 12 hours against 10^{-2} *M* chelating agent, followed by 12 hours against 3 changes of 10^{-2} *M* glycylglycine buffer, pH 7.3.
§ 8 hydroxy quinoline.

The effect of DPA on a known flavoprotein, the D-amino acid oxidase of hog kidney, was also examined. Employing DL-alanine as substrate with added catalase and FAD,[16] the rate of oxidation of D-alanine was unaffected by the presence of 0.02 *M* DPA.

DISCUSSION

Although DPA has been recognized as a major constituent of bacterial endospores since 1953,[8] little is presently known about either its state or its function in dormant spores. The DPA content of spores is not constant; for example, variations of 3–6 per cent for *B. cereus* var. *mycoides*,[17] 6–12 per cent for *B. cereus* var. *terminalis*,[1, 7] and 5–15 per cent for *B. megatherium*[8, 17] have been reported. The use of different assay methods for DPA may explain some of these variations; however, modifications of the sporulation medium greatly influences the DPA content of spores.[18] Knowledge of the state of DPA in dormant spores is equally incomplete. The isolation of the Ca DPA from germination exudates[8] and the demonstration that DPA strongly chelates Ca^{++}, Cu^{++}, Fe^{++}, and Mn^{++} and weakly chelates Mg^{++}[5] have led to various suggestions that DPA exists in spores in the chelate form. That DPA is distributed within the resting spore exclusively as the calcium salt is made unlikely on several grounds: (i) air-dried spores are devoid of the characteristic crystalline-powder X-ray diagram of calcium DPA;[5] (ii) Ca^{++}/DPA ratios of 0.021[19] to over 1[5] have been reported; (iii) hydrolysis by hot mineral acids is required to obtain total recoveries of DPA.[7, 17]

Although the mineral contents of spores are variable and to a certain extent the ions are interchangeable,[4] the need for acid hydrolysis to liberate free DPA implies that in dormant spores DPA exists in part in a bound inorganic or organic form. One such form is suggested by the isolation of the monethyl ester of DPA from the hydrolyzates of intact spores.[17] Although the nature and extent of the bound DPA forms are unknown, it seems clear from the above and from the eventual total liberation of DPA during germination and outgrowth[1, 7, 20] that the full chelation potential of DPA has not been realized in dormant spores.

It is not surprising that the introduction of a strong chelating agent in vivo or in vitro influences the metal balance and over-all metabolism of a system known to contain both metal-activated and metal-sensitive enzymes.[1] The stimulation of respiration from glucose in spore extracts upon the addition of either DPA or EDTA, as reported by Harrell[9] or in this paper, is undoubtedly due to their chelating properties. The system can be poisoned by many of the divalent metals known to be present in spores in high concentrations;[4, 5] the inhibition is overcome by the addition of DPA or EDTA. On the other hand, the metal requirement of this same system is further suggested by the stimulatory effect of 5×10^{-3} *M* Fe^{++}, Mg^{++}, and Ca^{++}. One would expect that, in the presence of excess free DPA, Ca^{++}, Cu^{++}, Ni^{++}, Fe^{++}, and Mn^{++} would be displaced from enzymes whose chelation stability constants for these metals was less than their affinities for DPA. Although undoubtedly various degrees of metal competition may exist between DPA and spore proteins, the slight stimulation reported here with a Ca^{++}-activated ATPase is insufficient to explain the over-all DPA effect in electron transport. The failure of DPA to inhibit ATPase suggests that the enzyme may largely retain its activating metal in the presence of DPA. Further studies on DPA effects on metal binding proteins, e.g., Mn^{++}-activated spore enzymes,[1] would seem warranted.

A second and perhaps more prominent function of DPA in electron transport, aside from the removal of inhibitory metals, is suggested by its effect on DPNH cytochrome c reductase. The enzyme, which had previously been dialyzed against several chelating agents, is specifically stimulated threefold by DPA and inhibited

by EDTA. This phenomenon is not unique to the dormant state, since similar observations were obtained with DPNH cytochrome c reductase of vegetative cells. Numerous models could be proposed to explain the DPA effect on this enzyme; however, an explanation of this phenomenon must await an analysis of the DPA-enzyme interaction.

The inhibition of DPNH cytochrome c reductase by EDTA clearly distinguishes this phenomenon from the stimulatory effect of EDTA on over-all oxygen uptake. It would thus appear that at least two sites are involved in the over-all stimulation of electron transport: (*a*) a site which is stimulated, probably via chelation, by EDTA and DPA and (*b*) a DPA specific stimulation. Until the nature of the native cytochromes and terminal respiratory systems of these spores are further characterized, it will be difficult to quantitate the contribution of these DPA-sensitive sites on regulating over-all electron transport.

The demonstration that DPA regulates electron transport in spore extracts suggests that it may play an important role in dormancy. An integrated biochemical description of dormancy demands a basis for converting the vegetative cell into the heat-resistant, quiescent state, as well as maintaining the dormant state. Of the various factors involved, heat resistance is correlated with the contents of Ca^{++},[4] DPA,[18] and possibly water,[20, 21] while the breaking of dormancy is stimulated by activation[1, 2] and by metabolizable germinating agents,[1, 2, 10] is dependent on metabolic activity,[2] and is sensitive to divalent metals.[1, 22] The early stages of germination are associated with the release of DPA,[1, 7] the appearance of respiratory activity, and the liberation of a number of dormant enzymes.[1]

The foregoing findings, the high content of divalent metals in spores,[4, 5] and the necessity of activating the electron-transport system of dormant spores for germination have suggested to us the following model for the regulatory role of DPA in electron transport. Activation leads to an increase in chelation potential by releasing bound forms of DPA. The liberated free DPA promotes the flow of electrons from substrate to molecular O_2 by (1) the removal of an inhibitory metal directly or indirectly affecting the system and by (2) a direct stimulation of DPNH cytochrome c reductase. We are currently attempting to test some of the predictions afforded by such a model.

SUMMARY

The rate of oxygen uptake from glucose or DPNH by cell-free extracts of *B. cereus* var. *terminalis* is stimulated by the addition of free dipicolinic acid and versene. Glucose oxidation was inhibited by Mn^{++}, Ag^{+}, Co^{++}, Zn^{++}, Cu^{++}, Hg^{++}, and Pb^{++}, the inhibition being reversed by DPA. Mn^{++} inhibition was reversed by ADP and inorganic phosphate.

A Ca^{++}-activated ATPase was detected in spore extracts whose activity was stimulated 30 per cent by 8×10^{-3} DPA. Attempts to demonstrate a dependence of respiration on ATPase activity or ADP levels was negative.

A soluble DPA-stimulated DPNH cytochrome c reductase was extracted from spores and vegetative cells. The enzyme is inhibited by versene and unaffected by 8 hydroxyquinoline. The specific stimulatory effect of DPA could not be attributed to the removal of an inhibitory ion.

The possible role of divalent ions and DPA in regulating the respiratory dormancy of spores is discussed.

* This investigation was supported in part by a research grant of the Wisconsin Foundation and by an institutional grant of the American Cancer Society. The authors wish to acknowledge the technical assistance of Mr. Ray Epstein and the helpful advice of Dr. Salih Wakil. The early phases of this program were conducted in co-operation with Dr. W. K. Harrell.

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[3] The following abbreviations are used in this paper: AMP, adenosine-5′-phosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; DPA, dipicolinic acid; DPNH, dihydrodiphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetic acid; FMN, riboflavin-5′-phosphate; FAD, flavin adenine dinucleotide; DEAE cellulose, diethyl-aminoethyl cellulose.

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*OBSERVATIONS ON THE FUNCTION OF PYRIDOXAL-5-PHOSPHATE IN PHOSPHORYLASE**

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Communicated October 2, 1958

The discovery that 4 moles of pyridoxal-5-phosphate are present per mole of muscle phosphorylase *a* and that this substance is the prosthetic group of the enzyme[1, 2] raises questions as to (1) its possible direct participation in the enzymatic reaction, (2) its mode of attachment to the protein, and (3) its importance