INDUCED RELEASE OF DIPICOLINIC ACID FROM SPORES OF *BACILLUS MEGATERIUM*[1]

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Conditions under which dipicolinic acid may be released from bacterial spores probably relate to the chemical state of the dipicolinic acid in the spore, to its physical location in the spore, and to its influence on the physiological properties of the spore. The latter aspect embraces the relation between release of dipicolinic acid and the viability of the spore, its heat resistance, and its refractility.

It is known that dipicolinic acid is released physiologically during germination of bacterial spores (Powell and Strange, 1953), and that it can be extracted by boiling dilute mineral acid (Perry and Foster, 1955). Dipicolinic acid in spores of *Bacillus cereus* var. *mycoides* is also released by mechanical disruption of the spores (Rode and Foster, 1956; Powell and Strange, 1955; Young, 1959). Because dipicolinic acid is important in physiological and biochemical studies of spores, a systematic survey of treatments was undertaken with the object of releasing dipicolinic acid and, in some cases, correlating the release with the viability of the spores. To distinguish the release of dipicolinic acid from spores by means other than the normal physiological process of germination, the term "induced release" is employed.

MATERIALS AND METHODS

Organisms and culture procedures. The strain of *B. megaterium* previously studied in this laboratory was employed. Spores were obtained by cultivation at 30 C in a liquid mineral salts-carbohydrate medium (Martin and Foster, 1958), substituting glucose for sucrose. Liquid cultures were shaken continuously during growth on a reciprocating mechanical shaker operating at 76 4-inch strokes per min. The cultures were allowed to incubate until well after maximal sporulation to take advantage of autolysis of the sporangia and the liberation of free spores. The spores were harvested by centrifugation and washed repeatedly (4 to 6 times) until microscopic examination of stained or unstained preparations confirmed that the content of vegetative cells and nonspore debris was negligible. Stock suspensions were stored at 4 C and used as needed. Viable spore counts were made by spreading 0.10 to 0.30 ml of appropriate dilutions of the spore suspensions on the surface of a nutrient agar supplemented with 0.1 per cent soluble starch in petri plates. Platings were done in replicates according to the quantitation requirements of the particular experiment. Maximal colony recoveries invariably were obtained after 16 to 30 hr of incubation at 37 C.

Dipicolinic acid measurements. These were performed spectrophotometrically by methods previously described (Perry and Foster, 1955; Martin and Foster, 1958). Practically all of the ultraviolet light absorbance of spore extracts prepared in various ways has been attributable to dipicolinic acid; measurement of optical density at the absorption maximum of calcium dipicolinate (270 mμ) is a reliable index of the extent of its extraction from suspensions of clean *B. megaterium* spores.

RESULTS

Mechanical rupture of spores. Special attention was given to the analysis of cellular debris for bound dipicolinic acid after breakage of the spores, and to the prevention of release of potentially bound dipicolinic acid by an enzyme activated or liberated during breakage of the cell. Spore suspensions were ground at 4 C in a Waring Blendor with Ballotini no. 10 glass beads, until very few intact cells remained. Analysis of the cell debris showed an insignificant amount of dipicolinic acid; all the dipicolinic acid of the original spores was in solution. The soluble dipicolinic acid was quantitatively dialyzable through cellophan and was identified as free

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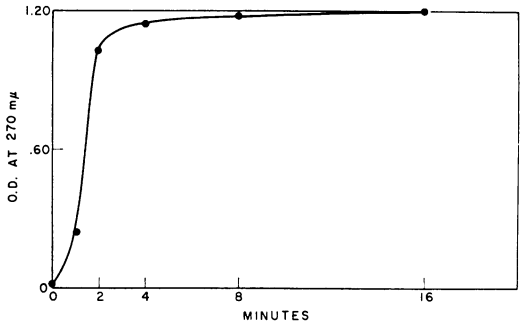

Figure 1. Thermal release of dipicolinic acid from spores of *Bacillus megaterium* at 100 C. The curve represents the dipicolinic acid content of the supernatant liquids. Optical density 1.20 represents release of all spore dipicolinic acid.

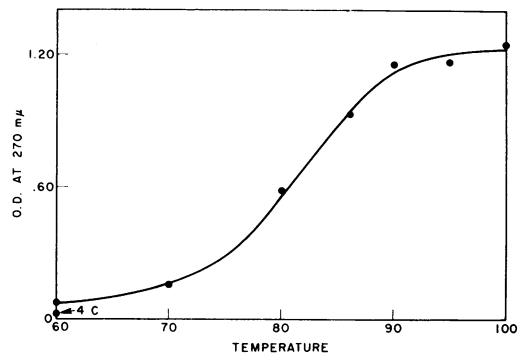

Figure 2. Thermal release of dipicolinic acid from spores of *Bacillus megaterium* in 20 min. The curves represent the dipicolinic acid of the supernatant liquids. Optical density 1.20 represents release of all spore dipicolinic acid.

dipicolinic acid by means of the ultraviolet absorption spectrum and by paper chromatography (Martin and Foster, 1958). Similar experiments with *B. megaterium* have been performed by P. H. Hodson in this laboratory. The breakage was done with a Mickle tissue disintegrator employing Ballotini no. 10 beads. The suspending liquid was 80 per cent ethanol, which virtually eliminates enzymatic action. The results were identical to those described above. In our experiments no evidence has been encountered for the kind of dipicolinic acid-amino acid complex recently described as occurring in extracts of broken *B. megaterium* spores (Young, 1959).

Thermal release of dipicolinic acid. Heating at 100 C for several minutes extracted all the dipicolinic acid from spores of *B. megaterium*. A typical experiment: one 4-ml sample of a suitable water suspension of spores was heated 15 min at 100 C and a second sample was allowed to stand unheated. After centrifugation, spectral analysis of the boiled extract showed the absorption spectrum characteristic of calcium dipicolinate. The unheated control showed no evidence of dipicolinic acid in its supernatant liquid; the optical density at 270 mμ was only 2.5 per cent of that of the boiled extract.

The residual cellular material in each treatment was analyzed by acid hydrolysis and ether extraction (Perry and Foster, 1955). Boiled spores had no dipicolinic acid, and unheated spores yielded dipicolinic acid equivalent to that found in the boiled extract. Whereas an initial acid hydrolysis was essential for maximal release of dipicolinic acid from *B. cereus* var. *mycoides*, a fact consistent with the existence of a bound form of this compound, it is clear that in *B. megaterium* spores all of the dipicolinic acid can be recovered free by boiling in water. If dipicolinic acid exists in spores in a bound form it must be labile. Autoclaving at 126 C for 10 min was found also to release dipicolinic acid from bacterial spores (Woese, 1959).

Kinetics of thermal release. Figure 1 shows that the major portion of the dipicolinic acid was released in 2 min at 100 C, and that 15 min ensured complete extraction. Figure 2 shows that the critical temperature for dipicolinic acid release from spores of *B. megaterium* was 70 C, using a 20-min heating period. The rate of release increased at temperatures above 70 C; 90 to 95 C were almost as effective as 100 C in the 20-min period.

A detailed study of the rate of dipicolinic acid release at various temperatures shows that there were important differences in the rates at the different temperatures, and that the difference in rate was marked even for the 10° increment between 90 and 100 C (figure 3). At 75 C the release was less than 5 per cent of that at 100 C; at 90 C it was 11 per cent of that at 100 C.

Thermal release of dipicolinic acid and loss of viability. Dipicolinic acid release and viable spore plate counts were made at various times on samples of a spore suspension heated at 40, 60, 75, 80, and 85 C, respectively. The results were qualitatively the same at all temperatures; as illustrations, data for 3 temperatures are plotted

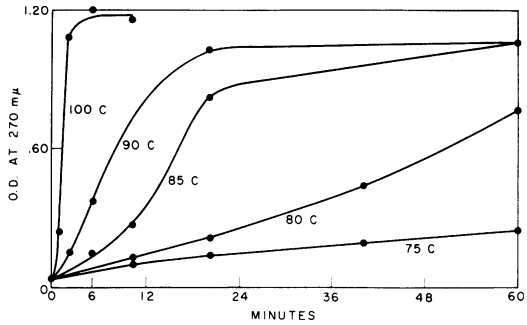

Figure 3. Temperature dependence of release of dipicolinic acid from spores of *Bacillus megaterium*. The curves represent the dipicolinic acid of the supernatant liquids. Optical density 1.20 represents release of all the spore dipicolinic acid.

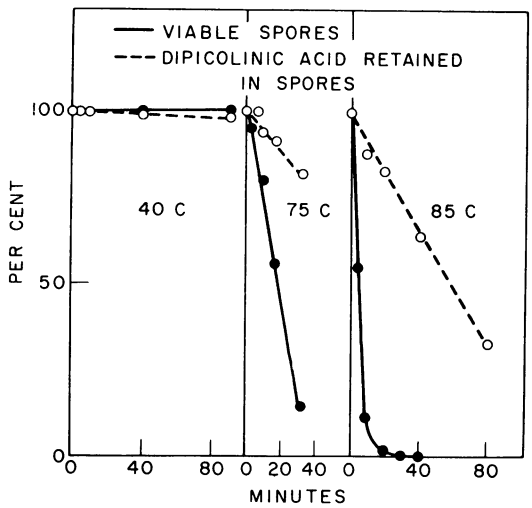

Figure 4. Relation between thermal loss of dipicolinic acid and loss of viability of spores of *Bacillus megaterium*.

in figure 4. The data support the conclusion that at sublethal temperatures dipicolinic acid was not extracted by water from spores of *B. megaterium*, and that at lethal temperatures the loss of viability occurred at a decidedly more rapid rate than loss of dipicolinic acid. The simplest interpretation of these data is that thermal loss of dipicolinic acid occurs only upon death (loss of viability) of the cell. No evidence has been obtained indicating thermal release of dipicolinic acid precedes or occurs independently of loss of viability.

No significant loss of dipicolinic acid has been found in water suspensions of clean spores stored at 4 C for as long as 6 months, nor has a loss been found when suspensions were shaken continuously for 24 hr at 37 C. Spontaneous germination (Powell, 1957) in clean spore suspensions has not been noted at any temperature.

Boiled spores which have lost all their dipicolinic acid remain refractile. Like normal spores, they are not stainable with ordinary vegetative cell stains, e. g., gentian violet or methylene blue.

Freezing and thawing. Five ml of a suitable suspension of spores in a polyethylene tube were alternately frozen in Dry Ice-ethanol (−80 C) and thawed in a 37 C water bath in rapid sequence a total of 8 times. There was no loss of viability or release of dipicolinic acid. Under the phase contrast microscope the refractility of the treated spores appeared normal. The resistance of spores to freezing and thawing may be a consequence of the low content or absence of water in resting spores (Powell and Strange, 1953).

X-ray irradiation. Six ml of a suitable suspension of spores in deionized water contained in a 50-ml beaker were exposed to 60,000 r during a 60 min period. The viability was barely reduced (3 per cent), indicating that the dosage employed was a threshold one. Woese (1959) found that *B. megaterium* is relatively resistant to X-ray inactivation. His data also indicate that the dosage we employed was threshold. The irradiated spores displayed normal refractility. Analysis of the clear supernatant liquid of the irradiated suspension indicated no significant release of dipicolinic acid had been induced by this irradiation treatment.

Ultraviolet irradiation. A suspension of spores of *B. megaterium* 2.5 mm thick in a petri dish was exposed to an ultraviolet lamp at a distance of 14 cm. After 5 min irradiation the viable count was 0.1 per cent of the control unirradiated suspension. No dipicolinic acid was released during this period; even after 2 hr irradiation none was released. However, in a second experiment where the dosage was increased (suspension depth = 0.7 mm, 8.5 cm from ultraviolet source, irradiation time 4 hr), a considerable quantity of dipicolinic acid was found in the supernatant liquid. The absorption spectrum of the supernatant was very similar to that of dipicolinic acid. Quantitative analysis of the dipicolinic acid in the unirradiated spores and in the spores recovered after the incubation revealed that 30 per cent of the original dipicolinic acid

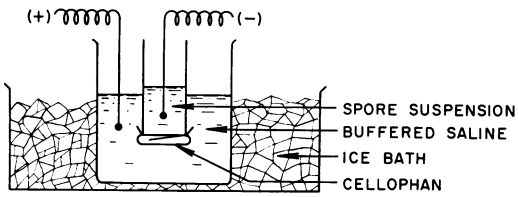

Figure 5. Apparatus employed for electrodialysis of dipicolinic acid from spores of *Bacillus megaterium*.

of the spores had been released by ultraviolet irradiation. Although the spores were nonviable at the time dipicolinic acid was released, a direct relation does not exist between killing by ultraviolet irradiation and its release. But the mechanism of dipicolinic acid release by prolonged ultraviolet irradiation is of interest and pertinence to the role that this compound plays in spores.

Electrodialysis. Quantitative release of free dipicolinic acid by mechanical breakage of spores and by relatively mild heating in water implies that the compound exists free in the spores as the salt or metal chelate, and that it is not bound in the usual primary valence sense. The ionic nature of dipicolinic acid suggested that even in the intact spore it might respond to an impressed electric potential. A suspension of intact *B. megaterium* spores was electrodialyzed in an apparatus schematically reproduced in figure 5. The inner chamber, containing 3 ml of the spore suspension in deionized water, was immersed in a beaker (outer chamber) containing 6 ml of 0.0066 M phosphate buffer in 0.2 per cent NaCl. A negative platinum electrode was placed in the inner chamber; the positive electrode in the outer chamber. The appartus was covered in an ice bath and was located in a cold room at 4 C.

An initial current of 20 (±2) milliamps was applied. Moderate but not excessive foaming developed in the inner chamber, and the spores migrated fairly rapidly to the cellophan membrane. The current was maintained for 7 hr varying from 20 to a final 28 milliamps. There was a progressive pH change; the inner chamber was pH 11.7 after 7 hr and the outer chamber pH 2.1. The temperature in the inner chamber did not exceed 39 C and in the outer chamber 30 C. At the end of the 7 hr period all the spores had collected at the cellophan surface where they formed a tough pellicle which could not be easily dispersed but which could be readily removed

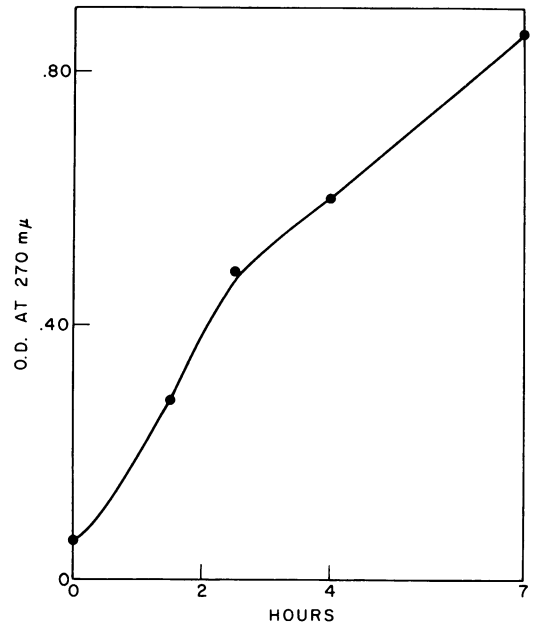

Figure 6. Release of dipicolinic acid from spores of *Bacillus megaterium* by electrodialysis. The curve represents the dipicolinic acid of the liquid in cathode chamber.

quantitatively. The liquid contents of the inner and outer chambers were clear and colorless at this time. Figure 6 shows the increase in optical density (at 270 mμ) of the solution in the outer chamber as a function of time. The absorption spectrum of this solution measured at pH 2.1 and at pH 6.9 was characteristic of dipicolinic acid. In this experiment not all dipicolinic acid was electrodialyzed from the spores. A rough indication of the amount removed from the spores by this treatment was obtained by measuring the residual dipicolinic acid in the spores and comparing that with the content of an equivalent amount of untreated spores. This was done by extracting the two spore suspensions in water at 100 C for 15 min, and then determining the optical density at 270 mμ of the extracts diluted equally. The extract of the spores not electrodialyzed had an optical density of 1.082 and that of the spores electrodialyzed for 7 hr had 0.774, indicating that approximately 28 per cent of the dipicolinic acid had been electrodialyzed in that experiment. In a second experiment the corresponding data were 0.730 vs. 0.262, indicating that 64 per cent of the dipicolinic acid had been electrodialyzed.

TABLE 1

*Release of dipicolinic acid from resting spores of Bacillus megaterium by various concentrations of ethanol at 56 C**

	Optical Density at 270 mμ with Ethanol Conc. (Per Cent):						
	95	80	60	40	20	10	0
Supernatant liquid	0.006	0.510	0.211	0.087	0.056	0.038	0.066
Residual spores†	0.659	0.094	0.402	0.551	0.603	0.543	0.530

* Treatments lasted 2 hr. Measurements were made on the clear supernatant liquids after centrifugation. Maximal amount of dipicolinic acid in these spores corresponded to optical density 0.614, determined by heating a sample at 100 C for 15 min.

† Centrifuged spores extracted at 100 C for 15 min and recentrifuged.

Quantitative studies of the viability of the electrodialyzed spores were not possible because of the pellicular state of the spores and lack of success in preliminary attempts to disperse these spores for plate counting purposes. However, when the material was streaked on recovery plates, confluent growth occurred at the streak sites indicating that at least some of the spores remained viable throughout the experiment. Control experiments have indicated that the change in pH of the solutions could not account for the release of the dipicolinic acid nor for a significant loss of viability of the spores. An extensive study of the electrodialysis of dipicolinic acid from bacterial spores is underway.

Organic solvents. The readily induced release of dipicolinic acid by the procedures described above suggest a superficial location of it in the spore, and that breaching the impermeability of the spore would allow rapid diffusion of the compound from the spore. In the expectation that lipids may play an important role in the impermeability of spores, a survey of the following organic solvents was undertaken to determine if they would facilitate the release of dipicolinic acid from spores of *B. megaterium*: acetone, 80 per cent acetone, ethanol, 80 per cent ethanol, methanol, 80 per cent methanol, acetone-ether (4:1), acetone-chloroform (4:1), acetone-*n*-hexane (4:1), ethanol-ether (4:1), ethanol-chloroform (4:1), ethanol-*n*-hexane (4:1), methanol-ether (4:1), methanol-chloroform (4:1), methanol-*n*-hexane (4:1), *n*-hexane, chloroform, ethyl ether. The solvent mixtures were added to samples of water-wet spores obtained by centrifugation of a water suspension of spores in tubes, followed by decantation of the supernatant liquid. One tube of each solvent suspension of spores was shaken continuously for 2 hr at room temperature (25 C) and another tube was heated in a water bath at 56 C for 2 hr. None of the solvents was pronouncedly more effective than the water control in releasing dipicolinic acid at room temperature. At 56 C, however, the following solvents were strikingly effective in releasing dipicolinic acid from the spores, whereas water was ineffective: 80 per cent ethanol, 80 per cent methanol, and 80 per cent acetone.[2]

A more detailed study was made of dipicolinic acid release from spores of *B. megaterium* by those three solvents. Ethanol was by far the most effective. The concentration was extremely critical for all three solvents; 80 per cent was optimal in each case. The results obtained for ethanol are shown in table 1. It is clear that 80 per cent ethanol extracted most of the dipicolinic acid from the spores. This was confirmed by the analysis for the residual dipicolinic acid in the spores recovered from the alcohol treatments.

In spite of the loss of the major fraction of their original dipicolinic acid, the refractility (phase contrast microscopy) of the spores from the 80 per cent ethanol treatment was not noticeably less than that of any of the other treatments, or of the normal untreated spores. It appears that the property of refractility of the spore is not dependent on dipicolinic acid of the spore.

The three solvents in various concentrations were tested for their ability to release dipicolinic

[2] Because of the absorbance of acetone in the ultraviolet, acetone supernatant liquids were evaporated to dryness, and optical density measurements were made on water solutions of the residues.

TABLE 2

Release of dipicolinic acid from spores of Bacillus megaterium by various chemical treatments

Expt. No.	Chemical Treatment	Temp	Time	Optical Density at 270 mμ of Hot Water Extract of Spores after Chemical Treatment*	Dipicolinic Acid Released from Spores by Chemical Treatment
		C	*hr*		%
1	None	4	64	0.808	0
	Phenol, 5%	4	64	0.094	88
	H_2O_2, 5%	4	64	0.172	79†
	HCl, pH 1.45	4	64	0.640	21
	Arquad T, 0.5% (surfactant)	4	64	0.100	88
2	None	37	2	1.040	0
	H_2O_2, 5%	37	2	0.823	21
	None	45	2	1.027	1
	H_2O_2, 5%	45	2	0.650	38
	None	56	2	0.998	4
	H_2O_2, 5%	56	2	0.032	97‡
	H_2O_2, 5%	56	0.5	0.592	43
	H_2O_2, 5%	56	1	0.177	83
	H_2O_2, 5%	56	1.5	0.043	96
	H_2O_2, 5%	56	2	0.034	97
3	None	4	90	1.878	0
	Phenol, 1%	4	90	1.433	24
	Phenol, 5%	4	90	0.430	77‡

* Centrifuged spores extracted in water 15 min at 100 C. Volumes were equal for all analyses. The following chemicals were also tested with essentially negative results: iodine, 200 ppm; NaOH, pH 12.2; $CaCl_2$, 0.1 per cent; ethanol, 50 per cent; methanol, 50 per cent; acetone, 50 per cent; *n*-propanol, 50 per cent. Commercial surface active agents (all tested at 0.5 per cent) were: Santomerse-3, Areskap-100, Sterox-6, and Sterox-5 (products of Monsanto Chemical Company, St. Louis, Missouri); Triton X-100 (Rohm and Haas Company, Philadelphia, Pennsylvania); Tween 80 (Atlas Powder Company, Wilmington, Delaware); Ethofat 142/20 and Ethofat 142/25 (Armour Chemical Division, Chicago, Illinois).

† In a second experiment at these low temperatures, using a different batch of spores, dipicolinic acid release was not observed in this treatment.

‡ The dipicolinic acid in these H_2O_2 and phenol supernatant liquids was determined directly as optical density 0.942 and 1.146, respectively. The residual H_2O_2 was decomposed by addition of a solution of catalase. The solution was then acidified with H_2SO_4 and extracted with ethyl ether. The ether extract was evaporated and the residue made to the original volume with water and its optical density determined. The excess phenol was extracted with ether at pH 7. The aqueous solution was then acidified with H_2SO_4 and the dipicolinic acid extracted with ethyl ether and analyzed as in the H_2O_2 treatment. The ultraviolet absorption spectra of the material extracted by H_2O_2, by phenol and by the surfactant Arquad T, were identical with that of dipicolinic acid.

acid at 4, 37, 45, and 56 C. In a 3-hr period, there was little if any release at the three lower temperatures. At 56 C, the release by 80 per cent ethanol was maximal; that by 60 per cent ethanol, 80 per cent methanol, or 80 per cent acetone was about ⅓ to ½ of maximal. The ultraviolet absorption spectra of the various solvent extracts, and the ether solubility of the ultraviolet-absorbing material at acid pH values indicated that the absorption was due to the content of dipicolinic acid (calcium chelate).

Miscellaneous chemical extractants. Table 2 describes the chemicals employed to induce a release of dipicolinic acid and their relative efficacy under various conditions. Spores of *B. megaterium* were added in suitable concentration to tubes of the various chemical solutions in a final volume of 4 ml. After thorough mixing, the tubes were capped with aluminum foil and held with periodic shaking under the various conditions described in table 2. The spores were then centrifuged and the loss of dipicolinic acid from the spores was determined by measuring the residual dipicolinic acid in the spores recovered and washed by centrifugation in water. Water extraction at 100 C for 15 min permitted recovery of the residual spore dipicolinic acid. Table 2 reveals that three chemicals, namely, phenol, hydrogen peroxide, and the surface active agent Arquad T, were outstanding in their ability to release dipicolinic acid from the spores under the conditions employed. The absorbance of the HCl supernatant may in part be due to deoxyribonucleic acid reported to be extruded from spores by this treatment (Robinow and Fitz-James, 1956).

The changes caused by hydrogen peroxide occurred rapidly at slightly elevated temperatures. Phenol was not studied at the elevated

temperatures, but judging from its effectiveness at 4 C over a 3- or 4-day period, one may assume that phenol-induced release of dipicolinic acid would be greatly accelerated at elevated temperatures. Spores that had lost all or most of their dipicolinic acid as a result of H_2O_2 or phenol treatment retained full refractility when examined with a phase contrast microscope.

An extensive study has subsequently been made of dipicolinic acid release from bacterial spores by surface active agents; the results of that study are being published separately.

SUMMARY

Factors inducing release of dipicolinic acid from washed clean spores of *Bacillus megaterium* were studied. Spore dipicolinic acid was released as free acid by mechanical breakage of the spores. Thermal release of dipicolinic acid was studied in detail. Practically all of the dipicolinic acid was released from intact spores by heating a few minutes at 100 C; 70 C was critical for the release. Loss of viability always exceeded the rate of thermal dipicolinic acid release indicating that death of the cells precedes the release. Repeated freezing and thawing of normal spores did not release dipicolinic acid or kill the spores, nor did exposure to X rays at a dosage of 60,000 r for 60 min. Ultraviolet irradiation released substantial amounts of dipicolinic acid, but only long after the spores had lost viability. Electrodialysis of spores through a cellophan membrane resulted in substantial movement of dipicolinic acid from the spores to the cathode chamber. Several organic solvents, e. g., 80 per cent methanol, 80 per cent ethanol, 80 per cent acetone, released substantial amounts of dipicolinic acid at 56 C, whereas water extracted none under the same conditions. Of a series of chemicals tested for induction of dipicolinic acid release, H_2O_2 (5 per cent), phenol (5 per cent), and a commercial surface active agent, Arquad T, were effective inducers of dipicolinic acid release during exposure at various temperatures. Spores that have lost their dipicolinic acid thermally in water or in solvents or in chemical reagents retained their characteristic refractility.

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