RAMAN SPECTROSCOPY *In Vivo*: EVIDENCE ON THE STRUCTURE OF DIPICOLINATE IN INTACT SPORES OF *Bacillus Megaterium*

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SUMMARY: Raman spectra of spores of *B. megaterium* are interpreted in terms of the predominant chemical component in the spores, the 2,6 pyridinedicarboxylate molecule (dipicolinate). The spectra show that the structure of dipicolinate within the spores is quite different from that of tridentate calcium dipicolinate, a previously proposed model for in-spore dipicolinate structure. Structural possibilities are discussed in light of the Raman evidence.

INTRODUCTION: Typical bacterial spores contain approximately 10% by weight of 2,6 pyridinedicarboxylate,* which, together with calcium ion, is imputed to be responsible for the high resistance of the spores to heat and other environmental stresses (1). The structure of DPA within the spores is therefore an important point, concerning which little evidence exists. Ultraviolet and infrared spectroscopic results on intact spores have been interpreted to indicate that DPA is present in spores as the calcium chelate (CaDPA) (2,3) or as dipicolinic acid (H_2DPA) or an ester thereof (4). The location of the bulk of the DPA within the spore has recently been shown to be the protoplast (5). As the core region is only a third of the volume of the spore (6) this means that some 30% of the weight of the spore protoplast, which also

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*Abbreviations: 2,6 pyridinedicarboxylate = DPA; dimethyl sulfoxide = DMSO.

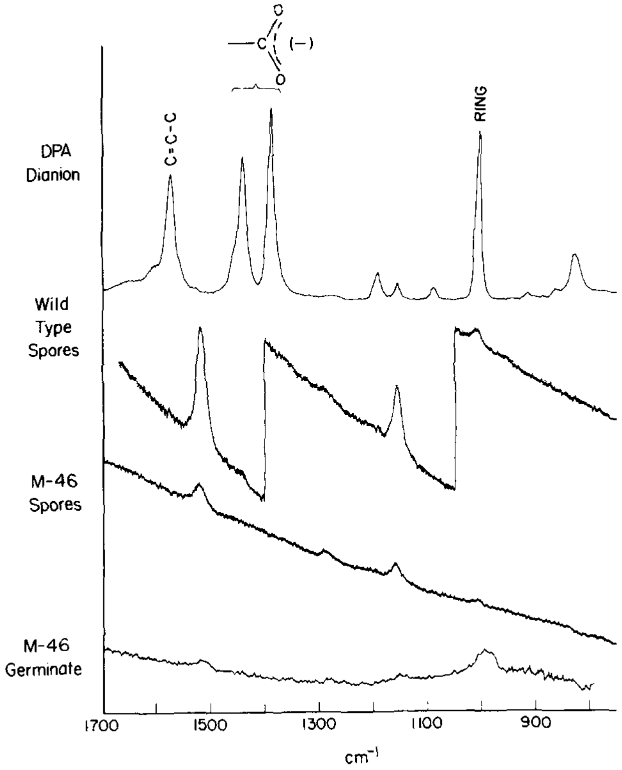

Figure 1. Raman spectra of spores and germinated spores of *Bacillus megaterium*, including the spectrum of the dipicolinate dianon in 1 *M* aqueous NaOH. The discontinuities in the spectrum of wild type spores are due to instrument baseline readjustments. Spectra were obtained using a Coherent Radiation CR-5 Ar^+ laser, a Spex 1401 double monochromator, and d.c. amplification of the signal from an ITT FW130 photomultiplier. Exciting wavelength was either 4880Å or 4579Å. Samples of the model compounds (Table I) were contained in 1 mm diameter capillaries, the solids being powder samples. Suspensions of the spores and germinate of *Bacillus megaterium* were filtered on 13 mm Millipore discs having 0.45 μm pore size. The discs were mounted on the Spex rotating sample accessory.

contains the spore nucleic acids and cytoplasmic proteins, is attributable to this one substance. We have obtained the Raman spectrum of intact spores of *Bacillus megaterium*. The spectrum is that of DPA in the form which predominates in the protoplast. The Raman evidence indicates that the DPA species in the spores is not the simple calcium chelate, or H_2DPA, or an ester or amide of DPA.

RESULTS AND DISCUSSION: Figure 1 shows the spectra of resting and germinated spores of *B. megaterium*. Two strains were used; a local strain (wild type) and a mutant strain M-46. The two spectra of the resting spores are similar, showing peaks at 1007, 1158, and 1520 cm^{-1} on top of a rising (fluorescent) background. (The spectrum of the M-46 spores is weaker than that of the wild-type spores because the former contains only about half as much DPA (7).) The spectrum of the germinated spores shows only a broad peak centered at approximately 995 cm^{-1}.

The spore spectra in Figure 1 are presented with the spectrum of the dianion of DPA in 1 *M* aqueous NaOH. The DPA dianion is one of several model DPA species examined for comparision with the spectra of the *B. megaterium* spores. The vibrations and assignments for these model species are given in Table I. Assignments were made by analogy to previous assignments for carboxylic acids (8-11,16) and pyridine (12).

The vibrations due to the aromatic ring of DPA vary little in frequency among the model species studied (Table I). The strongest of these ring vibrations is the symmetric "breathing" mode which occurs near 1000 cm^{-1}. In the spectra of the resting spores this vibration is seen as a weak feature at 1007 cm^{-1}. No other ring vibrations of DPA are observed in the resting spores. The primary chemical difference between resting and germinated spores is the absence of DPA in the latter (1). Therefore, the broad peak at 995 cm^{-1} in the spectrum of the germinated spores, which is obscured by the high background in the spectra of the resting spores, is probably due to the ring modes of the various

Table I

Raman Spectra and Assignments of Dipicolinate Species, 900-1800 cm^{-1}

Saturated H_2DPA in DMSO	Solid H_2DPA	Aqueous $DPA^=$	Solid CaDPA	Saturated Aqueous CaDPA	Assignment, Symmetry*
1753					Isolated C=O, A_1
1713	1614				Associated C=O, A_1
		1600	1628		1 + 6a**
1592			1589	~ 1580	C=C-C, A_1
1577	1576	1572	1570	~ 1580	C=C-C, B_1
1465	1447		1470		19a**
		1439	1445	1447	Ionized COO^-, A_1
		1386	1395	1397	Ionized COO^-, A_1
	1328				
	1298				Associated COOH, A_1
1270	1273				Associated COOH, A_1
1160	1182	1192	1194	1195	9a**
	1155	1155	1156		15**
		1087	1082		18a**
1007					Isolated C-O, A_1
998	999	1002	1017	1021	Ring, A_1

*Based upon polarization measurements for solutions, assumed for solids.

**Pyridine assignments, see Reference 12.

nucleic acid bases and aromatic protein side chains which are present (14,15). In contrast to the ring vibrations, the vibrational frequencies of the carboxylate groups of DPA differ substantially

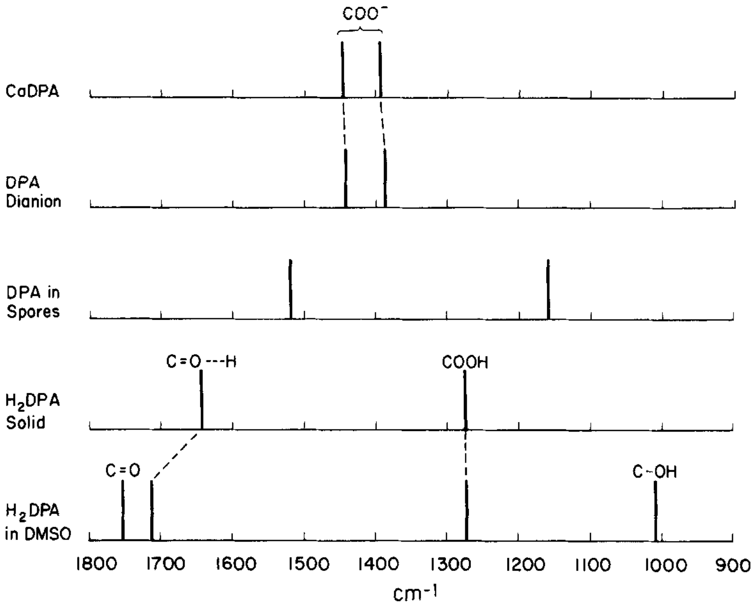

Figure 2. Frequency correlation diagram for carboxylate vibrations in DPA species, including DPA in spores of Bacillus megaterium.

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among the various derivatives (Table I). This is due to the varying degrees of polarization imposed upon the carboxylate groups by complexation, protonation, and hydrogen bonding.

We assign the intense bands at 1158 and 1520 cm^{-1} in the spectra of the resting spores to the two symmetric carboxylate stretching modes expected for DPA. Figure 2 shows the relationship of the carboxyl group frequencies among the DPA model species and in the spores. It appears that the DPA in the spore protoplast is subject to an interaction which causes the carboxyl groups to be polarized to an extent intermediate between $DPA^{=}$ and CaDPA on the one hand and H_2DPA on the other. The nature of this interaction is uncertain, but the Raman evidence is clear on the following point. The DPA species in these spore protoplasts is not the simple calcium salt. This conclusion is contrary to previous interpretations of infrared (2) and ultraviolet (3) spectra. The predominant DPA species in the spores is not H_2DPA or an ester or amide thereof. Any of these species would show a carbonyl stretching vibration near 1750 cm^{-1}. Intermediate carboxylate frequencies, such as those observed in the spores, have been observed in lanthanide carboxylates (8). It is possible that the frequencies in the spore Raman spectra reflect a mode of binding of calcium ions to DPA which is quite different from the tridentate coordination found in the calcium and strontium salts (18,19).

The intensity of the ring breathing mode of DPA in the spores is anomalously low compared to the carboxylate peaks. For the model compounds of Table I, the ring mode is usually the strongest feature in the spectrum. We interpret the intensity reduction in the spores as an example of Raman hypochromism (14), which suggests that DPA is involved in a ring-stacking interaction in the spore protoplast.

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