Ultraviolet-Absorption Spectra of Dry Bacterial Spores

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Abstract

Bailey, Glen F. (Western Regional Research Laboratory, Albany, Calif.), Saima Karp, and L. E. Sacks, Ultraviolet-absorption spectra of dry bacterial spores. J. Bacteriol. 92:984–987. 1955.—The possibility of obtaining reasonably satisfactory ultraviolet-absorption spectra of dry spores embedded in KBr has been demonstrated. Such spectra show the three peaks characteristic of calcium dipicolinate. The dipicolinate spectra are more distinct when reference pellets containing appropriate amounts of the analogous spore coats are employed. These spectra are considered evidence that some type of calcium-dipicolinic acid chelate accounts for at least part of the calcium and dipicolinic acid content of the spore.

The presence of large quantities (5 to 15%) of dipicolinic acid (DPA) in bacterial spores has led to considerable speculation that it plays an important role in conferring upon the spore its unique properties (Foster, 1959). The powerful chelating properties of DPA and the presence of approximately equal molar quantities of calcium have provoked speculation that Ca and DPA are combined in some molecular complex (Foster, 1959). There is considerable indirect evidence supporting this concept (Slepecky, 1961; Windle and Sacks, 1963), but direct evidence is lacking.

The powerful and distinctive ultraviolet-absorption spectra of the various chelates of DPA (Slepecky, 1961) have encouraged many investigators to consider the possibility of examining the ultraviolet-absorption spectra of intact spores to obtain evidence on the existence of such a Ca-DPA chelate. Unfortunately, the high refractivity of spores causes serious light-scattering at low wavelengths, which we have been unable to overcome by customary techniques, such as the use of opal plates (Shibata, Benson, and Calvin, 1954). Similarly, efforts to reduce scattering by suspending the spores in inert liquids of high refractive index (Barer, 1955) have generally failed because of ultraviolet absorption by the liquid.

This paper describes a technique capable of giving fairly well-resolved DPA spectra in spores dispersed in KBr, a solid having a refractive index very close to that of spores and exhibiting insignificant ultraviolet absorbance at wavelengths of interest. These spectra suggest that DPA is present as some form of calcium chelate in the intact spore. The hypothetical structure of Riemann (1961) may be one way in which it is chelated.

Materials and Methods

Spores. Lyophilized spores of Bacillus megaterium NRRL B-938, B. cereus T, and B. coagulans NCA 43P were prepared and stored as described previously (Sacks and Alderton, 1961). Clostridium bifermentans α ala #4 spores were prepared by cultivation on Trypticase Soy Broth (BBL) for 48 hr at 35 C. Spores were purified in a two-phase system by the method of Sacks and Alderton (1961), and the lyophilized spores were stored at 5 C. Spores of B. subtilis 15 u (obtained from Neva Snell of this laboratory) were prepared in the same way as the B. coagulans spores described previously (Sacks and Alderton, 1961), except that the lysozyme treatment was omitted. B. macerans 7X1 was prepared according to Sacks et al. (1964), except that the medium contained 5% agar instead of 10% agar.

KBr pellets. Spore samples were dispersed in 250 mg of infrared-grade KBr powder (100 mesh) by shaking for 2 min in a Wig-L-Bug dental amalgamator with a 55-mg steel ball pestle. Spore samples (0.25 to 0.50 mg) were weighed to ±2 μg.

The sample dispersed in the KBr was loaded in a uniform layer into a die (diameter, 12.7 mm) between optically finished, hardened-steel wafers (thickness, 3 mm), which served the double purpose of giving the pellets flat surfaces and of extruding them from the die without cracking. The die was closed, evacuated to <1 mm of pressure for 5 min, and pressed for 5 min at 10,500 kg/cm². Although this pressure is considerably

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higher than the minimum required to fuse the KBr powder into a solid pellet, it yielded pellets of lower and more uniform scattering. The pellets, after extrusion and weighing, were mounted in a 12.7-mm aperture for spectrophotometry.

“Spore coats” were prepared by dry-rupturing spores to >99% of total rupture (Sacks et al., 1964), washing them twice in cold glass-distilled water at 10,000 X g for 15 min, and lyophilizing the packed residue. These spore coats were then embedded in KBr as were the whole spores, and were usually employed as reference discs against the whole spores.

Spectrophotometry. Spectra were run with a Cary model 14 recording spectrophotometer with band width less than 1.2 mμ in the region of interest.

DPA complexes. CaDPA was prepared by dispersing 3.3 mmole of DPA (Aldrich) in 50 ml of deionized water and neutralizing by dropwise addition of 3.3 mmole of Ca(OH)\textsubscript{2} [245 mg of Ca(OH)\textsubscript{2} dispersed in 50 ml of water]. The solution was stored at 5 C. After 48 hr, the crystals were separated by membrane filtration and dried. Ca represented 15.7% by weight, as determined by the method of Williams and Wilson (1961), compared with a theoretical value of 15.45% for CaDPA-3H\textsubscript{2}O.

MgDPA was prepared by neutralizing 100 mg of DPA with fresh, saturated Mg(OH)\textsubscript{2}. After the pH reached 7.3, the preparation was filtered through a Millipore filter and air-dried.

K\textsubscript{2}DPA was prepared by neutralizing 100 mg of DPA with 1.0 N KOH. The solution was dried at room temperature, and the crystals were collected.

MnDPA was prepared as described by Windle and Sacks (1963).

Metal analysis. Potassium was determined by X-ray fluorescence. Mg was determined by the method of Young and Gill (1951). Mn and Ca were determined by emission spectroscopy (Windle and Sacks, 1963).

DPA analyses. DPA analyses were carried out by the method of Janssen, Lund, and Andersen (1958).

RESULTS

Figure 1A shows the spectrum of B. macerans in KBr when run against a standard KBr reference disc. The DPA peaks are clearly evident near 250, 272, and 262 mμ, but there is considerable background absorbance. Figure 1B shows the spectrum of the B. macerans coats run against KBr. All of the DPA has been released and extracted in water. The spectrum here mainly represents absorption by proteins in the coats, with some scattering. Figure 1C shows the dry-spore DPA spectrum obtained by running the whole-spore preparation against the corresponding coat preparation. This type of reference compensates for scattering and absorption by

![Fig. 1. Ultraviolet spectrum of Bacillus macerans 7X1 in KBr. (A) B. macerans run against clear KBr reference pellet; (B) B. macerans "coats" run against KBr reference pellet; (C) B. macerans whole spores run against "coat" reference pellet; (D) ultraviolet-absorption spectrum of aqueous extract of dry-ruptured spores of B. macerans, run against a water reference.](image1)

![Fig. 2. Ultraviolet-absorption spectra of various DPA salts and chelates, dispersed in KBr and run against clear KBr as a reference. Successive spectra are displaced 0.2 optical density units to avoid overlapping.](image2)
Table 1. Dipicolinic acid and cation content of spores

<table>
<thead>
<tr>
<th>Organism</th>
<th>DPA</th>
<th>Ca</th>
<th>Mn</th>
<th>Mg</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Per cent</td>
<td>Amt</td>
<td>Per cent</td>
<td>Amt</td>
<td>Per cent</td>
</tr>
<tr>
<td>Bacillus cereus T</td>
<td>6.94</td>
<td>41.5*</td>
<td>5.0</td>
<td>125*</td>
<td>0.32</td>
</tr>
</tbody>
</table>
| Clostridium biferm-
| 7.56 | 45.2   | 6.4    | 160    | 0.04  | 0.18  | 0.25    | 10   | 1.6     | 41  |
| B. macerans       | 9.12 | 54.6   | 2.0    | 50     | 1.4   | 25    | 0.53    | 22   | 3.8     | 97  |
| B. subtilis       | 8.88 | 53.1   | 3.9    | 97     | 0.46  | 8.4   | 0.17    | 7.0  | 1.8     | 46  |
| B. coagulans      | 9.10 | 54.4   | 2.3    | 57     | 1.2   | 22    | 0.54    | 22   | 0.65    | 17  |

*All amounts expressed as millimoles per 100 g.

non-DPA ultraviolet-absorbing materials. Figure 1D shows the aqueous extract obtained from the ruptured spores in preparing the spore coats. Allowance was made for the loss of weight due to soluble materials released upon preparation of the spores. Only about 60% as much solid matter is embedded in the coat preparations as in the whole-spore preparation, so both discs should represent the absorbance of approximately equal numbers of spores. Figure 1D shows that the DPA spectrum in the dry spore has the major features of the DPA in the aqueous extract. The peaks are displaced about 2 mλ, probably from the effects of the solid state as compared with the solution.

The spectra of various DPA salts and chelates embedded in KBr are shown in Fig. 2. These spectra are very similar to those of the same compounds in aqueous solution (see Slepecky, 1961). There are some slight wavelength shifts, probably resulting from the effects of the solid state. Otherwise, the spectra are very similar to those shown by Slepecky, except for the case of the Mg salt which shows some differences.

These spectra show that only two of the cations abundant in spores, Ca and Mn, will give a peak at 280 mλ. Mg shows a peak at 278 mλ and K shows no peak in this region. The 280-mλ peak of Mn is not nearly as prominent as that of Ca. This, coupled with the relative scarcity of Mn in B. macerans (Table 1), led to the conclusion that, in these spores, the DPA exists largely as a Ca chelate.

Spectra similar to that of B. macerans were obtained for B. coagulans, B. megaterium, B. subtilis, B. cereus, and C. bifermantans (Fig. 3), suggesting that Ca (or in some cases Mn) is the dominant DPA complex in dry spores. Uncompensated scattering raises the minimum near 250 mλ compared with the maximum near 280 mλ, as seen particularly in the curves for B. cereus and C. bifermantans and in the curve for B. macerans in Fig. 1. In the absence of such scattering, the 280-mλ peak would be slightly more prominent.

Table 1 shows the DPA and mineral content...
of the spores employed. *B. megaterium* and *B. coagulans* spores, which have a low Ca content, show a less prominent peak at 280 m\(\mu\) than do the spore species of higher Ca content (Fig. 3).

Spores which have been dispersed in KBr have poor viability, as evidenced by low plate counts when the discs are dissolved and plated. Preliminary experiments indicate that the spores are damaged chiefly during the dispersal in KBr, rather than in the pressing process.

**Discussion**

The spectra demonstrated by spores embedded in KBr are presumptive evidence that at least some of the Ca and Mn in spores is involved in a DPA complex. The peak at 280 m\(\mu\) is not as pronounced as it is in a water extract of the same spores, possibly because the Ca-DPA extracted from broken spores by water is not the same compound that exists in the spore. Riemann (1961) postulated that DPA exists in the spore as a protein-DPA-Ca complex, the ultraviolet-absorption spectrum of which can only be guessed. Other possible reasons for the discrepancy may be: (i) the large scattering involved in the solid state samples, and (ii) the different electrical environment (KBr vs. water) in which the samples exist.

If the spores recovered from the pellets have a low order of viability, as appears to be the case, a question regarding the significance of these results can be raised. Because water was absent during all stages of the process, it seems unlikely that mechanical damage of the spores would appreciably alter the ultraviolet spectra.

The height of absorption peaks obtained for dry spores embedded in KBr is only about 60% of theoretical, compared to aqueous extracts. This is probably a result of imperfect dispersal in the KBr (Otvos, Stone, and Harp, 1957).

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**Literature Cited**


