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ELECTRON PARAMAGNETIC RESONANCE OF MANGANESE(II) AND COPPER(II) IN SPORES

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SUMMARY

Paramagnetic resonance of manganese and copper have been observed in a number of different bacterial spores. The manganese spectra are different from those of corresponding vegetative cells and most other plant materials which have been investigated. The spectra consist of a single broad curve, that is 460–510 Gauss wide, with a sextet hyperfine pattern superimposed. Upon germination or autoclaving of *Bacillus megaterium* spores, most of the manganese is released and the residual cellular matter exhibits only the small sextet hyperfine spectrum. It is concluded that manganese in these spores is bonded in at least two different ways. The implications of the electron paramagnetic resonance results on the hypothesis of a metal–dipicolinic acid complex is considered. The electron paramagnetic resonance spectrum for copper in spores is consistent with bonding of copper to protein. Lyophilized clean spores do not exhibit a free-radical spectrum on prolonged storage in air.

INTRODUCTION

Bacterial spores contain much higher levels of divalent metals than do comparable vegetative cells[1]. Calcium is usually the most abundant, and a high calcium content is necessary for the formation of heat-resistant spores[2–4]. Manganese, although generally less abundant, is of equal interest because it is essential for sporulation[5]. Spores can also accumulate nickel, cobalt, zinc, and copper[4]. Most of these divalent metals appear to be interchangeable to a certain extent[4]. In addition to a high metal content, spores are also unique in possessing a high concentration of DPA, and there is indirect evidence which suggests that some of the metals may exist as DPA chelates in spores[6–9].

In this paper an account is given of an investigation of manganese and copper in clean spore preparations by means of EPR. An effort has been made to obtain information on the nature of these metals in spores from the EPR spectra of dormant

Abbreviations: EPR, electron paramagnetic resonance; h.f., hyperfine; DPA, dipicolinic acid; DPPH, diphenylpicrylhydrazyl.

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spores, spores treated in various fashions, and of some model compounds. The implications of this investigation on the metal–DPA hypothesis are discussed.

MATERIALS* AND METHODS

The EPR spectra were obtained at room temperature utilizing a recording X-band spectrometer of our own design. A Varian 6-in electromagnet and a 100-kc/sec, multipurpose cavity are used in this instrument. The first derivative of the absorption curve is recorded as a function of the applied magnetic field. The g values were measured by comparison with DPPH. The dry specimens were packed into quartz tubes 3 mm internal dia. Wet spores and aqueous solutions were placed in capillary 1.2–1.5 mm external dia. Where wet spores were used, the samples were packed by centrifugation.

Spores of *Bacillus megaterium* NRRL B-938, *B. cereus* Strain T, *B. coagulans* NCA Strain 43Γ, and *Clostridium bifermentans* (previously designated α-alanine No. 4) were prepared as described previously[10]. Spores of *B. subtilis* var. *globigii* were obtained from Fort Detrick through the kindness of Mr. N. E. LITTLE. All the above spore preparations were treated with a polyethylene glycol–potassium phosphate system to remove vegetative cells and debris[10]. *B. megaterium* QM B-1551 was obtained from Dr. H. LEVINSON. The lyophilized spore preparations were stored at 5°. DPA analyses were performed according to JANSSEN *et al.*[11].

Metal analyses were carried out by emission spectroscopy and manganese analyses were verified by periodate oxidation.

Manganese salts of DPA were prepared as follows: The 1:1 complex was made by adjusting 0.3 mmoles DPA (Aldrich Chemical Company) to pH 7.0 with 1.0 N NaOH in 5 ml glass-distilled water. An equimolar amount of a 1.0 M solution of the $MnCl_2$ was added slowly while maintaining a constant pH. The resulting crystalline precipitate was collected on a membrane filter and dried. The Mn–DPA crystals were white elongated, prismatic crystals with beveled ends. In polarized light they exhibited oblique extinction for all ordinary views. Analysis by periodate oxidation showed a 21 % Mn content as compared to a theoretical yield of 20.1 %.

The 1:2 complex was soluble in water and was prepared as above except that only half as much manganese was added. The final $Mn–(DPA)_2$ solution had a pale green color.

Germination of *B. megaterium* and *B. cereus* was carried out in the synthetic media described previously[10]. Spores were heat-shocked at 60° for 30 min prior to addition to the germination medium. After 15–20 min at 35° with aeration, the spores were removed by centrifugation, and the packed sediment examined by EPR.

In autoclave experiments spores were suspended (20 mg/ml) in water, and subjected to 121° for 1 h. They were then washed once in water, and the packed spores examined by EPR.

Vegetative cells of *B. cereus* were grown on medium G[12] in shake flasks at 30°, harvested at intervals, washed, and the packed cells examined by EPR.

* Reference to a company or product by name does not imply approval or recommendation of the product by the Department of Agriculture to the exclusion of others that may be suitable.

EXPERIMENTAL RESULTS

EPR spectra of dry, lyophilized spores of *B. megaterium* and *B. coagulans* that had been produced on manganese-rich media are shown in Figs. 1a and 2a. (Metal analyses and DPA levels are given in Table I.) The g value of 2.00 and characteristic sextet hyperfine pattern were considered presumptive evidence of manganese. The chief characteristic of these derivative spectra is the simple, broad curve which varies from 460 to about 510 Gauss in width between peaks. When the spores were suspended briefly in 1 N HNO_3[13,14], the broad curve was completely converted to the typical sextet h.f. pattern characteristic of manganous ion (Fig. 1b) thus confirming its identity. Washed vegetative cells of *B. cereus* grown on G medium showed only the usual sextet h.f. structure for manganese, commonly observed in plant tissues[15,16].

When dry spores were suspended in water, the spectrum was virtually the same as that obtained for dry spores (Fig. 3a), except that the h.f. pattern is more pronounced, relative to the broad curve. *B. megaterium* NRRL B-938 spores were allowed to germinate, and were then quickly centrifuged out of the germination medium. Their spectra revealed a narrow h.f. pattern (Fig. 3b); the broad peak had disappeared. Emission spectroscopy confirmed the fact that at least 85% of the manganese had been released into the medium during germination. The narrow h.f. spectrum had a component line width of about 10 Gauss and a h.f. interval of 91 Gauss. Autoclaving a suspension of *B. coagulans* (Fig. 2b) or *B. megaterium* for 1 h also resulted in the disappearance of the broad curve.

The h.f. pattern of the particulate residue had a hyperfine interval of about

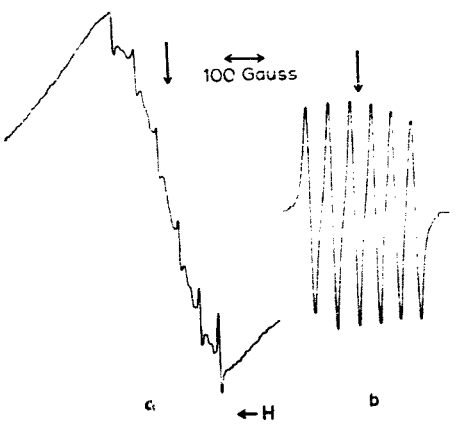

Fig. 1. (a) EPR spectrum for dry spore preparation of *B. megaterium*, NRRL B-938. Modulation amplitude 0.95 Gauss. The very sharp (about 10 Gauss) sextet h.f. pattern superimposed upon the broad derivative curve has only been observed for this strain. In this figure and the others $g = 2.00$ is indicated by the vertical arrow. The D.C. magnetic field is increasing to the left. The ordinate is dX''/dH, the first derivative of the imaginary part of the complex magnetic susceptibility. (b) *B. megaterium* spores suspended in 1 N HNO_3; sample centrifuged to pack spores. Instrument conditions, sample tube diameters, and packing conditions were different for dry and wet spore preparations, so these spectra can not be compared on a quantitative basis. Modulation amplitude 1.9 Gauss.

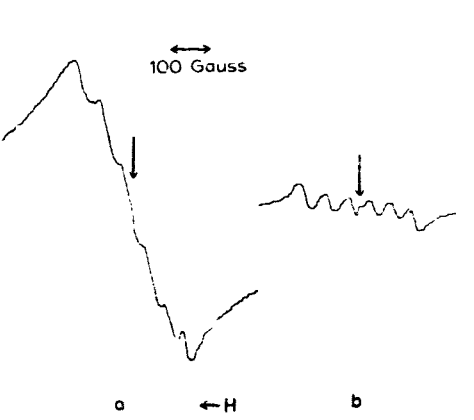

Fig. 2 (a) EPR spectrum for dry spore preparation of *B. coagulans*. A somewhat broader sextet h.f. pattern than observed for *B. megaterium* is superimposed. Modulation amplitude 0.75 Gauss. (b) *B. coagulans* after autoclaving for 1 h. Only the sextet h.f. pattern remains. The individual components are about 58 Gauss wide. Modulation amplitude 4.8 Gauss.

95 Gauss, and the line widths of the h.f. components average about 58 Gauss. These results suggest that the manganese spectrum is composed of at least two different manganese patterns superimposed and that the manganese responsible for the broad curve is associated with those portions of the spore that are released upon germination or autoclaving, whereas, the manganese giving the h.f. pattern is associated with the residue.

A broad manganese spectrum devoid of resolvable h.f. structure has been observed for manganese complexes formed with chelating agents such as nitrilotriacetic acid[17]. Inasmuch as DPA is a powerful chelating agent[7,18], the possibility that the broad manganese spectrum is due to a DPA chelate of manganese was investigated. The EPR spectrum of the polycrystalline powder, Mn-DPA, consists of a single curve about 1100 Gauss between points of maximum slope. However, such a broad line is to be expected from anisotropic interactions in the solid in which the concentration of manganese is of the order of 20 %. A 10 mM solution of $Mn-(DPA)_2$ (Fig. 4) gave a smooth curve about 502 Gauss wide which is quite similar in width to the spore spectra. When a 10 mM solution of $Mn-(DPA)_2$ was air-dried to yield a poly-

TABLE I

DPA AND METAL ANALYSES OF SPORES AND ASSOCIATED Mn EPR LINE WIDTHS

	DPA (%)	*Ca* ± 15%	*Mg* ± 10%	*Mn* ± 15%	*Cu* ± 50%	*W** ± 5 *Gauss*
B. megaterium NRRL B-938	9.6	2.1	0.92	0.88	0.017	461
B. megaterium QM B-1551	—	4.0	1.1	0.02	0.026	485
B. cereus Strain T	8.2	5.0	0.30	0.32	0.18	502
B. subtilis var. *globigii*	7.0	3.0	0.08	0.26	0.26	502
B. coagulans NCA 43P	—	3.0	0.43	1.18	0.01	508
C. bifermentans α-ala No. 4	10.0	4.5	0.30	0.02	0.1	—

* *W* is defined as the separation in Gauss between points of maximum and minimum deflection on the broad, smooth Mn curves.

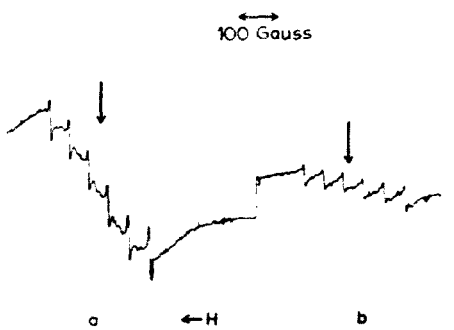

Fig. 3. Influence of germination on EPR spectrum of *B. megaterium* NRRL B-938. (a) *B. megaterium* suspended in water. Modulation amplitude 1.9 Gauss. (b) After germination. Only the sextet h.f. pattern remains; the narrow components are about 10 Gauss wide.

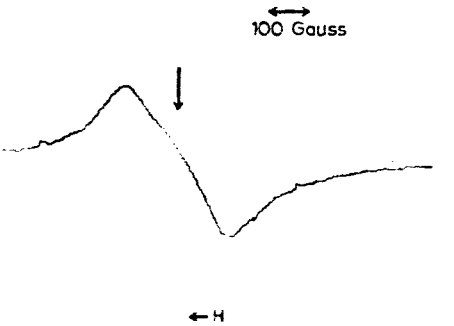

Fig. 4. EPR spectrum for a 10 mM solution of of $Mn-(DPA)_2$. Modulation amplitude 4.8 Gauss. In this and other Mn chelates giving similar spectra, the line width and separation of the Mn h.f. components are such that the resultant spectrum is a smooth, broad curve with no apparent h.f. structure[19,20].

crystalline powder, a spectrum similar to that of Mn-DPA was observed. When $Mn-(DPA)_2$ was treated with nitric acid the characteristic sextet h.f. of Mn^{2+} was obtained.

The EPR spectra of some Mn–protein systems were obtained for comparison. Aqueous solutions of egg albumin (10 %), gelatin (9 %), and lysozyme (10 %) at pH 7 containing 0.01 M $MnCl_2$ gave ionic-type spectra similar to those observed in plant tissue[15,16].

The EPR spectra for Cu(II) in spore preparations are easily recognizable from the asymmetrical shape of the spectrum, the h.f. structure, and g values. An example of such a spectrum is shown in Fig. 5 for *C. bifermentans*. In these spores the manganese concentration was not so high as to distort or obscure the Cu signal. At intermediate levels of manganese and copper, both spectra are evident as is the case for *B. subtilis* var. *globigii* spores (Fig. 6) and in *B. cereus* Strain T and *B. megaterium* QM B-1551. It is noteworthy that the Mn signal observed in this latter spore preparation is essentially the same as those previously described, in spite of its very low manganese concentration (see Table I). When these spores were autoclaved for 1 h and subsequently washed, no trace of the Cu signal remained.

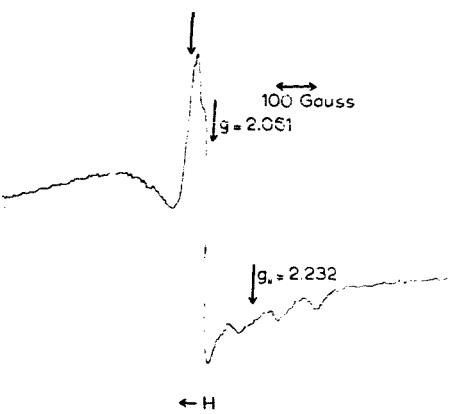

Fig. 5. EPR spectrum for the dry spore preparation *C. bifermentans*. The spectrum is due mainly to Cu(II). The largest peak has its absorption maximum at a g value of 2.061 and is due to lines centered at $g_\perp$. The right-hand portion of the spectrum is due to lines centered at $g_{\parallel} = 2.232$. The broad shoulder on the left is due to Mn(II). Modulation amplitude 4.8 Gauss.

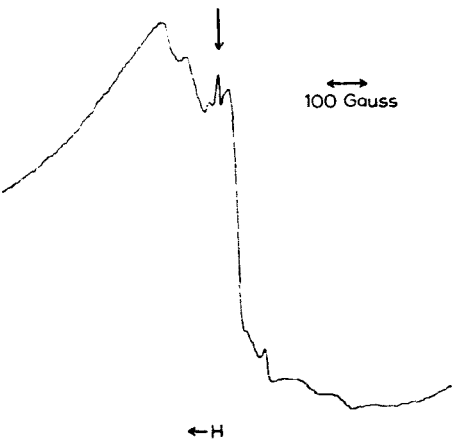

Fig. 6. EPR spectrum for the dry spore preparation *B. subtilis* var. *globigii*. This is an example of a spectrum in which Mn and Cu are both present in equal amounts. Modulation amplitude 1.2 Gauss.

DISCUSSION

The EPR spectra for Mn in most plant materials and bacterial vegetative cells consists of an almost completely resolved sextet h.f. pattern, similar in appearance to Mn^{2+} in aqueous solution. The Mn spectrum of bacterial spores is completely different. The germination and autoclave studies described above suggest that this is a composite spectrum consisting of a single smooth curve upon which is superimposed

a small ionic-type spectrum. Smooth, broad Mn curves of the type proposed for spores have been observed for some dilute Mn chelate solutions such as Mn–nitrilotriacetate and, in the present work, Mn–$(DPA)_2$*; thus the hypothesis of a composite spectrum is shown to be feasible. It is not proposed that Mn–$(DPA)_2$ is present, as such in the spore. However, there is considerable indirect evidence which suggests that Ca and Mn may be forming some type of DPA–metal complex in spores. The presence of nearly equal molar quantities of Ca and DPA[9,21,22], the mutual replaceability[4] of Ca and Mn, and the virtually simultaneous release of Ca, Mn and DPA upon autoclaving[9] or germination[6] are particularly relevant to the results of the present investigation. The EPR spectra may be understood in terms of a DPA–Mn chelate which exhibits a broad, smooth curve in the intact spore, and whose spectrum disappears following release of the Mn during germination or autoclaving. Although these results do not prove the existence of a DPA–metal chelate in spores, they are not inconsistent with such a hypothesis.

The Mn giving rise to the sextet h.f. pattern superimposed upon the broad signal had a h.f. interval which indicates almost complete ionic bonding[23,24]. This Mn usually remained associated with the sedimentable particulate matter after germination or autoclaving. The main constituent of the autoclaved residue is the proteinaceous spore coat[25]. The persistence of this h.f. spectrum in the washed residue suggests it represents Mn ionically bound to coat protein. The h.f. spectrum for *B. megaterium* NRRL B-938 is different from all of the other spores investigated in that it appears to contain a third Mn component. The extreme sharpness and intensities of the h.f. spectrum of this component closely resembles the "powder" spectrum for Mn(II) diluted in a diamagnetic solid[26]. At present we can offer no hypothesis to account for this spectrum in spores.

The EPR spectrum for Cu(II) in spores is very similar to the spectra observed in frozen solutions of Cu–protein complexes[27]. A comparison between the g values and the h.f. interval ($A = 159$ Gauss or 0.015 cm^{-1}) for spores with values for Cu–protein complexes[27] shows that the values for these parameters for spores are consistent with bonding of Cu to protein.

None of the lyophilized spore preparations grown and purified at this laboratory show free-radical spectra. This fact is noteworthy considering the development of prominent free-radical resonances by lyophilized vegetative cells stored in air[28,29]. The total absence of free radicals in clean spores, even after long storage in air may, perhaps, be considered further evidence of the extremely low level of metabolic activity in the dormant spore.

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* Such smooth curves, where the width between points of maximum slope is less than 700 Gauss, are indicative of some degree of covalent bonding[19,22].

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