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Use of Ultraviolet Radiation to Locate Dipicolinic Acid in *Bacillus cereus* Spores

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A novel method is described that allows a direct determination of the location of a portion of the dipicolinic acid (DPA) in spores. Ultraviolet (UV) irradiation is shown to cause cross-linkage of DPA to spore proteins which have the characteristics of membrane proteins. We suggest that DPA resides in the inner forespore membrane (IFSM) and spore cytoplasm (i.e., the spore protoplast). Only that portion of the DPA in the vicinity of the IFSM appeared to form UV-induced DPA-protein adducts.

Dipicolinic acid (DPA) was first discovered in bacterial spores by Powell (16) in 1953; it occurs in amounts of 5 to 15% of the spore's dry weight (12). Recent evidence (4-6, 8, 9, 15, 17) suggests that DPA is located in the protoplast rather than in the cortex (14). The difficulty in locating DPA lies in its ready release from spores upon germination and upon mechanical or chemical-enzymatic spore rupture. We reported that DPA sensitizes *Bacillus cereus* spores (6) and sporulating cells (5) to ultraviolet (UV) inactivation. This observation suggested that UV irradiation might provide a method of maintaining the DPA-spore association in broken spores by formation of covalent linkages between DPA and its molecular neighbors in the whole spore via UV-induced free radical attachment. In fact, evidence that less DPA is extractable from X-irradiated spores than from non-irradiated spores has already appeared (3). It was considered that mechanical breakage of UV-irradiated spores containing radioactive DPA and subsequent determination of the distribution of the "photochemically bound" radioactivity among major spore components should give a direct indication of its original location. In this paper our initial findings are described.

MATERIALS AND METHODS

Organism and media. *B. cereus* T strain HW-1, a gift of H. Orin Halvorson, was used throughout. This mutant strain is unable to synthesize DPA (21) but is able to incorporate added DPA during sporulation (7, 21, 23). The organism was grown and sporulated in modified G medium (18).

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Preparation of spores with radioactive DPA. Cultures in stage II of sporulation (5, 6) were supplemented with appropriate amounts of either [4-^{14}C]DPA, 300 μCi/mmol (synthesized by the Division of Radioisotopes, Australian Atomic Energy Commission), or [^{3}H]DPA, 2.8 mCi/mmol (The Radiochemical Centre, Amersham). Free spores were extensively washed with 0.05 M phosphate buffer, pH 7.0, and stored at 4 C. DPA was extracted from spores by the method of Lewis (10). Radioactivity was estimated as before (6). Thin-layer and paper chromatography of DPA extracts confirmed that the radioactivity resided only in DPA.

Irradiation. Vigorously stirred spore suspensions were irradiated with an 8-W germicidal lamp (254 nm) at room temperature, as previously described (6). All reported dosage levels were corrected for sample absorption (11). In the experiments where the effect of UV dose on the binding of DPA was studied, spores suspended in 30 ml of buffer (optical density at 254 nm of 0.8 to 0.9/cm) were irradiated. At the appropriate doses, 1-ml samples were removed and the DPA was extracted. Since each sampling reduced the layer depth of the spore suspension by about 3%, accumulated doses were calculated for each sampling interval. If corrected dosages had not been calculated, the large number of samples removed from each suspension would have resulted in a dosage error of over 30% in the last sample. Next, the ethanolic spore suspension (10) was membrane filtered (0.45 μm, Oxoid). Radioactivity remaining on the filter after ethanol and ether washes was taken as non-extractable DPA still in the spores. In every case, over 99% of the radioactivity present in the control (non-irradiated) spores was extracted (i.e., less than 1% retained on the filter).

Breakage of DPA-labeled spores and release of bound DPA. Spores containing either [^{14}C]DPA or [^{3}H]DPA were irradiated (1.2×10^{5} ergs/mm^{2}) and ruptured in 0.05 M phosphate buffer, pH 7.0, at 1 C by shaking with glass beads (0.1-mm diameter).

Samples (0.2 ml) were added to 0.8 ml of buffer containing the enzymes or reagents being tested for their ability to release bound DPA. One sample was immediately added to ice-cold trichloroacetic acid, to give a final concentration of 5%, and served as control (0% solubilized). In cases where an enzyme was not added, bovine serum albumin was included to give 100 μg/ml. All incubations, unless noted otherwise, were at 38 C for 60 min. After incubation, the samples were filtered through membranes (0.45 μm, Oxoid). Filters were washed with 5 ml of cold distilled water, 5 ml of cold 95% ethanol, and 1 ml of ether, dried, and assayed for radioactivity. Comparison of the percentages of total DPA photochemically bound as determined by (i) Lewis extraction, (ii) trichloroacetic acid insolubility, or (iii) retention on a membrane filter were (i) 39.1, (ii) 35.9, and (iii) 27.7, respectively (average of four experiments).

Density gradient centrifugation. Spores containing 3.18% [^{3}H]DPA were exposed to 2.4×10^5 ergs/mm^2, broken, and mixed with solid CsCl to give a final density of 1.67 g/cm^3. The 2.5 ml of solution overlaid with oil was centrifuged at 60,000 rpm (20 C) for 23 h in the SW65 rotor in a Beckman L2-65 ultracentrifuge. Gradient fractions were collected by pumping (0.6 ml/min) from the bottom of the tube onto Whatman 3 MM paper strips (2). The strips were then washed (cold 5% trichloroacetic acid, cold ethanol, and then ether), dried, sectioned, and assayed for tritium by liquid scintillation spectrometry.

Gel electrophoresis. [^{3}H]DPA spores were irradiated to 1.2×10^5 ergs/mm^2, broken, heated at 80 C for 1 min in 1% sodium dodecyl sulfate (SDS), and subjected to electrophoresis (2.5 mA/tube) in a 10% polyacrylamide gel with 0.3 M tris(hydroxymethyl)-aminomethane (Tris), 0.01 M ethylenediaminetetraacetic acid (EDTA), and 1% SDS (pH 7.85). Gels were then frozen with dry ice and sectioned into 2-mm slices, and the slices were dissolved overnight at 50 C in 30% H_2O_2 (19, 22); scintillation fluid (6) containing 20% ethanol (19) was added and the radioactivity was measured.

Preparation of crude spore membranes. Nonradioactive washed spores were treated with alkaline SDS-dithiothreitol reagent to extract coat proteins (1). After removal of the reagent by repeated washing with 0.05 M Tris-10^{-2} M $MgCl_2$, pH 7.6, spores were broken by two treatments with glass beads. Large spore fragments were removed by low-speed centrifugation. The supernatant solution containing cytoplasmic protein and membrane fragments was centrifuged at 120,000 × *g* for 2 h in a Beckman L2-65 ultracentrifuge. The pellet was taken up in the Tris-magnesium buffer, clarified by low-speed centrifugation, and then centrifuged at 120,000 × g. The final pellet was taken up in the Tris-EDTA-SDS buffer described above. Before application to the gel, a sample was treated at 80 C as above.

RESULTS

Selection of an appropriate system. Our method for estimating the location of DPA in spores depends on the ability to measure unambiguously UV-induced DPA adducts, both in whole spores and in mechanically disrupted spores. Since many DPA adducts are possible, chemical methods of identification would be less specific and sensitive. Thus, we chose to use spores containing radioactive DPA. Our method also required that all of the radioactivity in the spores reside in DPA. Because methods of obtaining wild-type spores specifically labeled only in their DPA are not available, we used a mutant of *B. cereus* T that is unable to synthesize DPA (21) but is capable of incorporating exogenously supplied DPA (5–7, 21, 23) during forespore development. Suspensions of DPA-labeled spores were irradiated, and the amount of DPA rendered non-extractable was determined. As previously reported (6), UV-irradiated spores neither became phase dark nor released DPA or other UV-absorbing material during irradiation. Thus, we assumed that the irradiation procedure did not alter the original location of DPA in the spore (i.e., did not induce migration of DPA).

Because the assay for DPA adducts depends on following DPA radioactivity, one must be certain that the radioactive label does not migrate from the DPA molecule during irradiation. We considered two such possible complications: (i) photochemical decarboxylation, which could lead to ^{14}C migration if carboxyl-labeled [^{14}C]DPA were used, and (ii) tritium exchange if [^{3}H]DPA were employed. To avoid these possible complications, initial experiments were done with [^{14}C]DPA labeled in the 4- position of the pyridine ring. However, as will be seen, [^{3}H]DPA gave essentially the same results as the [4-^{14}C]DPA, and thus tritium exchange was not a problem.

DPA binding in UV-irradiated spores. Spores (0.1% DPA, dry weight) labeled with [4-^{14}C]DPA were irradiated, and the non-extractable DPA was estimated (Fig. 1). UV irradiation "bound" a large fraction (about 35%) of the total DPA. Extractable radioactivity from the spores exposed to over 1.5×10^5 ergs/mm^2 showed on the chromatograph as two principal peaks, one at the position of DPA and the other in the α-picolinic acid region (data not shown). About 90% of the extractable radioactivity was in DPA. Due to the limited quantities and relatively low specific activity of the [4-^{14}C]DPA, the experiment was repeated by using spores labeled with [^{3}H]DPA (specific activity about ×10 higher than [4-^{14}C]DPA). We obtained a result similar to that in Fig. 1 (data not shown), except that the maximum percent of total DPA bound by irradiation was

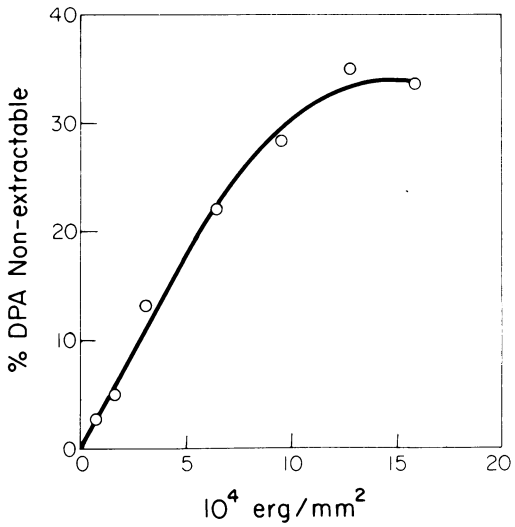

FIG. 1. *Binding of [4-^{14}C]DPA by UV irradiation.*

less than one-half that obtained with [4-^{14}C]-DPA spores. As will be apparent later, this difference was dependent upon the greater DPA content of the [^{3}H]DPA spores (0.6%). Chromatography of the extractable tritium from irradiated spores indicated that DPA accounted for about 90% of the radioactivity. This is in agreement with the [4-^{14}C]DPA findings.

The UV-induced binding of DPA in spores containing a range of [^{3}H]DPA contents is shown in Fig. 2. The actual amount of DPA bound per milligram of spores increased with the DPA content of the spores. In each spore crop the increment of DPA bound decreased above 15 $\times$ 10^4 to 20 $\times$ 10^4 ergs/mm^2. The maximum efficiency of DPA binding (maximum initial slope) was reached with about 1% DPA content. When the data were expressed as the percentage of the total DPA content of the spores that is bound (Fig. 3), it was seen that both the slope and the maximum percent bound decreased with increasing DPA content.

Distribution of bound DPA. Most of the bound DPA was found associated with the broken spore fragments retained on membrane filters. In some experiments, however, a variable portion of the bound DPA was lost from the spore fragments. This resulted, in part, from some endogenous enzymatic degradation of the spore fragments since it was accelerated by warming from 0 to 38 C (Table 1) and since about half of the released radioactivity was no longer precipitable with cold trichloroacetic acid. This endogenous activity was not inhibited with 10^{-3}M EDTA.

To show that binding of DPA occurred only during irradiation and not during breakage, non-radioactive wild-type spores containing over 10% DPA were irradiated to 1.35 $\times$ 10^5 ergs/mm^2, mixed with non-irradiated [^{3}H]DPA mutant spores (3% DPA), and broken with beads. In addition, a control mixture of non-irradiated wild-type and non-irradiated [^{3}H]DPA mutant spores was also broken. Radioactivity in the insoluble fraction accounted for 1.10 and 0.87% of the total radioactivity for irradiated and control mixtures, respectively. Since the percentage of [^{3}H]DPA that was released from these spore fragments was the same as for non-irradiated controls (99%), we concluded that DPA was not bound during breakage.

Because bound DPA was associated with the spore fragments, it was possible to screen a number of reagents for their ability to solubilize the bound DPA. Irradiated spores containing [4-^{14}C]DPA (0.1%) and [^{3}H]DPA (3.18%) were broken and then treated with the reagents listed in Table 1. After treatment, samples were filtered and the radioactivity remaining on the filters was determined. All figures in the table

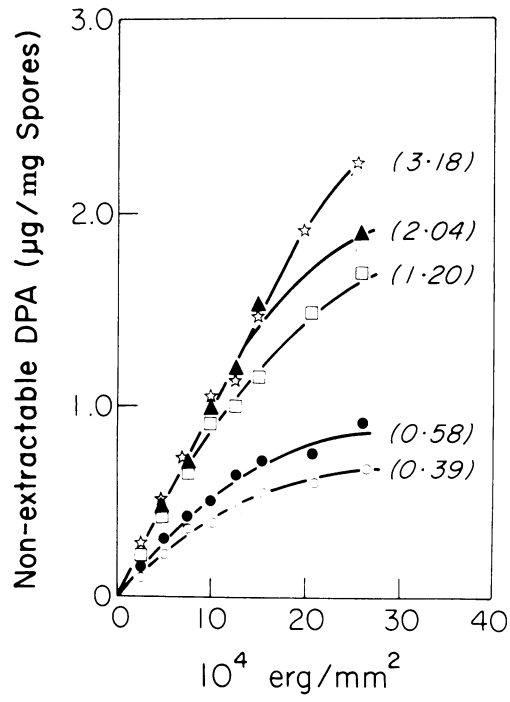

FIG. 2. *Effect of spore DPA concentration on [^{3}H]DPA bound by UV irradiation. Numbers in brackets refer to the percent DPA (dry weight) of each spore crop.*

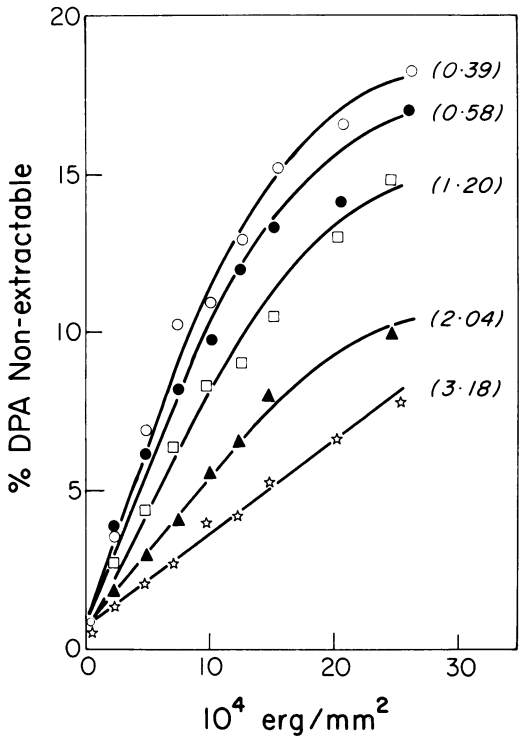

FIG. 3. *Percent of total [^{3}H]DPA bound by UV irradiation in spores with different DPA contents. Numbers in brackets refer to the percent DPA (dry weight) of each spore crop.*

are compared with the radioactivity in the cold trichloroacetic acid-insoluble fraction obtained for each spore crop. In the [^{14}C]DPA experiment, endogenous activity caused a 15% loss of label in 60 min at 38 C. Hot trichloroacetic acid released 19% of the label, and SDS and Pronase released about 80% each. The other enzymes were ineffective. Similar results were obtained with the [^{3}H]DPA spores. The results of two experiments are shown. In one experiment about 40% of the radioactivity was released when disrupted spores were incubated at 38 C for 60 min in phosphate buffer; at 0 C only 3% was released. In both experiments SDS and Pronase increased final solubility to 70 to 80%. Lysozyme, ribonuclease (RNase), and deoxyribonuclease (DNase) were ineffective (the percentage released was of the same order as the buffer control at 38 C). In both [^{3}H]- and [^{14}C]DPA spores, radioactivity solubilized by hot trichloroacetic acid or Pronase was not reprecipitated by cold trichloroacetic acid. In contrast, SDS-released radioactivity was insoluble in cold trichloroacetic acid. The same distribution of radioactivity was found in the [^{3}H]DPA and [^{14}C]DPA spores, even though their overall DPA content differed 30-fold.

Because DPA sensitized spores of *B. cereus* T (wild-type or DPA mutant) to UV inactivation, and since thymine photoproducts were more abundant in high DPA than in low DPA spores (6), we expected to observe DPA-deoxyribonucleic acid (DNA) adducts in UV-irradiated spores. Control experiments on spores whose DNA had been specifically labeled with [^{3}H]thymine indicated that in non-irradiated spores 80 to 90% of the DNA was released from the spore upon breakage, whereas in irradiated ($>10^5$ ergs/mm^2) spores 5 to 20% less DNA was released. About 80% of the released DNA, in both cases, was insoluble in cold trichloroacetic acid. The data in Table 1 suggest that a DNase-sensitive adduct was not formed. The absence of a high-molecular-weight DPA-DNA adduct is further substantiated by the following experiment. If DPA-DNA adducts were formed during irradiation, one should be able to demonstrate, after centrifugation in a CsCl gradient, DPA radioactivity in the region of spore DNA density (1.695 g/cm^3). As shown in Fig. 4, the results indicate that DPA-DNA adducts were not formed; the trichloroacetic acid-insoluble radioactivity assumed a position near the top of the gradient, and no peak of radioactivity was seen in the middle-lower portion (density >1.67 g/cm^3). Similar results were also

TABLE 1. *Effect of various treatments on bound DPA radioactivity*

Treatment	Radioactivity solubilized (%)		
	[^{14}C]DPA	[^{3}H]DPA	
		I[a]	II
Buffer			
0 C	0	0	3
38 C	15	3	39
Cold[b] trichloroacetic acid, 5%	0	0	0
Hot[c] trichloroacetic acid, 5%	19	23	—[d]
SDS, 1%	79	68	77
Pronase, 100 μg/ml	84	74	81
Lysozyme, 100 μg/ml	12	—	28
RNase, 100 μg/ml	7	—	31
DNase, 100 μg/ml	10	—	42

[a] Experiment number.
[b] 0 C.
[c] 85 C, 30 min.
[d] Not determined.

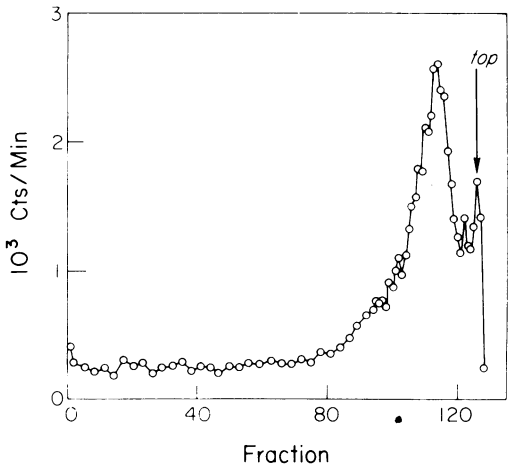

FIG. 4. *CsCl density gradient centrifugation of UV-induced DPA adducts.*

obtained if the sample was treated with Pronase before centrifugation or if the spores were actually broken in the presence of CsCl. Thus, if DPA-DNA adducts were present, they remained associated with the spore fragments (Fig. 4) in a DNase-resistant form (Table 1).

The results in Table 1 and Fig. 4 suggested that DPA became cross-linked to a structural protein component in the whole spore. Two such proteinaceous structural components of the spore are the coat layers and the inner and outer forespore membranes (IFSM and OFSM, respectively). DPA has been shown not to reside in the spore coat of wild-type *B. cereus* (1). To determine whether extraction of coat protein caused a marked loss of DPA in mutant spores, [^{14}C]DPA spores were extracted for 60 min at 38 C with an alkaline SDS-dithiothreitol reagent (1). The extract was assayed for release of DPA. Non-irradiated and irradiated ($>10^5$ ergs/mm^2) spores released 3.5 and 5.7% of their DPA, respectively. Because a marked loss of DPA was not seen, DPA does not reside in the coat proteins of mutant spores. As noted, this is consistent with the data for wild-type spores (1).

The IFSM and OFSM appear, therefore, as the most likely sites for the location of the photochemically active DPA. SDS-polyacrylamide gel electrophoresis of irradiated, broken, SDS-treated spores indicated that radioactivity banded in a number of positions (Fig. 5). Non-irradiated spores gave no evidence of radioactivity in the gels. DPA formed adducts with at least seven proteins. Most of the DPA-protein adducts corresponded to stained protein bands observed in polyacrylamide gels of crude spore membrane preparations (Fig. 5).

DISCUSSION

The results show that DPA is photochemically bound to proteins in the spore. From the data in Fig. 2, it can be estimated that about 3 μg of bound DPA per mg of spores in a reasonable estimate of the maximum binding to be expected in mutant spores containing 4% DPA (the maximum level obtained by mutant spores in our work). This represents about 8% of the total DPA. If a similar relationship exists between DPA content and UV-induced binding in wild-type spores, about 3% of the total DPA in spores containing 10 to 12% DPA would bind. Furthermore, if DPA is distributed uniformly throughout the protoplast in the normal spore, then the amount of DPA photochemically bound (3%) would approximate the ratio of volumes of the binding site to the spore protoplast.

The decrease in DPA bound with increased spore DPA contents suggests that at the lower concentrations (less than 0.4%) the DPA is more efficiently bound and/or a greater proportion of DPA is near a photochemically reactive site. At high DPA concentrations a greater proportion of the DPA is probably distributed away from the binding site. The binding site, however, continues to accept a diminishing proportion of DPA molecules over the total range of concentrations studied.

We previously reported (6) that mutant spores containing 0.05% DPA were over twice as resistant to UV inactivation as mutant spores with 4% DPA, and that the greater part of the difference in UV sensitivity between spore types

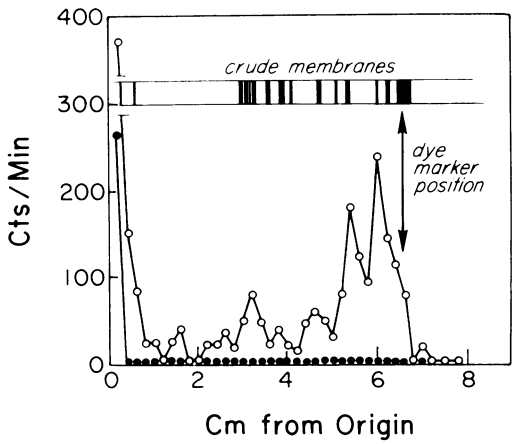

FIG. 5. *Polyacrylamide gel electrophoresis of [^{3}H]DPA-protein adducts. Coomassie brilliant blue-stained gel pattern of a B. cereus spore membrane preparation is included for reference. In all gels shown, bromophenol blue served as marker dye. Irradiated spores* (○); *non-irradiated spores* (●).

could already be seen in spores with 1% DPA. Thus, the concentration for both increased UV sensitivity (6) and for saturation (maximum binding slope) of binding occurred over the same low DPA range. Both wild-type and mutant spores exhibited an increase in UV sensitivity as their DPA content increased (6), although the wild type exhibited a greater increase in UV sensitivity for a given increase in DPA than did the mutant. Hence, it appears that DPA occupies the same site in mutant and wild-type spores. Mutant spores without DPA are markedly more sensitive to heat and more difficult to germinate than either mutant spores with DPA or wild-type spores (7, 23); thus, DPA confers similar characteristics on both types. We suggest, accordingly, that the data reported here on the mutant are at least qualitatively representative of the situation in the wild type.

Since the DPA rendered non-extractable by UV irradiation occurred in the particulate fraction retained on the membrane filters, the soluble cytoplasmic contents of the spore do not constitute the binding site. DNase and RNase failed to release bound DPA, and DPA-DNA adducts were not seen in CsCl gradients. If nucleic acids formed adducts with DPA, they were in a nuclease-resistant form in spore fragments. The limited solubilization of radioactivity by hot trichloroacetic acid could indicate the presence of such nuclease-resistant adducts. In addition, some DPA-protein adduct linkages may be hot acid labile. Although some endogenous release occurred during incubation in buffer, neither lytic action during breakage of spores nor added lysozyme released DPA. Thus, it is unlikely that DPA occurs in the cortex. As already mentioned, spore coats are not involved in DPA binding.

Pronase solubilized 80% of the radioactivity into a trichloroacetic acid-soluble form. SDS released a similar amount of DPA. Detergent-released radioactivity was trichloroacetic acid insoluble. The low density of the DPA adducts as judged by the behavior in CsCl also suggests the presence of DPA-protein adducts. The data indicate that the proteins involved in the binding of DPA are most likely associated with the IFSM or OFSM. We have suggested that UV sensitization of spores by DPA occurred through transfer of energy to DNA (6). Such a transfer requires a DPA to DNA proximity within 10 nm. In the mature spore the DNA is in a peripheral position just inside the IFSM. Because the range of DPA concentrations most effective in UV sensitization (6) and in DPA binding is similar in mutant spores, we suggest that the binding site is close to the DNA and is active in UV sensitization. The DNA-distal location of the OFSM therefore rules it out and suggests the IFSM as the binding site. Electrophoresis of SDS-solubilized DPA-protein adducts in polyacrylamide gels indicated that a number of different proteins were capable of forming adducts with DPA (Fig. 5). Thus, the possible association of DPA with membrane structural protein (and perhaps membrane-bound proteins) in normal spores may be a relatively nonspecific one (with the possible exception of a membrane-bound DPA permease system). Earlier work (1, 6) also supports the conclusion that DPA is present in the IFSM.

The cytoplasmic volume (28×10^7 nm^3), based on a prolate spheroid model, is about 30 times greater than the IFSM volume (about 1.1×10^7 nm^3). This is in agreement with the proposed ratio of the volumes of the DPA binding site and the total volume populated by DPA in the spore given above. Evidence from studies with cortexless mutants (4, 15), with spores produced in the presence of different carbon sources (8), and from calcium location (17) and β-attenuation studies (9) indicates that DPA is probably confined to the cytoplasm. Our results are consistent with these findings.

Finally, the basis for the apparently low photochemical reactivity of the bulk of the spore DPA is not known. The concentration of DPA in normal spores is 1 to 2 M, a value far in excess of its aqueous solubility of about 4×10^{-2} M. Perhaps the combination of a high DPA content and a low free water content (see reference 4) of the cytoplasm enhances quenching of photochemically excited species.

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