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Isolation and Characterization of Two Distinct Fractions from the Inner Membrane of Dormant *Bacillus megaterium* Spores

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Two distinct membrane bands were obtained after sucrose velocity gradient centrifugation of crude inner membranes from dormant *Bacillus megaterium* spores disrupted under conditions which minimized endogenous enzyme action. These two inner membrane fractions (termed LD and HD) contained similar amounts of total and individual phospholipid species. However, LD and HD differed significantly in phospholipid/protein ratios (4.3 and 0.47 mg/mg, respectively), equilibrium densities (1.12 and 1.18 g/cm^3), NADH oxidase specific activity (<0.01 and 0.13 μmol/min · mg), and content of specific proteins. In contrast, crude membranes prepared in identical fashion from germinated spores gave only a single inner membrane band (termed G) on sucrose velocity gradients. G had a phospholipid/protein ratio of 0.98 mg/mg, an equilibrium density of 1.16 g/cm^3, and an NADH oxidase specific activity of 2.1 μmol/min · mg. Essentially all of the proteins present in LD or HD or both were found in G, consistent with the latter membrane being derived from a mixture of LD and HD. No evidence was found suggesting that there is significant degradation of dormant spore inner membrane protein upon spore germination.

Dormant bacterial spores contain two distinct membranes, termed inner and outer membranes (4). The inner membrane directly surrounds the spore core or protoplast, whereas the more peripheral outer membrane is separated from the inner membrane by the peptidoglycan cortex of the spore (4). These two membranes differ from each other in lipid and protein content and undergo a number of significant changes upon spore germination (3, 4, 24). The changes in the inner membrane are particularly dramatic and include (i) a 10- to 20-fold activation of membrane-bound respiratory enzymes (23, 27), (ii) a large, probably transient increase in membrane permeability to ions such as H^+, K^+, and Na^+ (25), (iii) increases in membrane fluidity (7, 24), and (iv) phospholipid turnover (9), although the magnitude of this turnover has not been reported. A recent report has also suggested that a large amount of the inner membrane protein of dormant spores of *Bacillus megaterium* KM is degraded upon spore germination and that this may account for up to one-half of the protein degraded during spore germination (23).

The amount of dormant spore protein hydrolyzed early in spore germination is significant, ranging from 7 to 20% of the total spore protein in different species (8, 20). In contrast to the report cited above (23), other studies have indicated that the great majority of the spore protein degraded is a group of low-molecular-weight spore-specific proteins found in the spore core (20). Because of our interest in proteolysis during spore germination, we decided to reexamine the question of membrane protein degradation during spore germination, taking careful precautions to ensure that the membranes we analyzed were as free from contaminants as possible and that enzymatic action during spore breakage and membrane isolation was minimized. Our studies provided no evidence for significant membrane protein degradation during spore germination but revealed a hitherto undescribed heterogeneity in the inner membrane of dormant spores.

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MATERIALS AND METHODS

Reagents and spores. All reagents and enzymes used were purchased from Sigma Chemical Co.

The organism used in this work was *B. megaterium* QM B1551 (originally obtained from H. S. Levinson, U.S. Army Natick Laboratories, Natick, MA). Cells of this organism were grown and sporulated at 30°C in supplemented nutrient broth, and dormant spores were harvested, washed, and stored as previously described (21). Stripped dormant spores, from which coats and outer membrane have been removed, were prepared by extraction with dithiothreitol and sodium dodecyl sulfate at pH 10 as described by Vary (26). Spore germination was preceded by a heat shock (20 min, 60°C) of the spores (25 mg/ml) in water, followed by cooling in ice. Spores (20 mg/ml) were germinated in 0.1 M glucose and 50 mM Tris-hydrochloride (pH 7.5). After 10 min at 30°C, >95% of the spores had germinated, as determined by observation in the phase-contrast microscope. The spores were centrifuged (5 min, 10,000 × *g*), and the pellet fraction was disrupted (see below).

Disruption of spores. Dormant, stripped dormant, or germinated spores (0.5 to 1 g [dry weight]) were disrupted by being shaken with 50 g of glass beads (0.15 to 0.18 mm) for three 30-s pulses in a Braun MSK homogenizer with compressed CO_2 as a coolant, as described by Wilkinson et al. (27), in 7 ml of 50 mM Tris-hydrochloride (pH 7.5) containing 0.25 mM phenylmethylsulfonylfluoride and 10 μg of RNase A per ml (buffer A). After breakage was >85% complete, as determined by microscopic examination, the slurry was diluted with ice-cold buffer A (50 ml), and the glass beads were allowed to settle in a cylinder. The supernatant fluid was decanted, and the glass beads were washed with cold buffer A (50 ml). The final volume of the combined, decanted supernatant fluid was about 100 ml. Alternatively, stripped dormant spores (0.5 to 1 g) were treated with lysozyme (12 min, 30°C) and sonicated with glass beads as described by Racine and Vary (15), with the addition of 0.25 mM phenylmethylsulfonylfluoride. All subsequent steps were at 4°C.

Purification of membrane fractions. The initial steps in the purification of the spore inner membrane fraction were essentially identical to those described by Wilkinson et al. (27). The crude disrupted spores were centrifuged (5 min, 3,000 × *g*) to remove unbroken spores and remaining glass beads. The majority of the spore integument fraction (spore coat, cortex, and entrapped outer spore membrane) was removed by two further centrifugations (5 min, 27,000 × *g*), and the crude inner spore membrane fraction was collected by high-speed centrifugation (60 min, 105,000 × *g*) and washed once in 100 mM Tris-hydrochloride (pH 7.5) containing 10 mM $MgCl_2$. The washed membranes were suspended in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) pH 7.5 (buffer B) containing 5% (wt/wt) sucrose and run for 16 h on a sucrose velocity step gradient as described by Racine and Vary (15). Fractions (1 ml) were collected from the top; pooled membrane fractions were diluted with buffer B to less than 10% sucrose, sedimented at 105,000 × *g* for 2 h, and resuspended in buffer B.

For further purification on equilibrium sucrose gradients, membranes were resuspended in buffer B containing 50% (wt/wt) sucrose. Gradients in buffer B were layered in steps as described by Osborn et al. (14) with 0.5 ml of 60% sucrose, 1.5 ml of 55% sucrose, and 1.5 ml of 50% sucrose containing pooled membrane fractions from the velocity gradient, followed by 1.5-ml layers of 45, 40, 35, 30 and 25% sucrose. Gradients were centrifuged at 4°C for at least 72 h at 36,000 rpm in an SW41 Ti rotor.

Analytical methods. Protein was usually determined by the method of Lowry et al. (11) with the modifications described by Seto-Young and Ellar (23). In certain samples, protein was determined by reaction with ninhydrin after alkaline hydrolysis or by amino acid analysis after acid hydrolysis (22). Phospholipids were extracted from disrupted spores or membrane fractions by the procedure of Rothman and Kennedy (17), and from intact spores by the method of Bertsch et al. (1). Lipid phosphate was determined by the method of Chen et al. (2). A weighted average for total phospholipids of *B. megaterium* spores of 0.894 mg of phospholipid per mmol of lipid phosphate was used to calculate phospholipid weights (1). RNA concentrations in sucrose velocity gradient fractions were determined by precipitating samples in cold 10% trichloroacetic acid, washing the pellet twice with 95% ethanol, extracting it twice for 15 min at 90°C in 5% trichloroacetic acid, and determining the absorbance at 260 nm of the hot trichloroacetic acid-soluble material. No DNA was detected in these fractions by using the diphenylamine reaction (18). NADH oxidase activity was measured spectrophotometrically at room temperature by the oxidation of NADH in 50 mM Tris-hydrochloride (pH 7.5) (27). One unit of NADH oxidase is defined as the amount which oxidizes 1 μmol of NADH per min. Thin-layer chromatography of lipids was performed on silica gel G plates (Analtech) which were developed in chloroform-methanol-water (65:25:4). Chromatograms were stained with both iodine vapor and molybdate spray. Sodium dodecyl sulfate-containing acrylamide slab gels were prepared, and samples for analysis were treated as described by Laemmli and Favre (10). Gels were stained with silver nitrate by the method of Merril et al. (12).

RESULTS

Two methods have been used to isolate inner membranes from dormant bacterial spores. One uses mechanical breakage of spores followed by differential centrifugation to obtain crude inner spore membrane (23, 27), whereas the other first removes spore coats, disrupts spores with lysozyme, isolates crude inner membranes by differential centrifugation, and further purifies the membranes by sucrose velocity gradient centrifugation (15). The results from the latter purification procedure clearly indicate that inner spore membranes purified only by differential centrifugation are impure, being contaminated with RNA, hexosamine (spore cortex), and probably fragments of spore coats (15). However, we were concerned that lysozyme lysis of dormant spores might mimic spore germination in some ways and thus prevent isolation of a true dormant spore inner membrane. Consequently, we chose to routinely break spores mechanically at low temperatures to preclude, in so far as was possible, endogenous enzyme action, and then to purify crude membranes extensively via sucrose gradient centrifugation.

Purification of germinated spore membranes in this fashion resulted in two bands being visible to the naked eye upon sucrose velocity gradients (Fig. 1A) as reported by Racine and Vary (15). The lower band (I) was extremely turbid and contained high levels of hexosamine (data not shown); it presumably represents small pieces of integument not removed by differential centrifugation (6, 15); the upper band (G) was the germinated spore membrane (see below). Crude dormant spore inner membranes also gave a predominant integument band upon sucrose velocity gradients, but surprisingly they gave two visible bands (LD and HD) in the region in which the germinated spore membrane ran (Fig. 1B).

Chemical analyses of samples across the velocity gradients revealed that the fractions (G, HD, LD, and I) which contained the visible pigment (λ_{max}, 485 nm) also contained the great majority of the phospholipid, although there was a pigment shoulder above LD which had little phospholipid (Fig. 2). We routinely recovered ~50% of the spore phospholipid in the crude membranes which were applied to these velocity gradients; of this phospholipid, 55 to 65% was in the LD plus HD fractions, and 70% was in the G fraction (Fig. 2). These recovery values are similar to those reported

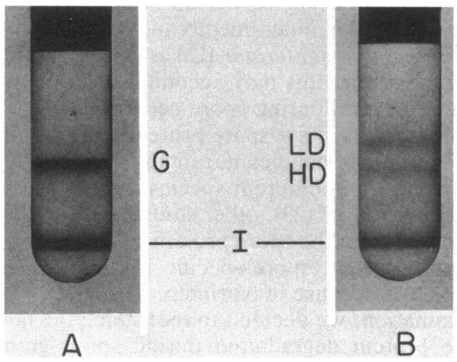

FIG. 1. Photograph of crudely purified germinated (A) and dormant (B) spore inner membranes run on sucrose velocity gradients. Dormant or germinated spores (0.5 g) were disrupted with the Braun homogenizer, and the crude membrane fraction was isolated as described in the text and run on a sucrose velocity gradient for 16 h at 4°C. The membrane bands contained a red pigment and were readily observed with the naked eye. They were photographed by using panchromatic film with a green (X1) filter. LD, Light dormant spore inner membrane; HD, heavy dormant spore inner membrane; G, germinated spore membrane; I, integument fraction containing small fragments of spore coat, cortex, and outer spore membrane.

by others (15). Analysis of sucrose velocity gradients of 10 independent inner dormant spore membrane preparations showed that, on the average, the phospholipid in the LD fraction contained only slightly more than half (55%) of the phospholipid in both the LD and HD fractions (Table 1). Thin-layer chromatography of the phospholipids extracted from LD, HD, and G showed that all three fractions contained the same phospholipid species in proportions similar to those previously reported (1, 15), but they contained no detectable neutral lipid (data not shown). Neutral lipid has been reported to be a major component of the spore outer membrane (3, 4).

NADH oxidase cosedimented with the G and HD fractions but was present in very low levels, if at all, in LD (Table 1). Moreover, the specific activity of NADH oxidase in the G fraction was much greater than that in HD, which is similar to the reports of Ellar and co-workers of a 10- to 20-fold increase in NADH oxidase specific activity in the crude membranes of spores after germination (3, 23, 27).

Although it was clear that a significant amount of protein

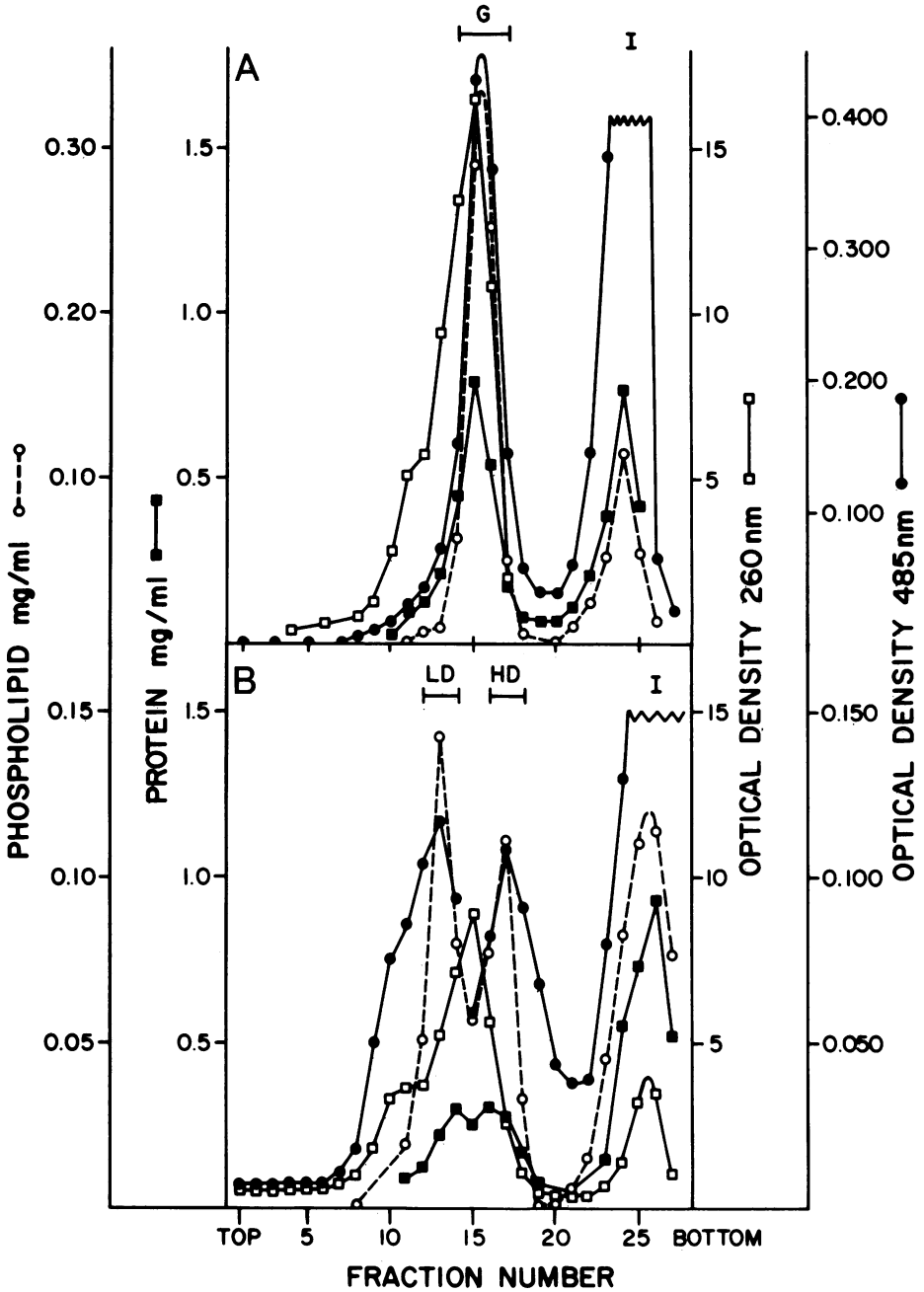

FIG. 2. Sucrose velocity gradient profile of crude inner membrane from germinated (A) or dormant (B) spores. Dormant or germinated spores (0.5 g) were disrupted with a Braun homogenizer; crude inner membranes isolated by differential centrifugation were run on a sucrose velocity gradient, and fractions were analyzed as described in the text. The brackets over the various peaks denote representative fractions pooled for detailed analysis or further purification.

TABLE 1. Properties of velocity gradient-purified membrane fractions from dormant and germinated spores[a]

Fraction	Component-mg per g of dormant spore:			Phospholipid/protein ratio (mg/mg)	NADH oxidase (U/mg of protein)
	Phospholipid[b]	Protein[c]	RNA		
LD	1.08	0.70	0.44	1.55 (0.97–2.11)	<0.01
HD	0.92	3.55	0.59	0.26 (0.20–0.37)	0.13
G	2.26	4.26	1.46	0.53 (0.43–0.78)	2.2

[a] Membrane fractions were isolated and purified through the velocity gradient step and analyzed as described in the text. Typical fractions pooled for analysis of various membrane fractions are given in Fig. 2A and B. Values are the averages of six separate membrane purifications, and the values in parentheses are the range of values obtained from the six separate membrane purifications.

[b] Total phospholipid is 9.7 mg/g of disrupted dormant spore, of which 50% was recovered in the crude membrane fraction applied to the velocity gradient.

[c] Values determined by Lowry assays; data have been corrected for contamination of fractions by ribonucleoprotein.

in the crude membrane fraction was due to contaminating integument fragments (I band), accurate determination of the amount of protein in the other membrane bands was difficult because the protein patterns did not exactly follow the phospholipid or pigment profiles in these gradients (Fig. 2). This was almost certainly due to the presence of RNA, probably ribonucleoprotein, which sedimented close to the membrane fractions (see profiles of the optical density at 260 nm, Fig. 2) as noted previously by Racine and Vary (15). When the RNA content of the various fractions was determined and the RNA associated protein (67% of the weight of the RNA) was subtracted from each fraction, it became clear that HD and LD had significantly different phospholipid/protein ratios, with G having an intermediate value (Table 1).

These differences in phospholipid/protein ratios were confirmed by further purification of these fractions by equilibrium density gradients (Fig. 3). HD and G fractions gave only single visible pigment bands in the gradient, with significant amounts of UV-absorbing material at the bottom of the gradient (Fig. 3B and C). Since this latter material had a ratio of absorbance at 260 nm/absorbance at 280 nm of about 2, it is undoubtedly ribonucleoprotein, which is known to sediment to the bottom of such gradients (16). The LD fraction gave two visible pigment bands on equilibrium gradient centrifugation: one at the position of HD, which undoubtedly represents contamination of the initial LD fraction with HD, and a second peak at a lower density, which is the purified LD (Fig. 3A). The crude LD fraction also gave significant RNA at the bottom of the gradient.

Chemical analysis of these gradients showed that only phospholipid and protein cosedimented with the pigment bands, that RNA was found only near the bottoms of gradients, and that little other protein could be found except that sedimenting with the membrane fractions or RNA (Table 2). The phospholipid/protein ratios of purified LD and HD differed by a factor of 9, with G having an intermediate value twice that of HD (Table 2). Similarly, LD had an equilibrium density much lower than that of HD, with G having an intermediate value (Table 2). The disparity between the phospholipid/protein ratios determined before and after the equilibrium gradient purification may be due in part to the correction for ribonucleoprotein applied to the velocity gradient-purified membranes but also to the different protein assays used in the different experiments (Tables 1 and 2).

Analysis of proteins present in LD and HD by sodium dodecyl sulfate-acrylamide slab gel electrophoresis revealed that LD had fewer major protein bands than HD (Fig. 4, lanes 1 and 3). The majority of the prominent LD protein bands were present in HD, and this was confirmed by electrophoretic analysis of a mixture of LD and HD (Fig. 4, lane 5). However, there were some protein bands which were present in LD at significantly higher levels than in HD and vice-versa (Fig. 4, lanes 1, 3, 4, 5, and 6). Comparison of protein profiles of the LD, HD, and G fractions showed that most prominent protein bands in HD and LD were present in G, and conversely, that the major protein bands in G were present in HD, LD, or both (Fig. 4).

Analysis of spore membranes prepared by other methods. Since purification of inner membranes from dormant spores broken with the Braun homogenizer had given the unexpected finding of two distinct fractions, we felt it was imperative to purify and analyze inner membrane from spores (i) which had their coats and outer membrane removed (stripped) and (ii) which were first stripped and then disrupted by lysozyme lysis (15). Sucrose velocity gradients of crude membranes isolated from stripped dormant spores which were broken with the Braun homogenizer showed essentially identical amounts of LD and HD as found in gradients of membranes from unstripped spores (Fig. 1B and 2B), but the amount of material in the I band was reduced more than fivefold (data not shown). The absence of a prominent integument band on sucrose velocity gradients of crude membranes of stripped spores has been noted previously (15). Similarly, crude membranes from stripped dormant spores treated with lysozyme followed by sonic disruption had little or no integument band but also had only a single major membrane band in the LD-HD region as reported previously by Racine and Vary (15) (Fig. 5). The phospholipid/protein ratio of this membrane fraction (as determined by Lowry assays and after correction for ribonucleoprotein) was 0.36, and the NADH oxidase specific activity was 1.1, similar to values reported previously (14) and closer to the values determined for G than to those for HD or LD (Table 1). The density of the membrane band isolated as described above has been reported to be 1.165 g/cm^3 (16), similar to the value we found for the G band.

DISCUSSION

The data presented in this communication do not support an earlier report (23) that 50% of spore inner membrane protein is selectively degraded upon spore germination. Thus, the phospholipid/protein ratio of the purified G membrane fraction is between the values obtained for the two purified dormant fractions HD and LD. If we assume that 50% of dormant spore inner membrane spore inner membrane/phospholipid is in the HD and LD fractions respectively and that these membranes combine to form the G membrane, this would generate a membrane with a phospholipid/protein ratio of 2.4, which is higher than the value obtained for the G fraction. Similarly, electrophoretic analysis of the proteins from purified dormant and germinated membranes revealed no obvious losses of major membrane proteins. Although it is certainly possible that there is degradation of a small amount of membrane protein or a parallel degradation of membrane protein and phospholipid upon spore germination, we found no evidence for the twofold increase in the phospholipid/protein ratio of the inner spore membrane upon germination as reported previ-

ously (23). For this earlier report, researchers analyzed only crude inner spore membranes, and as seen in Fig. 2, much of the protein in these crude membranes is not associated with the membrane fraction but rather with the integument fraction. The fact that the combination of LD and HD would give a higher phospholipid/protein ratio than found in the G membrane would actually suggest that there is significant phospholipid degradation upon spore germination. Indeed, such phospholipid degradation has been reported (4).

Certainly the most striking finding in this study was that fractionation of crude dormant spore membranes gave two distinct membrane fractions (HD and LD) where the germinated membranes gave only one fraction (G). HD and LD are clearly distinct entities based on differences in (i) phospholipid/protein ratios, (ii) equilibrium densities, and (iii) levels of specific proteins, in particular, NADH oxidase. Since HD and LD are well separated by sucrose velocity gradient centrifugation, it is also possible that these fractions differ significantly in particle size; however, we have not measured this parameter.

Although the dormant spore contains two distinct membranes, the inner and outer, we believe LD and HD are derived from the spore inner membrane for the following reasons. (i) Previous work has indicated that most of the spore outer membrane is removed by the differential centrifugation steps involved in preparation of the crude inner membrane fraction (3, 4); (ii) stripped dormant spores with much of their outer membrane removed (15, 26) gave yields of LD and HD identical to those of unstripped spores; (iii) spore outer membrane is reported to have a different lipid

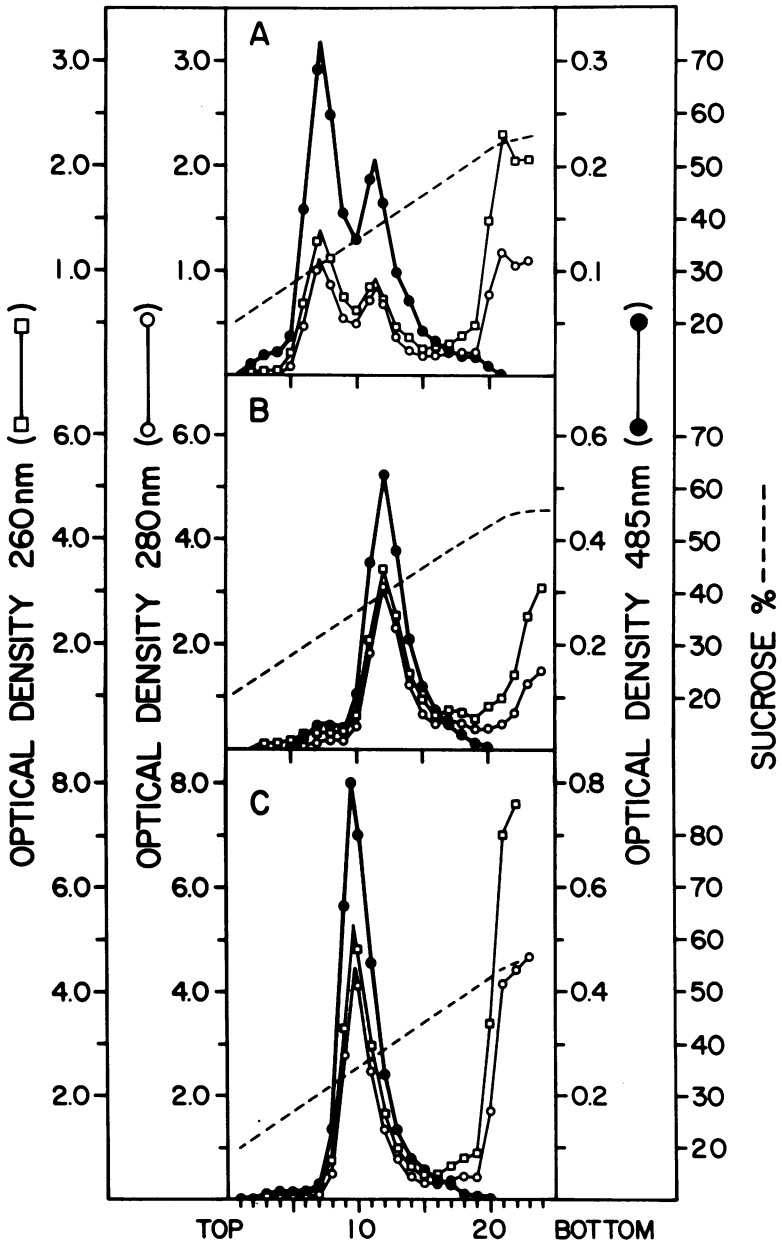

FIG. 3. Sucrose equilibrium gradient centrifugation of membrane fractions from velocity gradients. LD (A), HD (B), and G (C) membrane bands were isolated, concentrated, run on a sucrose equilibrium gradient, and analyzed as described in the text.

TABLE 2. Properties of membrane fractions purified by equilibrium density gradient centrifugation[a]

Peak	Fractions pooled	Phospholipid (mg/ml)	Protein (mg/ml)[b]	RNA (mg/ml)	Phospholipid/protein ratio (mg/mg)	Density (g/cm^3)
	(Fig. 3A)					
	1–5	<0.01	<0.01	<0.02		
LD →	6–8	0.66	0.153	<0.02	4.3	1.122
	9–10	0.07	0.10	<0.02		
(HD)	11–13	0.10	0.20	<0.02	(0.5)	(1.175)
	14–17	0.06	0.10	<0.02		
	18–23	<0.01	0.021	0.32		
	(Fig. 3B)					
	1–5	<0.01	0.03	<0.02		
	6–10	0.15	0.10	<0.02		
HD →	11–13	0.34	0.725	<0.02	0.47	1.181
	14–16	0.09	0.19	<0.02		
	17–20	0.02	0.08	<0.02		
	21–24	<0.01	0.30	0.45		
	(Fig. 3C)					
	1–8	<0.01	0.02	<0.02		
G →	9–11	0.426	0.434	<0.02	0.98	1.157
	12–16	0.05	0.06	<0.02		
	17–19	<0.01	0.04	<0.02		
	20–23	<0.01	0.16	0.24		

[a] Designated fractions were pooled from gradients shown in Fig. 3, diluted to <10% sucrose with buffer B containing 0.25 mM phenylmethylsulfonylfluoride, sedimented at 105,000 × *g* for 3 h, resuspended in 1 ml of buffer B, and analyzed as described in the text.

[b] Protein was determined by reaction with ninhydrin after alkaline hydrolysis as described in the text. Values for the peak fractions were determined by amino acid analysis, which gave values similar to those determined by reaction with ninhydrin after alkaline hydrolysis. However, these values were 25 to 40% lower than those found with the Lowry assay.

composition than inner membrane (4, 7), yet LD and HD had the same composition; and (iv) spore outer membrane is reported to lack red pigment (3), yet both LD and HD contained red pigment.

If, as we suggest, both LD and HD are derived from the dormant spore inner membrane, are these two fractions an artifact of preparation? We believe this is unlikely because preparation of membranes from germinated spores in an identical manner gave only a single inner membrane band, as also seen in vegetative cells (data not shown). Although only a single major inner membrane band is obtained from dormant spores broken with lysozyme at 30°C (reference 15 and Fig. 5), it seems likely that the latter procedure could allow, and may indeed involve, spore germination-like reactions which alter dormant spore membrane features. In contrast, breakage with the Braun homogenizer was carried out at low temperatures and without promoting spore cortex hydrolysis. These differences between the two procedures of dormant spore membrane isolation may explain the large differences in spore membrane properties (such as NADH oxidase specific activity) reported in the literature (3, 16, 23, 27).

Given the conclusions noted above, probably the major question concerning the findings reported in this paper is the significance of the separate LD and HD fractions of the dormant spore inner membrane. Since the phospholipid/protein ratio of the G fraction is intermediate between LD and HD and the proteins found in LD and HD are found in G,

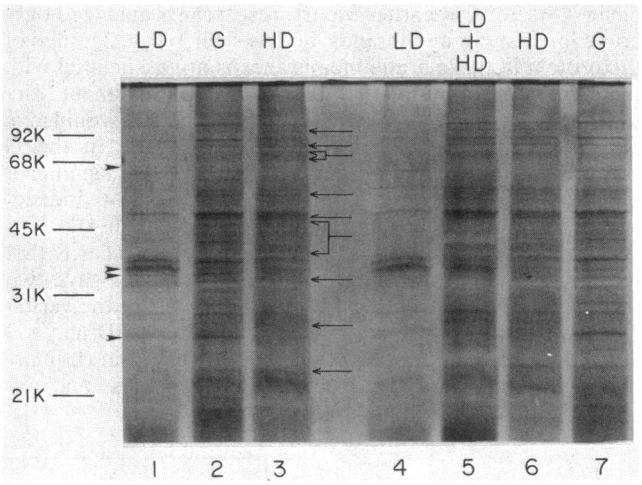

FIG. 4. Sodium dodecyl sulfate-acrylamide gel electrophoresis of protein in various spore inner membrane fractions. Membrane fractions (indicated by the letters above each lane) purified through the equilibrium gradient step were analyzed on a 12.5% acrylamide slab and stained with silver nitrate as described in the text. The amounts of protein applied to each lane were: LD, 3.8 μg; G, 4.3 μg; HD, 7.3 μg; and LD and HD, 3.8 μg of LD and 7.3 μg of HD. Arrowheads (➤) indicate protein bands seen in LD but not HD. Arrows (→) indicate protein bands seen in HD but not LD. The positions of molecular weight markers are shown to the left of the gel, labeled by their respective molecular weights.

this suggests (although does not prove) that the inner membrane (G) of the germinated spore may arise by the fusion of LD and HD. During the first minutes of spore germination, the volume of the spore core bounded by the inner membrane increases about 2.5-fold (5, 19), and thus, the membrane surface area must increase almost twofold. This change can take place in the absence of protein and phospholipid synthesis (5, 19, 25), and it seems unlikely (although not without some precedent [13]) that twofold changes in mem-

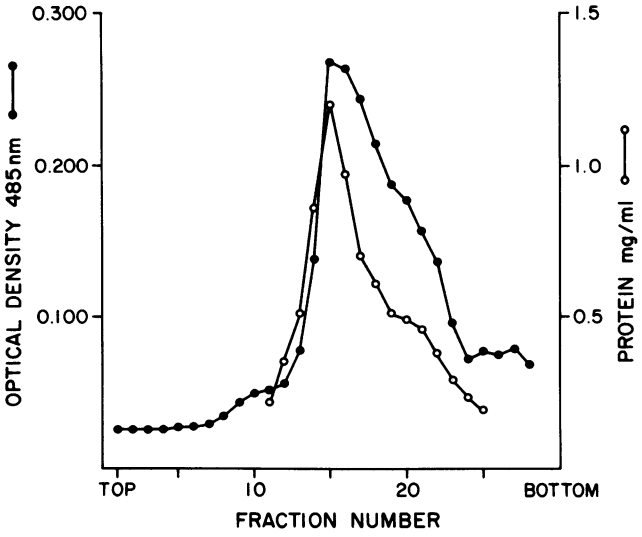

FIG. 5. Sucrose velocity gradient of crude inner membranes from stripped dormant spores disrupted with lysozyme. Crude inner membranes were prepared, run on a sucrose velocity gradient, and analyzed as described in the text.

brane surface area could be generated by compression or relaxation of a preexisting membrane. However, if ca. one-half of total spore inner membrane phospholipid is in a membrane (perhaps HD) enveloping the dormant spore core, with the remaining 50% (perhaps LD) either as invaginations of the enveloping membrane or as internal vesicles, one can readily imagine how the surface area of the membrane surrounding the spore core could increase by fusion of LD and HD. If on the other hand, the spore inner membrane surface area does increase twofold upon germination solely due to the relaxation of a pressure exerted on the inner membrane in the dormant spore, HD and LD may only represent two domains of the inner membrane which fuse upon spore germination. In either case, the fusion of HD and LD early in germination would predict that there be a significant change in spore membrane structure and composition at this time, and as noted in the introduction, there is much evidence for such an event. Although there is no direct evidence, such as from electron microscopy, that there are two distinct domains in the inner spore membrane, our isolation of two such membrane fractions suggests that they indeed exist and should be looked for. Obviously, the confirmation of their existence in vivo could have a marked influence on models of dormant bacterial spore structure, permeability, and resistance.

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