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AN INVESTIGATION OF MEMBRANE FLUIDITY CHANGES DURING SPORULATION AND GERMINATION OF *BACILLUS MEGATERIUM* K.M. MEASURED BY ELECTRON SPIN AND NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

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Summary

Changes in membrane and macromolecular fluidity which may accompany the differentiation processes of sporulation and germination in *Bacillus megaterium* K.M. are examined by electron spin and nuclear magnetic resonance spectroscopy. No change in membrane lipid fluidity is observed in isolated forespores up to stage VI. Between stage VI and release of mature spores, the ESR spectrum of doxylstearic acid spin labels becomes polycrystalline. This change in spectral fluidity is completely reversed during germination and is paralleled by the rapid release of Ca^{2+} from the spore. NMR studies also show that the mature spore has reduced macromolecular mobility and an increased non-exchangeable water pool compared with vegetative cells.

Introduction

Bacterial sporulation is a process of cell differentiation in which vegetatively growing bacteria undergo a well-defined series of cytological and biochemical changes, to yield a dormant endospore [1]. Attempts to study the structure and biochemical basis of dormancy and resistance by traditional methods, requiring prior cell breakage, are likely to disrupt the cellular architecture

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Abbreviations: 5-NS, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinoxyl; 12-NS, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinoxyl; 16-NS,2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinoxyl.

which is responsible for creating and maintaining dormancy. For this reason several non-destructive physiochemical techniques such a nuclear magnetic and electron spin resonance, differential thermal analysis, electron-probe X-ray microanalysis and dielectric studies are likely to provide important information on the conformation of spore structures, the fluidity of membrane lipids, the state of spore water and the degree of mobility and location of spore ions. No gross changes in lipid fluidity were observed when nitroxide spin resonance probes were used in this way to study membrane lipid changes during differentiation of the eukaryotes *Dictyostelium discoideum* [2] and *Fusarium sulphureum* [3] into spores and chlamydospores respectively.

In comparison with dormant forms of eukaryotes, bacterial spores exhibit almost no detectable metabolism and show remarkable tolerance of heat, radiation and chemical agents. The biochemical and biophysical basis for these spore properties remains unexplained, but the high levels of calcium and pyridine 2,6-dicarboxylic acid in the dormant spore, together with its low water content [4] and reduced ionic mobility [5] may be involved in creating and maintaining the dormant state. Among the characteristic changes which occur during the transition from an actively-growing vegetative cell to a spore are sporulation-specific changes in membrane structure and function [6—9]. Distinctive changes are also observed in the spore membrane during germination [1]. The use of ESR spin labels and NMR offers a novel and sensitive method of investigating these membrane changes during spore morphogenesis without unduly perturbing the dormant cell.

Materials and Methods

Chemicals. 12-NS (2-(10-carboxydecyl-2-hexyl-4,4-dimethyl-3-oxazolidinoxyl) was prepared by the method of Waggoner et al. [10]. 16-NS (2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinoxyl) was a gift from Dr. J.C. Metcalfe, Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, U.K. 5-NS (2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinoxyl), was purchased from Syva Associates, Palo Alto, CA. Dipicolinic acid was obtained from Koch Light Laboratories Ltd., Colnbrook, U.K. All other chemicals were obtained from Sigma Chemical Company, St. Louis, MO.

Organism. The organism used in these studies was a sporogenic strain of *Bacillus megaterium* K.M. [11]. The conditions for growth and sporulation were as previously described [11] except for the use of the modified sporulation of Wilkinson et al. [12].

Preparation of cell fractions. Forespores were isolated from sporulating cells by a method involving protoplast formation with lysozyme followed by brief sonication [11]. Prior to protoplasting, cells were washed once with 50 mM citrate buffer, pH 6.5, and once with deionised water. Vegetative cell membranes, spore inner membranes and spore integuments were prepared as previously described [6]. Lipids from membranes, integuments, freeze-dried cell soluble fractions and whole cells were extracted and subsequently purified using a Sephadex G-25 column [6,13]. Phospholipids were prepared by acetone precipitation [14].

Analytical methods. Protein was estimated by the method of Lowry et al. [15] with 1% (w/v) sodium dodecyl sulphate included in the sample volume to aid in solubilisation of the preparation. Total phosphorus was determined by a modified Bartlett procedure [16]. Phospholipids were separated by two-dimensional chromatography as previously described [11].

Heat activation. Spores were activated by heating in deionised water at 70°C for 30 min and used immediately.

Preparation of samples for ESR experiments. Incorporation of 12-NS into vegetative cells and sporulating cells involved either the addition of $7 \cdot 10^{-5}$ M spin label at the time of culture inoculation or at Stage IV of sporulation. Incubation of vegetative cells, forespores, spores and cell fractions with nitroxide spin labels was accomplished by first adding an appropriate amount (final concentration 10^{-4} M) of spin label in chloroform to a dry glass tube and removing the solvents in a stream of nitrogen gas. The aqueous sample was then added and incubated for 5 min at room temperature to allow the spin label to partition into the lipid phase. Purified lipid samples were dried and resuspended in 5 mM $MgCl_2$, 50 mM Tris-HCl buffer, pH 7.4, prior to incubation with label.

Recording and treatment of ESR spectra. ESR spectra were recorded as the first derivative on a Varian E4 spectra X-band spectrometer (Varian, Palo Alto, CA) fitted with an E 257-9 variable temperature accessory. Fluidity measurements were determined from the spectra of 16-NS by the rotational correlation time τ_0 [17,18]. The spectra of 12-NS and 5-NS show increasing asymmetry, and fluidity measurements from these spectra were more conveniently expressed as the order parameter S_3 [19—22]. Unless otherwise stated $K_3Fe(CN)_6$ was routinely added at a concentration of 0.1 M to all aqueous samples prior to ESR measurements [3,22,23].

Location of spin label probe. Spores were isolated from cultures which had been incubated with $7 \cdot 10^{-5}$ M 12-NS from stage IV of sporulation. Spore membrane, integument and soluble fractions were prepared from these and total lipid extracts obtained as described above. One tenth of the total sample of disrupted spores was lipid extracted to determine total spore lipid. Quantitation of label in each fraction was determined by ESR measurements using a standard solution of 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl, in methanol.

Spore germination measured by ESR. Spin probes were used at a final concentration of 10^{-4} M and were dried from chloroform solvent onto glass tubes prior to the addition of spore suspensions and germinants. Germination was carried out in 10 mM potassium phosphate buffer, pH 7.0, at 30°C using heat-activated spores (100 mg dry wt./ml) and 10 mM L-alanine as germinant. Samples were withdrawn from the germination suspension at appropriate time intervals and immediately made 0.1 M with $K_3Fe(CN)_6$ before transfer to the quartz sample cell for ESR measurement at 30°C. The change in the height of the high-field line of 16-NS was taken as a measure of the amount of fluid lipid due to germinated spores in the sample. For measurement of Ca^{2+} release during the above germination, 100 μl samples were withdrawn from the germination suspension, mixed with 1.9 ml of ice-cold 10 mM potassium phosphate buffer, pH 7.0, and filtered through a Millipore membrane with pore size 0.45 μm. The filtrate was further diluted with two volumes of deionised water

before quantitation of Ca^{2+} by atomic absorption spectrophotometry.

Preparation of samples for NMR experiments. Vegetative cells and spores were washed three times with deionised water at 4°C, twice with 50 mM KCl at 4°C and recovered by centrifugation. The pellets were then suspended in the minimum volume of water and freeze dried over P_2O_5. These spores or vegetative cells were subsequently resuspended in ^2H_2O (100 mg dry wt./ml) and again freeze dried over P_2O_5. This procedure was repeated twice more to remove all water from the system. Immediately prior to use, cells or spores were suspended in ^2H_2O to give a final suspension density of 100 mg dry wt./ml. Purified spore lipids was dried under nitrogen and suspended in ^2H_2O at 10 mg/ml. The sample was sonicated using a 0.5 inch sonic probe (Dawe Instruments Ltd., London), until a homogeneous dispersion was obtained. Where specified, $CaCl_2$ was added to give a final concentration of 1 mM. Spectra were measured on 0.5 ml samples of this suspension in a 5 mm NMR tube.

Spore germination measured by NMR. Spore suspensions in ^2H_2O at 100 mg dry wt./ml were prepared and heat activated as described above. KCl and L-alanine, freeze dried from ^2H_2O, were added to give a final concentration of 50 mM and 10 mM respectively, and NMR spectra were recorded continuously from 2 ml of this suspension contained in a 12 mm NMR tube at 30° C for 30 min.

Recording and treatment of NMR spectra. NMR spectra were recorded on a Varian XL 100 FT spectrometer operating at 100 MH in the fourier transform mode, fitted with a Varian XL 100 variable temperature accessory. A capillary of pyrazine was used as a reference. The same capillary was used for all samples. Spin-lattice relaxation times (T_1) were determined using the null method of Carr and Purcell [24].

Interaction of ions with monolayers of spore and vegetative cell lipids. A surface balance similar to that described by Cadenhead and co-workers [25—28] was used in thes studies to obtain continuous surface pressure measurements as a function of surface area. The lipids were spread from hexane-ethanol solutions onto a Teflon trough (40 cm × 15 cm × 1.5 cm) filled to the brim with 1 mM sodium phosphate buffer, pH 7.0, made with doubly-distilled water. The surface area was altered by moving the Teflon barriers at constant rate with a motor-controlled worm drive. The barriers took about 4 min to traverse the whole length of the trough. Surface pressures were measured with a roughened mica Wilhelmy plate suspended from the arm of a Cahn recording microbalance. The output from the balance and the change in surface area were fed into a pen recorder. The monolayer was compressed to a surface pressure of approx. 30 dynes/cm and explanded again to the original surface area, recording the changes in surface pressure and area continuously in both compression and expansion. The monolayer was then compressed to a surface pressure of 10 dynes/cm and the change in surface pressure recorded as successive aliquots of $CaCl_2$ were added to the sub-phase up to a final concentration of 0.9 mM. The sub-phase was stirred mechanically during these additions. The monolayer was then expanded to the original area, compressed to a surface pressure of approx. 30 dynes/cm and expanded again, recording continuously the changes in surface pressure and area. Surface pressure molecular area curves for the monolayer in the presence and absence of $CaCl_2$ were constructed from

the combined data for compression and expansion. In some cases pyridine 2,6-dicarboxylic acid was added to the sub-phase as detailed in the text. Preliminary experiments showed that such successive expansions and compressions of the monolayer had no significant effect on the force/area curve.

Results

When the growth of vegetative cells become limited by the depletion of an essential metabolite, certain strains of Bacilli undergo a morphological and biochemical differentiation which results in the production of a dormant spore. On the basis of electron microscopic observations it is possible to divide this morphogenesis into a number of stages [29—31] which are illustrated diagrammatically in Fig. 1. Stage III is the earliest stage at which forespores can be isolated and is characterised by the formation of a new intracellular compartment (forespore) which exists within the mother cell as a discrete cell bounded by two membranes. During stage IV and early V a cortex (peptidoglycan) develops between these two membranes [1,32,33]. Spore coat protein is deposited at the spore periphery largely during stage V when the forespores can be seen as phase white bodies within the surrounding sporangium, by phase-contrast microscopy. Throughout vegetative growth and prior to stage III, the intracellular Ca^{2+} levels are extremely low. At the end of this stage there is a rapid increase in calcium accumulation which results in the uptake of

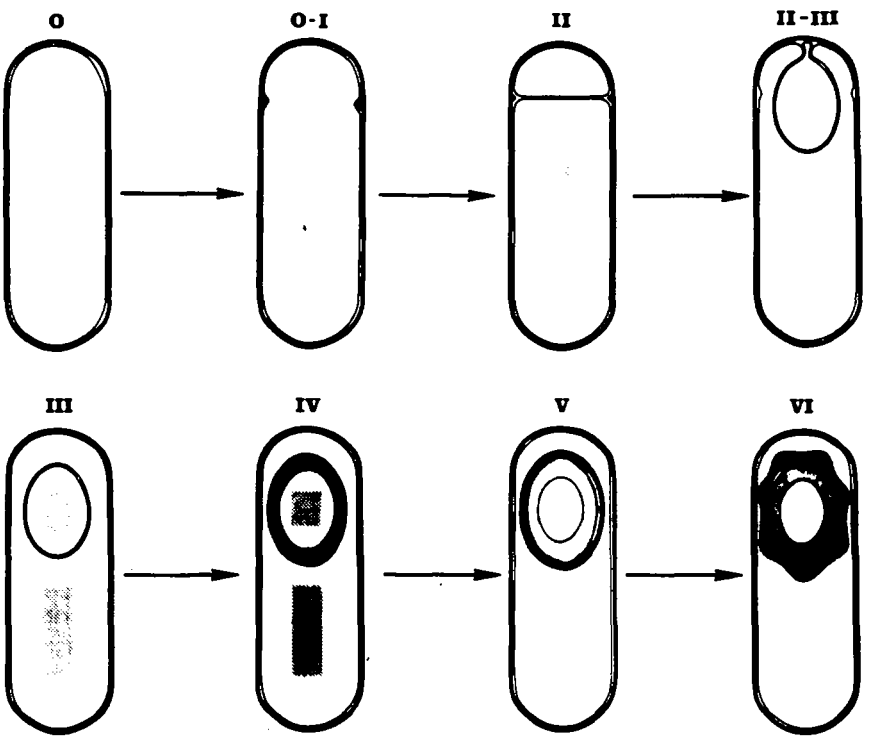

Fig. 1. Generalised diagram of the major morphological events occurring during sporulation. Individual stages are indicated by Roman numerals.

almost all the Ca^{2+} contained in the sporulation medium during the remaining sporulation stages [34]. The accumulated Ca^{2+} continually moves into the forespore compartment [35] so that in the mature spore it accounts for 2% on average of spore dry weight and exceeds the mole sum of all the other inorganic spore cations [36]. Stage VI is the final maturation stage during which the spore becomes typically heat resistant and dormant before being released by lysis of the mother cell. The mature spore may remain dormant over long periods but retains the ability to respond rapidly to chemicals which trigger spore germination and subsequent development of a new vegetative cell.

Incorporation of 12-NS into sporulating cultures of Bacillus megaterium K.M.

$7 \cdot 10^{-5}$ M 12-NS was found to be a critical concentration for incorporation into sporulating cultures. Above this level the sporulating cells lysed and below this level very little signal was detected. Only the free acid was used, as the methyl ester was not incorporated at any level below its lethal concentration (also $7 \cdot 10^{-5}$ M). Several authors have reported inhibition of bacterial functions by dilute solutions of nitroxide probes and by metabolisable and non metabolisable fatty acids [37—41]. We observed that levels of 12-NS that are lethal to sporulating cells do not affect exponentially-growing cells and that between stage 0 and stage IV of sporulation, very low levels (less than 10^{-6} M) of 12-NS are lethal. No ESR signal was observed from spectral analysis of spores immediately after isolation from cultures in which 12-NS had been added to the medium at stage IV of sporulation. A strong ESR signal was observed however if these spores were either stored on ice for several days, or treated immediately with 0.1 M $K_3Fe(CN)_6$ to prevent biological reduction of the spin label [3,22,23] which has been a major difficulty in analysis of in vivo systems [42—44].

12-NS location within the spore

Recovery of label from spore fractions totalled 68% of that recovered from intact spores and this compared with an 80% recovery of lipid phosphorus. 68% of the total label incorporated was found in the spore integument fraction, 10% was located in the inner-membrane, 10% in the soluble fraction and 10% was extracted from the glass homogeniser beads used to disrupt the intact spores. Lipid extracts of each fraction were separated into neutral lipids and phospholipids and no label was detectable in any phospholipid fraction. Thin-layer chromatography (TLC) of total lipid fractions showed no change in the overall phospholipid composition as compared to that published by Ellar et al. [11]. The low levels of 12-NS in the inner spore membrane may have resulted from the partitioning of label released into the medium after homogenisation.

As a result of the lack of any biosynthetic incorporation of 12-NS directly into phospholipids, the effect of brief incubation of spores, vegetative cells and cell fractions with label was examined. In all cases spectra were identical to those obtained from spores or cells grown in the present of label.

Vegetative cell lipid and spore inner-membrane lipid fluidity

Fig. 2 shows Arrhenius plots for purified vegetative cell lipid and spore inner membrane lipid with 5-NS, 12-NS and 16-NS. The increase in motional

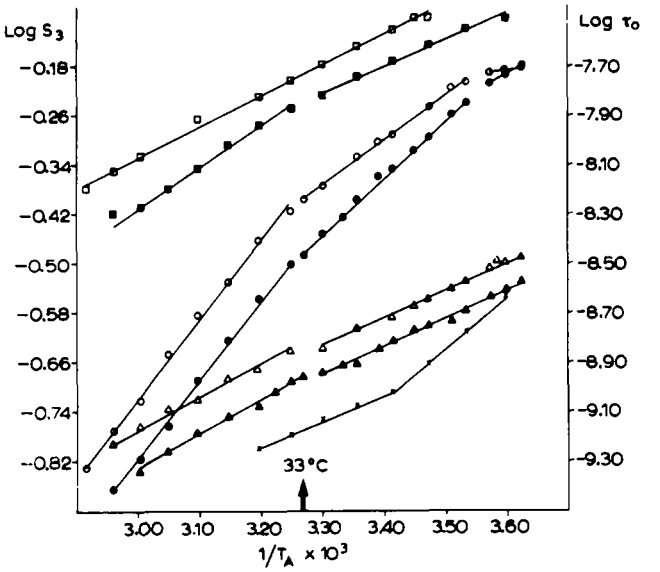

Fig. 2. Arrhenius plots of spin-labelled lipid. Open symbols denote lipid extracted from spore inner membrane; closed symbols denote lipid extracted from vegetative cell membrane. □———□ and ■———■, 5-NS plotted as log S_3; ○———○ and ●———●, 12-NS plotted as log S_3; △———△ and ▲———▲, 16-NS plotted as log τ_0; X———X, 16-NS intercalated with unextracted vegetative cell membrane for comparison.

freedom from 5-NS to 16-NS (label situated at the carboxyl and methyl end of stearate, respectively) is clearly demonstrated. It is also noticeable that spore inner-membrane lipid consistently forms less fluid bilayers than vegetative cell lipid. The fatty acid composition of spores and vegetative cells is very similar [11] but spore phosphatidyl glycerol and cardiolipin (diphosphatidylglycerol) comprise 24% and 35%, respectively, of total spore lipid compared to 67% and 4%, respectively of vegetative cell lipid [11]. This increase in cardiolipin appears to have resulted in an intrinsically less fluid lipid bilayer in vitro but does not alter the lipid transition temperature (33°C) when compared to vegetative cell lipid and this may again reflect the similarity in fatty acid composition. It should be stressed however that this is a transition temperature of isolated lipid and that preliminary experiments with 16-NS added to vegetative cell membranes and whole vegetative cells have indicated a lower transition temperature of approx. 20°C (Fig. 2), and a higher degree of molecular freedom.

Membrane fluidity changes in intact forespores during sporulation

The ESR spectra obtained from mature spores and forespores at stages III, IV and VI (Fig. 3) show no change in membrane fluidity occurs between stage III and stage VI. Between stage VI and final release of mature spores however, a complete immobilisation of spore lipid occurs. The spectrum obtained is equivalent to a polycrystalline or powder spectrum of the nitroxide [45]. It was anticipated therefore that a return to the vegetative cell spectrum might occur during the process of germination in which the spore develops into a new

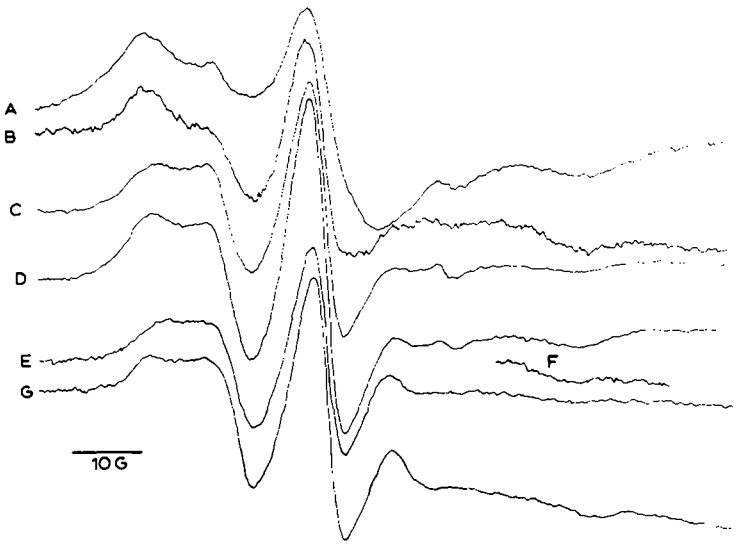

Fig. 3. ESR spectra of 12-NS intercalated with: A. Dormant spores prepared and suspended in deionised water (100 mg/ml). Mod. amp. 1.6, gain 1250; $K_3Fe(CN)_6$ included as described in text. B. Dormant spores (100 mg/ml) prepared and suspended in 50 mM Tris-HCl buffer, pH 7.5, and sonicated as described in text. C. Stage VI forespores prepared and suspended in 50 mM Tris-HCl buffer, pH 7.5. Mod. amp. 0.8, gain 2000. D. Stage VI forespores prepared as in C above but without the use of lysozyme as described in text. E. Stage V forespores prepared in 0.6 M sucrose, 16 mM Mg_2SO_4, 0.1 M potassium phosphate buffer, pH 6.3, and suspended in 0.6 M sucrose, 50 mM Tris-HCl buffer, pH 7.5. Mod. amp. 1.6, gain 2500. F. Stage V forespores prepared as in (E) with mod. amp. 1.6, gain 5000. G. Stage III forespores prepared and suspended as in (E) above. Mod. amp. 1.25, gain 3200.

vegetative cell [46]. Since previous work in our laboratory [47] had indicated that under certain conditions forespores prepared with the aid of lysozyme could undergo germination-like changes, it was important to ensure that the observed absence of fluidity changes in forespores isolated between stage III and VI was not the result of such premature germination. In control experiments to eliminate this possibility, stage VI forespores, prepared without lysozyme, and mature spores were subjected to the same sonication procedures. As shown in Fig. 3 no changes in ESR spectra were observed after these treatments and therefore the change from a fluid to a powder spectrum between stage VI and release of the mature spore does not appear to be an artefact of preparative procedure.

The progressive accumulation of manganese during sporulation [48,49] provided another parameter for monitoring the integrity of forespore preparations. The manganese spectra of spores have been investigated by Windle and Sacks [50] who demonstrated a single broad resonance, 460—510 Gauss wide, with a superimposed sextet hyperfine pattern. During spore germination most of the manganese is released [51] and shows a typical aqueous manganese spectrum of six equally spaced lines (Stewart, G.S.A.B., unpublished results). All forespore preparations used were devoid of a free manganese signal and were considered therefore to be ungerminated.

At approximately the same stage of sporulation that Ca^{2+} uptake begins, the cell begins to synthesise pyridine 2,6-dicarboxylic acid. This compound is

unique to sporulation and its synthesis continues in parallel with Ca^{2+} accumulation. The possible involvement of Ca^{2+} and pyridine 2,6-dicarboxylic acid in the membrane fluidity changes between stage VI and release was investigated by addition of 0.25 M calcium chloride and 0.025 M sodium dipicolinate, pH 7.0, to all forespore preparations. This concentration of calcium and pyridine 2,6-dicarboyxlic acid had no effect on the ESR spectrum of forespores.

Fluidity changes during germination of dormant spores.

Fig. 4a shows the ESR spectral changes of 16-NS during germination of spores with L-alanine. The change in fluidity from a powder spectrum at one minute, to isotropic freedom of the label at ten minutes is paralleled by the release of calcium from the spores (Fig. 4b). Similar results were obtained using 12-NS, although the spectral parameters were more difficult to interpret because, as discussed later, this label was present in two environments. 12-NS spectra of the dormant and germinated spore and integuments and inner-membrane fractions from the germinated spore are shown in Fig. 5. Powder spectra equivalent to the dormant spore spectra were obtained from both dormant and germinated spore integument fractions. By contrast, the spectra from dormant and germinated spore inner-membranes were very similar to those of the intact germinated spore. The 12-NS spectrum of stage VI forespores emphasises that the change in the fluidity of the spore membrane between stage VI and maturity is entirely reversed during germination.

Reduction of ESR signals in vegetative cells and forespores

As described earlier, $K_3Fe(CN)_6$ was used routinely to prevent biological reduction and to reoxidise nitroxide spin label. Apart from protecting the lipid bound label from reduction, $K_3Fe(CN)_6$ was also used to achieve a marked reduction in the free label (aqueous) signal from dormant spores. This was a result of spin exchange between the nitroxide and the paramagnetic iron. The rate of diffusion of label from lipid to aqueous environments was sufficiently low to permit almost a complete removal of the aqueous signal by this method. In the absence of $K_3Fe(CN)_6$ the reduction of label by vegetative cells and forespores can give a qualitative indication of their relative metabolic activities. Vegetative cells were observed to destroy the ESR signal even in the presence of ferricyanide, while isolated vegetative cell membrane reduced the label over a period of 3—4 min in the absence of ferricyanide. Up to stage V, forespores reduced the spin label at the same rate as isolated vegetative cell membrane but stage VI forespores required between 8 and 10 min to render the label undetectable at concentrations of 10^{-4} M. We consider that the different rates of nitroxide reduction during sporulation may reflect a decrease in the metabolic activity of forespores as they reach maturity.

Although intact dormant spores had no capacity to reduce the nitroxide label at room temperature, when these spores were heat activated at 70°C for 30 min, significant reduction of ESR signal was observed. 12-NS is stable in organic solvents at temperatures up to 90°C [43] and hence intrinsic lability of the label is not considered to be a contributing factor to its destruction. Recent work from our laboratory [52] has shown that there is no detectable metab-

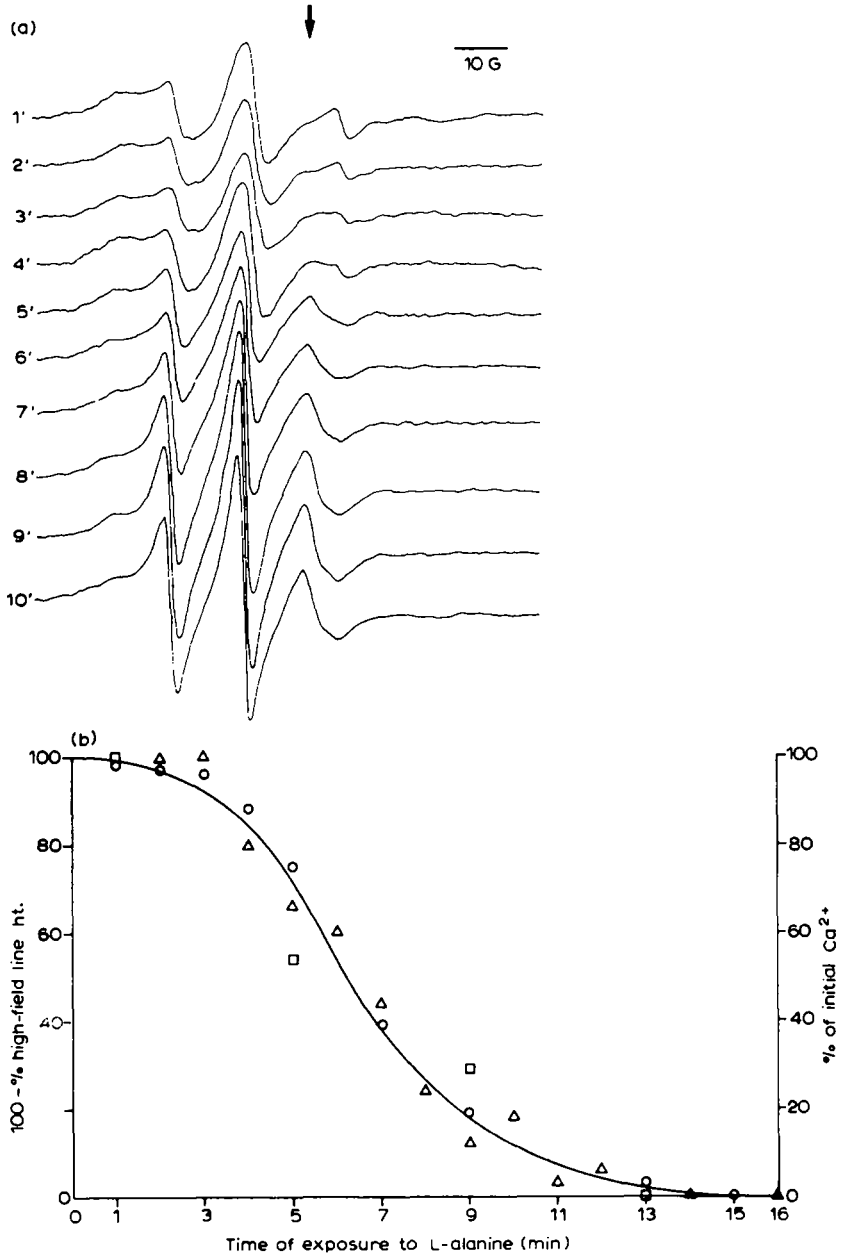

Fig. 4. (a) ESR spectrum of 16-NS intercalated with germinating spores. Heat shocked spores were germinated at 100 mg/ml in 10 mM potassium phosphate buffer, pH 7.0, and 10 mM L-alanine. ESR spectra of 16-NS intercalated with the above germinating spores were measured at Mod. amp. 1.6, gain 1000, scan range 200 set on 3220. The time of exposure to L-alanine is indicated in the left hand column. (b) Ca^{2+} release and lipid fluidity changes during spore germination. ○, Ca^{2+} release; △, spectral change in 16-NS high-field line height; □, change in the order parameter S_3 for 12-NS.

olism associated with heat activation in our strain of bacillus and therefore we conclude that the reduction of signal in the activated spore may be due to an increase in available SH groups in the spore coat. Baldassare et al. [53] have

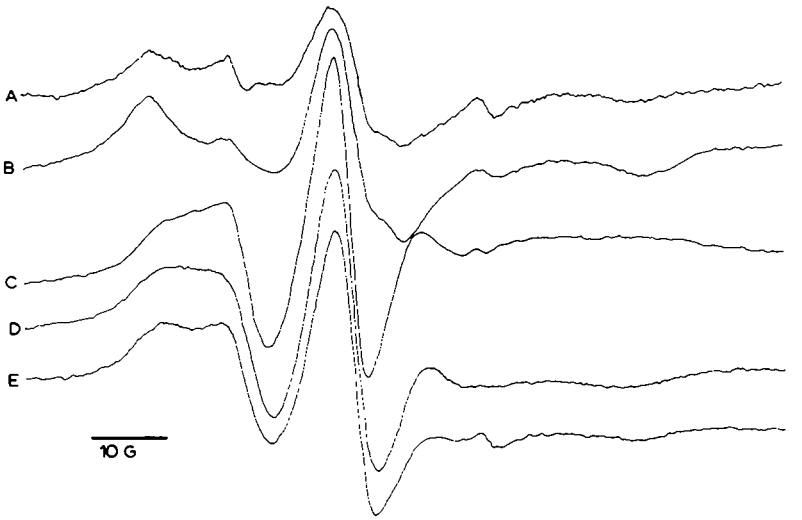

Fig. 5. ESR spectra of 12-NS intercalated with: A. Dormant spores (100 mg/ml in 10 mM potassium phosphate, pH 7.0). Mod. amp. 1.6, gain 1250. B. Germinated spore integuments (32 mg/ml in 50 mM Tris-HCl buffer, pH 7.5), prepared as described in text. Mod. amp. 1.6, gain 2000. C. Germinated spore inner membrane (54 mg/ml in 50 mM Tris-HCl buffer, pH 7.5), prepared as described in text. Mod. amp. 0.8, gain 800. D. 20 min germinated whole spores (100 mg/ml in 10 mM potassium phosphate buffer, pH 7.0). Mod. amp. 1.6, gain 1250; $K_3Fe(CN)_6$ included as described in text. E. Stage VI forespores prepared and suspended in 50 mM Tris-HCl buffer, pH 7.5. Mod. amp. 0.8, gain 2000.

shown nitroxide reduction by membrane SH groups in *E. coli* and Keynan et al. [54] describe an increase in free SH groups on activation of bacterial endospores. Setlow et al. [55,56] have made an extensive study of SH groups in bacterial sporulation and germination.

Ionic interactions with monolayers of spore and vegetative cell lipids

It has been shown that the addition of divalent cations to the sub-phase, leads to contraction (decrease in surface pressure) of monolayers of acidic phospholipids such as cardiolipin [57], phosphatidylserine and phosphatidic acid [58]. Since spores contain a significantly higher proportion of acidic phospholipids than vegetative cells [11,58] and the presence of calcium in the spore is strongly implicated in the maintenance of dormancy and resistance, we investigated the interactions of various cations with monolayers of spore and vegetative cell lipids. Fig. 6 shows the effect of successive additions of calcium to the sub-phase on the surface pressure of monolayers of spore and vegetative lipids. Monolayers of both types of lipid show some contraction in the presence of calcium, but the contraction of the spore lipid monolayer is 3 times greater than with the vegetative lipids. The total change in surface pressure of the spore lipid monolayer (4.7 dynes/cm) is very similar to the value of 5 dynes/cm observed by Papahadjopoulos [59] with pure phosphatidylserine monolayers. In both systems the contraction is completed at calcium concentration in the sub-phase of approx. 1 mM.

Fig. 7 shows a surface pressure/area curve for a spore lipid monolayer in the presence and absence of calcium (0.9 mM $CaCl_2$) in the sub-phase. The monolayer shows a 9—15% contraction in the presence of calcium. This is very close

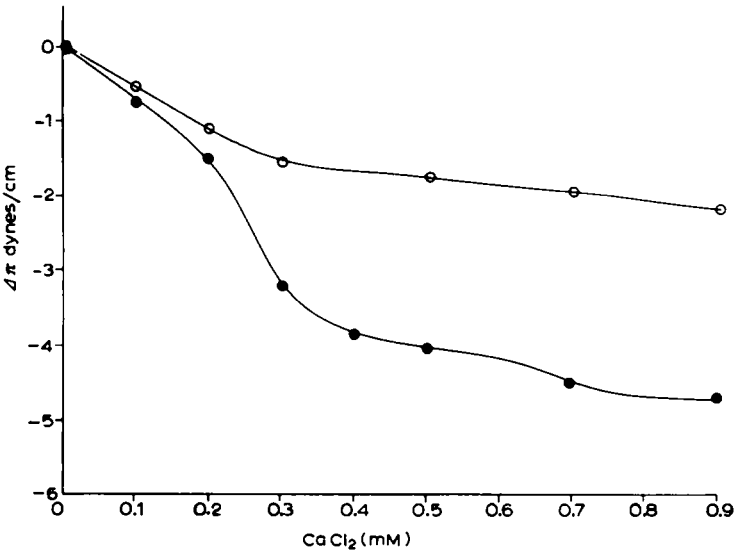

Fig. 6. Effect of calcium on the surface pressure of spore (●———●) and vegetative cell (○———○) lipid monolayers. A constant amount of spore or vegetative cell lipid dissolved in hexane-ethanol was spread as a monolayer on a 1 l Teflon trough filled to the brim with 0.02 M sodium phosphate buffer, pH 7.0. The mono-layer was compressed to give a surface pressure of 10 dynes/cm and the change in surface pressure recorded as successive aliquots of $CaCl_2$ solutions were injected into the subphase which was stirred mechanically. Each point plotted represents the mean of two independent experiments.

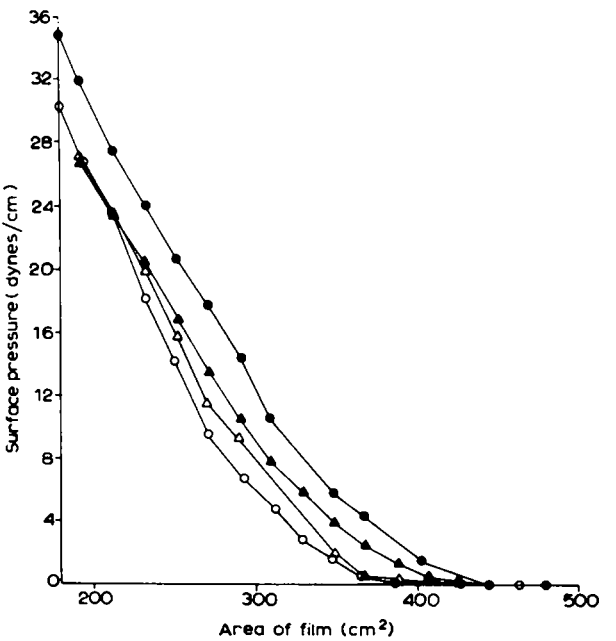

Fig. 7. Surface pressure vs. area curve for spore and vegetative cell lipid. ○———○, spore lipid without calcium; ●———●, spore lipid plus 0.9 mM calcium; △———△, vegetative cell lipid without calcium; ▲———▲, vegetative cell lipid plus 0.9 mM calcium.

to the 10—13% contraction of a pure cardiolipin monolayer on addition of calcium (0.01 M $CaCl_2$) observed by Shah and Schulman [57]. The contraction is greatest at surface pressures around 10 dynes/cm and decreases as the surface pressure is increased, since at high pressures the cross-sectional area of the hydrocarbon chains becomes limiting. Fig. 7 also shows the surface pressure/area curve for a monolayer of vegetative cell lipid in the presence and absence of calcium (0.9 mM $CaCl_2$). This shows a very much reduced contraction (maximum 4%) compared with that observed with the spore lipid.

Studies on spores and vegetative cells by NMR

NMR has been used extensively to study conformation changes in macromolecules and molecular motion in membrane systems [60,61] and the technique has been shown to be applicable to intact chromaffin granules [62] and micro-organisms [63]. Fig. 8 shows the NMR spectrum of spores and vegetative cells suspended in ^2H_2O at 30°C. The spectra are dominated by the peak at approx. 4.7 ppm due to ^2H_2O and ^2HHO. The spores and vegetative cells had been freeze dried extensively from ^2H_2O during preparation, and further washing with 99.9% ^2H_2O had no effect on the size of this peak, suggesting that it is due to non-exchangeable or 'bound' water in the spore. It is possible to quantitate this non-exchangeable water by integrating the area under the peak and comparing it with the area under the peak for a known quantity of pyrazine present in a capillary in the sample tube. The pyrazine is calibrated against a known amount of SDS and therefore knowing the number of protons in the pyrazine reference, the number of protons in the water peak can be calculated and hence the amount of water. The results are shown in Table I.

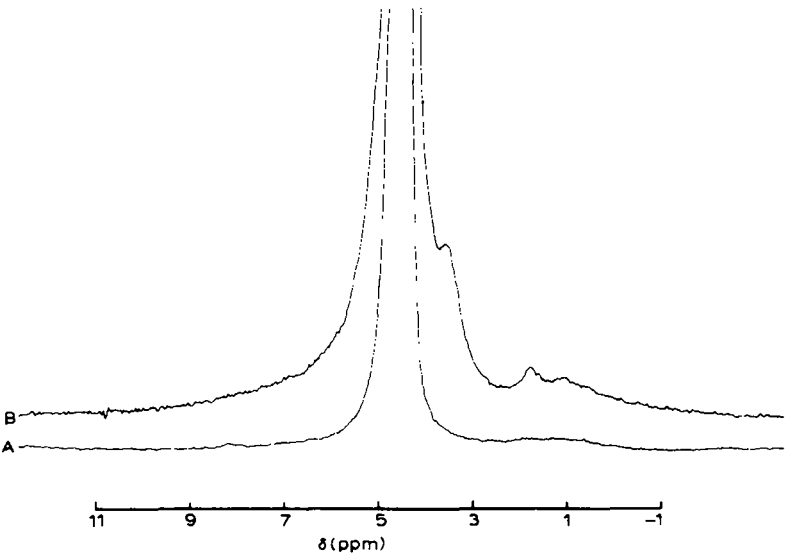

Fig. 8. NMR spectrum of spores and vegetative cells. A. Spores were suspended in ^2H_2O at 100 mg/ml and the spectrum taken on 2 ml of this suspension in a 12 mm NMR tube at 30°C. B. Cells were suspended in ^2H_2O at 40 mg/ml and the spectrum taken on 2.0 ml of this suspension in a 12 mm NMR tube at 20°C.

TABLE I

QUANTITY AND RELAXATION OF NON-EXCHANGEABLE WATER IN SPORES AND VEGETATIVE CELLS

Sample	Non-exchangeable water (% dry weight)	Relaxation time (s) X 10^2
Vegetative cells 30°C	1.4	2.02
Spores 30°C	4.6	2.46
Spores 70°C	—	7.24

The relaxation time for the non-exchangeable water in vegetative cells and spores was measured, for the latter both at 30°C and 70°C. The results are shown in Table I.

NMR spectral changes during spore heat shock and germination

To investigate the changes which might take place in spore components during heat shock and germination, the NMR spectrum of spores was recorded during these processes (Fig. 9). No major change in the NMR spectrum was observed when spores were heated to 70°C, which had been shown to be the critical temperature for activation of *B. megaterium* spores [64]. During spore germination there is a dramatic appearance of peaks in the 3.9 and 0.9—2.5

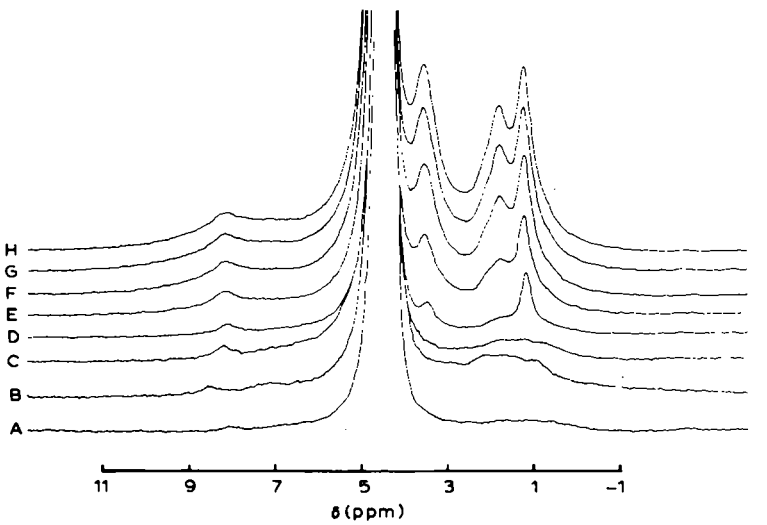

Fig. 9. NMR spectrum of spores during germination. Spores were suspended in ^2H_2O at 100 mg/ml and spectra taken on 2 ml of this suspension in a 12 mm NMR tube. A. Dormant spores at 30°C. B. Spores at 70°C after heating at 70°C for 30 min. C. Spores cooled to 30°C immediately after heating as in (B) above. D. Spores cooled to 30°C immediately after heating at 70°C for 30 min and after the addition of germinants (final concentration 10 mM L-alanine, 50 mM KCl) within 5 min of cooling. Spectrum accumulated 0—5 min after addition of germinants. The peaks at 1 ppm and 3.4 ppm are due, at least in part, to L-alanine. E. As (D) above but accumulated 6—11 min after addition of germinants. F. As (D) above but accumulated 12—17 min after addition of germinants. G. As (D) above but accumulated 18—23 min after addition of germinants. H. As (D) above but accumulated 24—29 min after addition of germinants.

ppm regions of the spectra. These peaks may be attributed at least in part to the germinant and the molecules released by germinating spores since the same spectra are observed in the supernatant after spore germination. Proteolysis has been shown to take place during this time period [65] and also release of cortex peptidoglycan fragments [66].

NMR spectra of spore lipids

The experiments on the interaction of calcium with monolayers suggest that the binding of calcium to the phospholipid head groups of the spore membrane could constrain the molecular mobility of these components in the spore membrane. We therefore investigated the effect of calcium on the NMR spectrum of sonicated aqueous dispersions of spore lipids at various temperatures. The NMR spectrum of spore lipids in C^2HCl_3 is shown in Fig. 10. The spectrum is consistent with the published lipid composition of this spore [11]. The doublet nature of the $-CH_3$ peak at 0.87 ppm is characteristic of the presence of branched chain fatty acids. The absence of any peak at 3.36 ppm confirms the absence of phosphatidylcholine from the spore and the virtual absence of an olefinic proton peak at 5.32 ppm is consistent with very low levels of unsaturated fatty acids. Fig. 11 compares the spectra of sonicated aqueous dispersions of spore lipid at various temperatures. At all temperatures, calcium causes considerable broadening of the peaks, implying a reduction in molecular motion. At no stage were there any signs of the lipid precipitating from the dispersion in the presence of calcium.

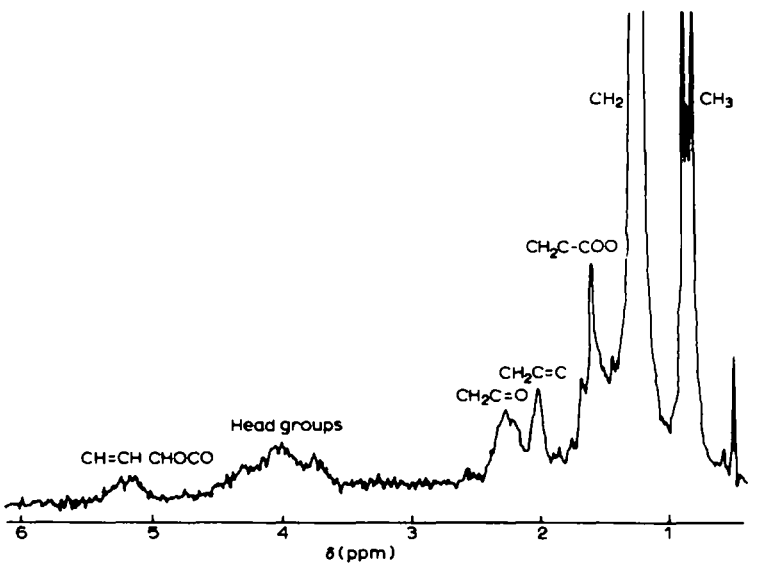

Fig. 10. NMR spectrum of spore lipids in C^2HCl_3. Asignments were made with the aid of Finer et al. [79] and standard chemical shift correlation tables. The peak at δ 0 ppm from tetramethylsilane was used as an internal reference.

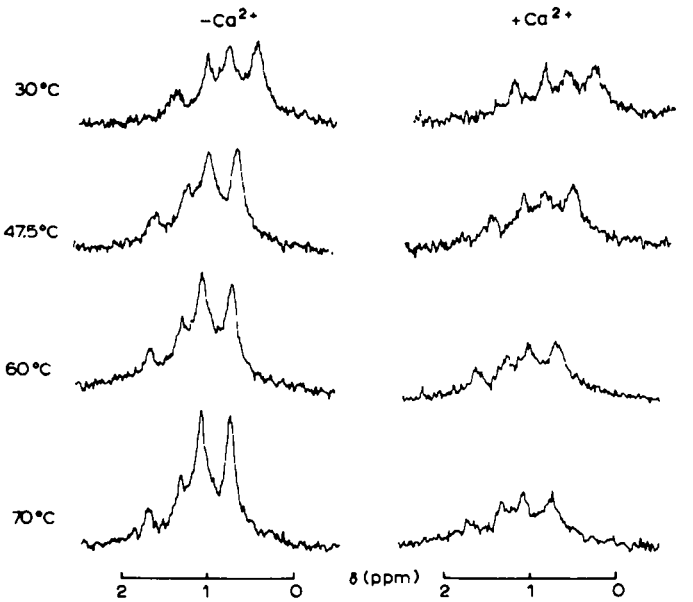

Fig. 11. Effect of Ca^{2+} on the NMR spectrum of a sonicated dispersion of spore lipids in ^2H_2O at different temperatures. Ca^{2+} concentration 0.9 mM.

Discussion

Nitroxide spin labelled fatty acids have been used to probe the membrane phospholipids of *Neurospora* [43,38,67], *Mycoplasma* [44], *Chlamydomonas* [68], *Clostridia* [69] and *Bacilli* [70]. Surprisingly in most of these cases no significant spectral differences were observed when the label was incorporated biosynthetically into the membrane phospholipid or by intercalation of fatty acid spin labels, although differences were reported in the case of studies with *Neurospora* [43]. In this work we have been unable to incorporate 12-NS biosynthetically into the phospholipids of *Bacillus megaterium* spores. 12-NS has been used in two reports [69,70] to investigate membrane lipid fluidity in prokaryotes but in neither case was 12-NS shown to be incorporated (biosynthetically) into phospholipids. Chan et al. [70] did report the successful incorporation of 5-NS into membrane phospholipids of a thermophilic bacillus, but they gave no indication of the amounts of original label recovered in the phospholipid fraction.

Spin probes based on stearate may not be ideal probes for incorporating into Bacillus membranes since in these organisms, stearate constitutes only 5% of total fatty acid [11,70]. Incorporation of a nitroxide derivative or a major constituent fatty acid such as C 15:0 br. might be more successful.

Data from ESR using 5-NS, 12-NS and 16-NS reveal that the lipids in dormant spore membranes are in a highly ordered, rigid, state throughout the width of the bilayer in marked contrast to lipids in vegetative cell membranes. Spectra indicating totally immobilised spin labels at all levels of the fatty acid backbone are more usually suggestive of binding to protein [2]. The polycrystalline spore spectra may therefore reflect a highly-ordered lipid environ-

ment, or label bound to spore coat protein. In vivo incorporation of 12-NS shows that the bulk of the label is indeed associated with the spore integument fraction containing the coat protein, but this fraction also includes the outer spore membrane comprising 34% of the total spore lipid [71]. Until the spin probe can be incorporated biosynthetically into the membrane phospholipid of these sporulating organisms however, this type of investigation remains a useful addition to the whole cell studies. The best indication of the site of 12-NS in the spore can be obtained from a consideration of the spectra of 12-NS in forespores and germinated spores. Stage VI forespores show increasing fluidity from 5-NS to 16-NS characteristic of spin label in lipid bilayers [2,72] and the same type of spectra is found in germinated spores. It seems possible that between stage VI of sporulation and final maturation, a contraction or dehydration of forespore membrane, possibly brought about by an interaction of calcium with acidic phospholipids [73—75], could change the lipid-protein structure of the membrane to an essentially crystalline form. Since no signals were obtained from any spore component (Fig. 8), the NMR spectra also suggest that these components may all exist in an essentially immobile or dehydrated state. The dramatic change during germination from the polycrystalline spin label component of the dormant spore to the more fluid environment characteristic of vegetative cells, is accompanied by the release of Ca^{2+} (Fig. 4b). This again implies a role for Ca^{2+} in the immobilisation of spore membrane. As alternative explanations for the above results, the label found associated with the membrane prior to stage VI or sporulation may be translocated to a protein (possibly coat protein) site or precipitated as the calcium salt in the dormant spore and subsequently returned to the membrane during spore germination. Such a reversible translocation of label would only occur if the intercalated label were expelled from its membrane location by the type of phospholipid crystallization which we describe above. Because the isolated integument fractions from both dormant and germinated spores show polycrystalline type spectra, we cannot disregard the possibility that a change in permeability of the developing forespore precludes incorporation of spin label either by in vivo or in vitro routes and that this permeability barrier is removed early in germination.

The absence of any fluid components in the dormant spore, as measured by ESR spectroscopy, prompted the investigation of possible fluidity changes occurring during the sporulation process. Between stage III and VI, forespores develop a peptidoglycan cortex, a proteinaceous coat and accumulate up to 2% of their dry weight as calcium and approximately 10% of their dry weight as an equimolar concentration of pyridine 2,6-dicarboxylic acid. Despite this accumulation of calcium there is no apparent change in the lipid fluidity of intact isolated forespores as shown by ESR measurements (Fig. 3). The stage VI forespore is heat resistant, chemically resistant, radiation resistant, osmotically stable and capable of responding to germinants to produce a viable vegetative cell [47]. Because of its similarity to the dormant spore, very little additional development must occur during the process of release from the mother cell. The role of calcium in the imposition, maintenance and loss of spore dormancy has been extensively investigated (see Ref. 76 for review). At least up to stage VI, calcium accumulation into the forespore is by facilitated

diffusion [1] down a concentration gradient possibly maintained by the chelation of accumulated calcium with pyridine 2,6-dicarboxylic acid within the forespore compartment [1]. It is possible that the free concentration of calcium in the forespore, prior to stage VI, is insufficient to cause any perturbation of membrane fluidity. If after stage VI a small amount of calcium or other divalent cation is accumulated in excess of pyridine 2,6-dicarboxylic acid it would bind to the acidic phospholipids of the spore resulting in a decrease in lipid fluidity as described for model membrane systems and lipid monolayers (Refs. 75,77—79 and in this paper). Such a change in membrane fluidity would have a considerable effect on membrane function; for example, an abrupt increase in the rate of carrier-mediated transport at the ordered to fluid transition has been observed [77,80]. Permeability to water and non-electrolytes [81] and translational motion of membrane components [82] also depends on the degree of membrane fluidity. A fluid to ordered lipid transition induced by the binding of calcium to the negatively-charged lipids of spore membrane(s) at a late stage in development, could contribute to dormancy by inactivating membrane-bound enzymes and rendering the spore core impermeable to nutrients, ions and water. Providing calcium can be removed from the phospholipid head groups [83] the release of calcium from the spores would be expected to return the membrane lipid to a fluid environment. This change is indeed observed during spore germination when the change from an ordered to fluid environment, measured by ESR spectroscopy of 16-NS, is paralleled by the release of Ca^{2+} from the spore (Fig. 4a and 4b).

The water content of spores is difficult to determine precisely, but data from a number of sources suggest that spores are dehydrated compared to vegetative cells [4]. Calorimetric studies by Maeda et al. [84] had previously revealed the presence of non-exchangeable water in spores. This is confirmed by the NMR results reported here which also show that there is three times more of this non-exchangeable water in spores than in vegetative cells.

The value of approx. 10^{-2} s obtained for the relaxation time of the non-exchangeable water in spores and vegetative cells is notable. In recent years a number of studies on the magnetic relaxation of proton, deuterium and oxygen-17 in water in biological tissue have been made with a view to understanding the state of water in living systems. The spin-lattice relaxation time T_1 of the protons in pure water is approx. 3 s at 25°C [85,86]. The proton relaxation time for water in biological tissues is generally observed to be approximately one fifth of this [85—92]. Two explanations have been advanced to account for this decrease in relaxation time: (i) All the cell water is in an ordered or 'semi-crystalline' state [88]. (ii) The cell water is comprised of two fractions in fast exchange, a small fraction (2—20% of total) directly associated with proteins, membranes and other macromolecules in the cell, which has a considerably lower relaxation time than free water, and a second larger fraction comprising the rest of the cell water which has a relaxation time similar to free water [85,89—92]. The bulk of the available evidence supports this model. Studies on the temperature dependence of T_1 show an order of magnitude fall at around −80°C [90—92]. At this temperature the bulk of the cell water freezes, leaving only the small macromolecule associated fraction, which does not freeze above −90°C, to contribute to the overall T_1 value. The value for T_1

below −80°C is very similar to the T_1 value observed for water adsorbed on collagen fibres [93,94] and muscle proteins [85]. Fung et al. [11] have shown that values calculated for T_1 on the basis of such a two phase model, where the combined T_1 is a weighted average of those for the two fractions, agree closely with the experimentally-observed values and account well for the observed frequency and temperature dependence of T_1.

The relaxation times observed here for the non-exchangeable water in spores and vegetative cells are of the same order as those observed for water adsorbed to macromolecules though rather lower than average. The increase in T_1 of approx. 3-fold on heating to 70°C is consistent with the observed temperature dependence of T_1 for adsorbed water molecules [91,92]. It has also been shown that the proton T_1 for water in biological and macromolecular systems decreases as the percentage water content decreases [85,86,91]. Thus the values observed for the T_1 of the non-exchangeable water in spores and vegetative cells suggest that this water could be adsorbed to macromolecules and is in a relatively anhydrous environment.

It must be emphasized however that these are only preliminary measurements and that vegetative cells and particularly spores are complex systems whose heterogeneity can readily introduce artefacts into relaxation measurements. In particular, as already mentioned, spores contain manganese (approx. 0.18% dry wt. [36]), which is paramagnetic and known to affect relaxation measurements.

Due to the particle size (approx. 1 μm) and probable anisotropy of the membrane lipid, no high resolution proton NMR spectra could be observed for membrane lipids in both vegetative cells and dormant spores. The use of carbon or phosphorus NMR may enable more information to be obtained about the state of phospholipids during sporulation and germination.

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