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USE OF ELECTRON SPIN RESONANCE TO STUDY BACILLUS MEGATERIUM SPORE MEMBRANES

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SUMMARY: Membranes from dormant and heat-activated spores were labelled with the fatty acid spin probe 5-doxyl stearate and analyzed using electron spin resonance spectroscopy. Membranes from dormant spores were slightly less fluid above 23° than membranes from heat-activated spores. Also L-proline caused a much larger increase in the upper transition temperature than did D-proline when added to membranes from heat-activated spores. Thus a compound known to trigger germination in this strain may interact stereospecifically to alter the biophysical properties of the spore membranes.

INTRODUCTION: The mechanism of breaking the dormant state of bacterial spores is not known. In B. megaterium QM B1551, rapid germination occurs if the spores are first heat-activated followed by the addition of a stereospecific compound like L-proline (1). One model to explain these processes suggests the spore membrane(s) may be involved (2), and to study this possibility we have used electron spin resonance (ESR)[4] spectroscopy. We report here what appears to be the first demonstration of biophysical changes in spore membranes that result from heat-activation and L-proline.

MATERIALS AND METHODS: B. megaterium spores were grown in supplemented nutrient broth, harvested and stored as previously described (3). All references to spore weights are on a dry weight basis. Spores (50 mg/ml) were

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[4]Abbreviations: ESR, electron spin resonance; 5-DS, 5-doxyl stearate; HEPES, N-2-hydroxyethylpiperazine-N'-2'-ethane sulfonic acid.

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extracted with sodium dodecyl sulfate-dithiothreitol and then washed as previously described (4). The details of the isolation and characterization of spore membranes will be described elsewhere (Racine, F.M. and Vary, J.C., in preparation) but briefly the methods were as follows. Sodium dodecyl sulfate-dithiothreitol extracted spores (4) were lysed in 0.1 M HEPES (pH 7.5) containing lysozyme (0.5 mg/ml), RNase (2.5 µg/ml) and DNase I (2.5 µg/ml) at 30° for 12 min followed by sonication (8 times for 30 s each) at 0° as previously described (4). Spore membranes were isolated by methods similar to those used for the isolation of E. coli cytoplasmic membranes (5). Membranes from heat-activated spores were obtained by first heating spores (50 mg/ml) at 60° for 10 min, followed by centrifugation at 5,000 x g for 10 min and then lysis as described above. The final membrane preparation in 10 mM HEPES (pH 7.5) contained 10-20 mg of protein/ml as determined by the method of Lowry et al. (6) and was stored on ice for further analysis.

All ESR studies were done with freshly prepared membranes (0.4 ml) to which 4-5 µl of 30 mM 5-DS was added. The labelling techniques that were used have been previously described (7). ESR spectra of such spin labelled preparations allow the determination of the hyperfine splitting parameter, $2T_{\parallel}$, which reports the local fluidity of the membrane lipids (8). High values of $2T_{\parallel}$ reflect low fluidity. All ESR studies were carried out with a Varian Century Line ESR spectrometer, model E-112, equipped with a variable temperature controller. An external calibrated thermistor probe (Omega Engineering, Inc., Stamford, CT) was used to monitor the temperature of the sample. The data ($2T_{\parallel}$ vs. temperature) were analyzed by an iterative least squares program to be described elsewhere (Coughlin, R.T., Brunder, D.G., and McGroarty, E.J., in preparation). Briefly, a B-spline (9) was used to provide a smooth fit for the ESR data and points of inflection were used to group data. Regression lines were calculated for each group and then plotted. This analysis allowed the determination of break points. Such breaks in the temperature dependence of $2T_{\parallel}$ have been correlated with lipid phase separations or lipid phase transitions from gel to liquid crystalline lipid states (7). All temperature dependent ESR parameters were shown to change reversibly up to 46°. For each set of data, at least 3 independently isolated membrane preparations were used.

RESULTS AND DISCUSSION: Membranes isolated from either dormant or heat-activated spores had the same phospholipids and in the same ratios as whole spores, similar to previously published data for total phospholipids in this strain (10). The membranes contained no peptidogylcan, a distribution of about 20 proteins ranging from 13,000 to 130,000 daltons, several respiratory associated enzyme activities and a unique carotenoid (unpublished).

The temperature dependence of $2T_{\parallel}$ in membranes from dormant spores is illustrated in Fig. 1. Of particular importance are the transition temperatures where the slopes of the lines change at 6° and 26° suggesting a change in the relative ratio of gel and liquid crystalline lipid. When the same experiment was done with membranes from heat-activated spores (Fig. 2a) the transition temperatures were slightly different, 7° and 23°. The more

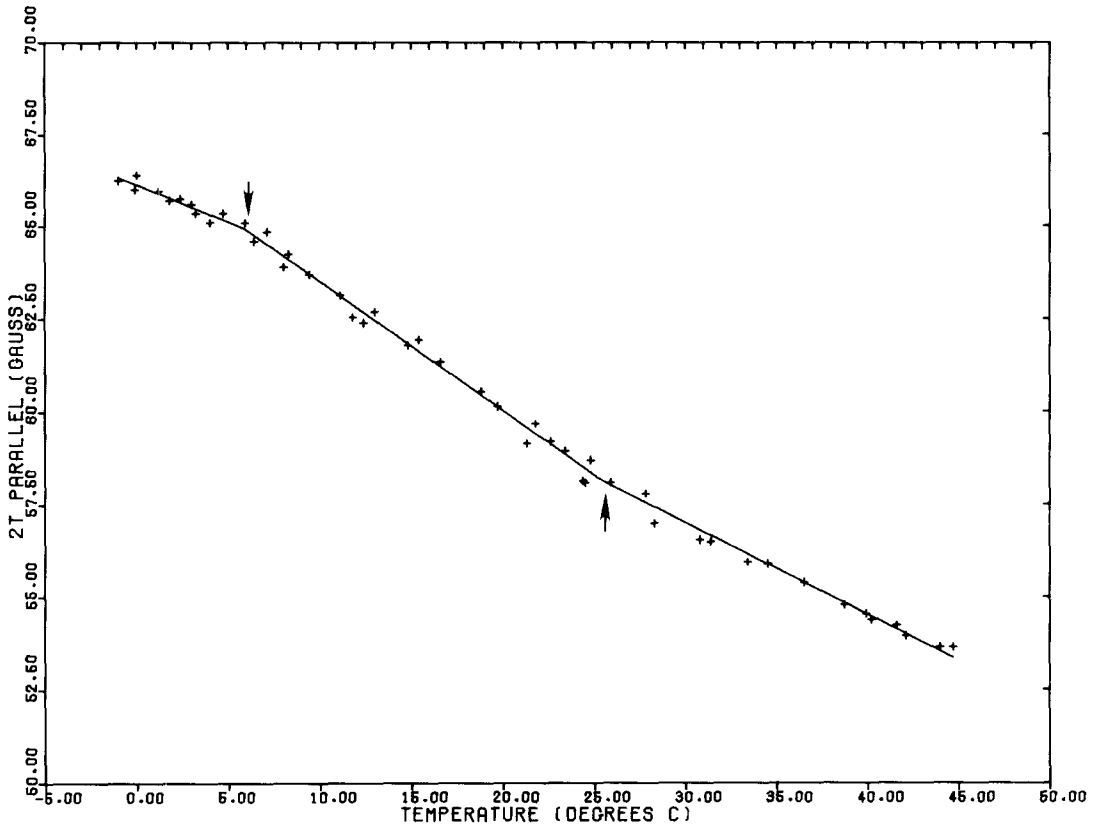

Fig. 1. Temperature dependence of $2T_{\parallel}$ in dormant spore membranes. Membranes were isolated and ESR spectra recorded at the indicated temperatures in the presence of 5-DS as described in Materials and Methods. Arrows indicate transition temperatures.

noticeable difference, however, is that above the upper transition temperature membranes from heat-activated spores exhibit a greater slope (-0.29 gauss/^{o}C) than do dormant spore membranes (-0.25 gauss/^{o}C). This implies that above the upper transition, membranes from heat-activated spores are more fluid than those from dormant spores. We feel that this change in fluidity should be interpreted with caution with respect to any possible functional role. But it is apparent that there is a physical difference between membranes from dormant and heat-activated spores which to date has never been reported.

Finally, we tested the effect of adding in vitro a known trigger reagent, L-proline, to membranes from heat-activated spores. The results (Fig. 2) show

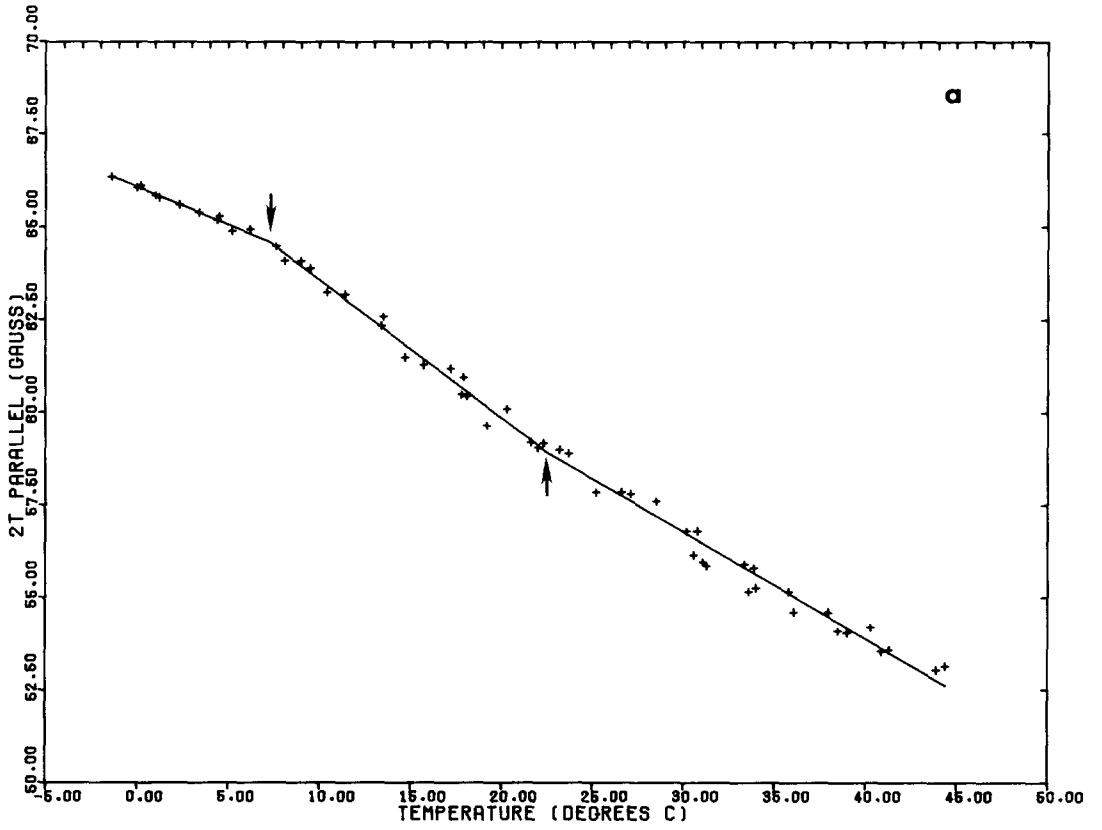

Fig. 2. Effect of L-proline. Membranes from heat-activated spores were isolated and analyzed as described in the legend of Fig. 1. in the absence (a) and presence (b) of 30 mM L-proline.

a dramatic change in the upper transition temperature from 23^{o} before to 31^{o} after the addition of L-proline. When the same experiment was done with D-proline (30 mM) which cannot trigger germination (1), a much smaller shift occurred in the upper transition temperature (from 23^{o} to 27^{o}). Preliminary experiments suggest that the addition of L-proline to dormant spore membranes caused no dramatic changes in the temperature dependence of $2T_{\parallel}$. In all of these experiments the low transition temperature did not change significantly. From these results, it is apparent that L-proline interacts with the membrane and causes a change in the supramolecular structure. We have no evidence that the transition temperature shift to 31^{o} is fortuitous or significant with respect to 30^{o} being optimal for triggering germination. The

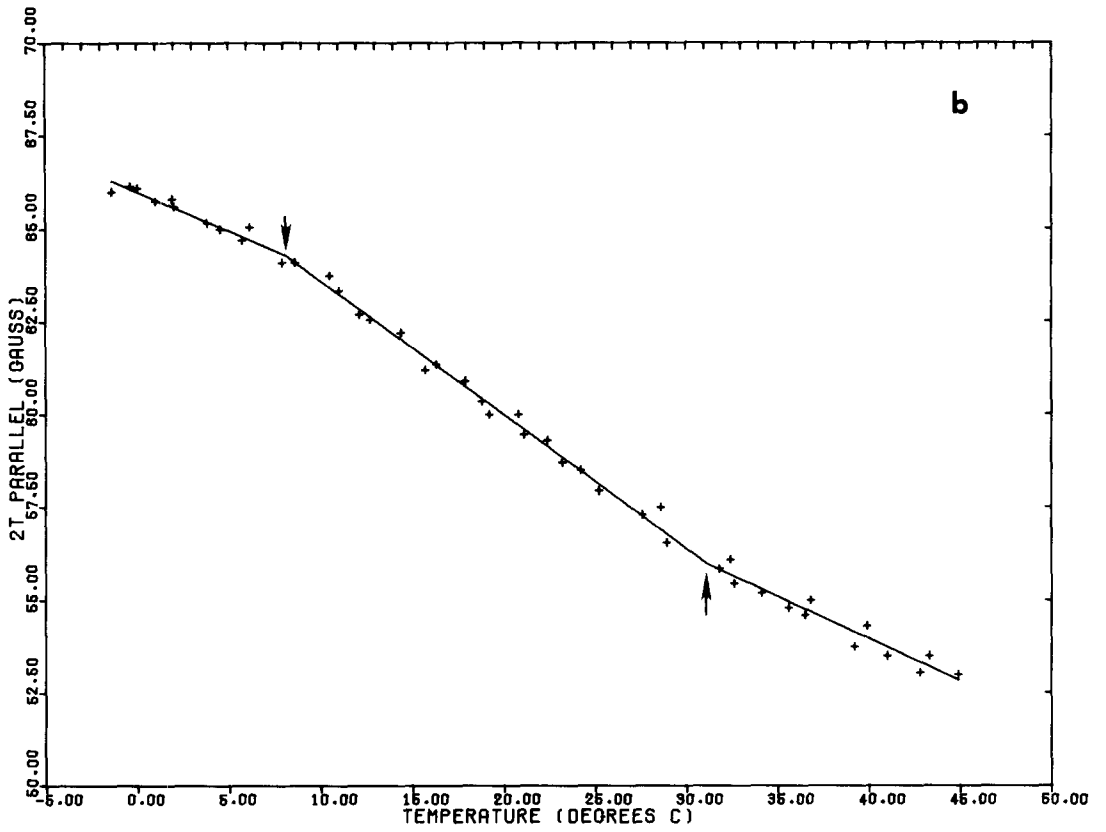

Fig. 2 (continued).

important point is that L-proline may interact with membranes from heat-activated spores in a stereospecific manner to cause a biophysical change in the spore membrane.

While these data do not explain the mechanism of triggering germination, techniques described here provide us with a useful tool to further analyze other trigger compounds, membranes isolated at different times during sporogenesis and to determine the role of spore membranes in development. Of particular interest are the recent data with a proline affinity analog (Rossignol, D.P. and Vary, J.C., submitted) which indicates a possible method to isolate the proline trigger site. Using these present and other biophysical techniques, we hope to study the interactions of L-proline with the trigger site both in vitro and in vivo.

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REFERENCES:

1. Rossignol, D.P. and Vary, J.C. (1979) J. Bacteriol. 138, 431-441.
2. Vary, J.C. (1978) in Spores VII (Chambliss, G. and Vary, J.C. eds.) 104-108, American Society for Microbiology, Washington, D.C.
3. Shay, L.K. and Vary, J.C. (1977) Biochim. Biophys. Acta 538, 284-292.
4. Vary, J.C. (1973) J. Bacteriol. 116, 797-802.
5. Schnaitman, C.A. (1970) J. Bacteriol. 104, 890-901.
6. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
7. Janoff, A.S., Haug, A. and McGroarty, E.J. (1979) Biochim. Biophys. Acta, in press.
8. McFarland, B.G. (1972) Chem. Phys. Lipids 8, 303-313.
9. Dierckx, P. (1975) J. Comp. Appl. Math. 1, 165-184.
10. Bertsch, L.L., Bonsen, P.P.M., and Kornberg, A. (1969) J. Bacteriol. 98, 75-81.