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MEMBRANE STUDIES OF *STREPTOCOCCUS PYOGENES* AND ITS L-FORM GROWING IN HYPERTONIC AND PHYSIOLOGICALLY ISOTONIC MEDIA

AN ELECTRON SPIN RESONANCE SPECTROSCOPY APPROACH

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SUMMARY

Electron spin resonance spectroscopy (ESR) was used to compare the lipid organization, thermal stability and the physical state of the membrane of a human pathogen, *Streptococcus pyogenes* and its osmotically fragile L-form with this same L-form now adapted to grow under physiologically isotonic conditions (physiological L-form). Comparison of the hyperfine splittings of a derivative of 5-ketostearic acid spin label, I(12, 3), after incorporation into the membrane, revealed that the lipid chain rigidity of these membranes is in the order physiological L-form > osmotically fragile L-form > streptococcus. The signal intensity (of the center magnetic field line) versus temperature analysis showed two transitions for these membranes. The first with melting points of 45, 26 and 36 °C and second transition at 70, 63 and 60 °C for the physiological L-form, osmotically fragile L-form and streptococcal membranes, respectively. This same order of membrane lipid chain rigidity was seen from the cooperativities obtained for each of these systems from analysis based on the expression for an *n*-order reaction. The I(12, 3) and other probes with the paramagnetic group close to the methyl end of the molecule suggested that this difference in lipid chain rigidity between these organisms resides in the environment closer to the lipid head group region rather than in the hydrophobic lipid core. Another major finding was the binding of I(12, 3) at two or more different sites in each of the membranes examined. This change in lipid chain rigidity now provides an explanation to account for the survival of a previously osmotically fragile L-form in physiologically isotonic media by focusing on changes in the physical nature of its membrane. In so doing, it adds to and reinforces the speculation of the potential survival in vivo and involvement in pathogenesis of osmotically fragile aberrant forms of bacteria.

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INTRODUCTION

A nonreverting, osmotically fragile L-form from *Streptococcus pyogenes* is devoid of a rigid cell wall similar to that of the parent organism from which it was derived [1]. It has long been known that changes in pH and temperature affect this L-form far more drastically than they do the parental streptococcus, suggesting a structural difference in the L-form membrane [2, 3]. Publications from this laboratory have shown that the lipid content and composition of the membrane of this L-form differs from that of its coccus [4, 5]. Also, that this L-form contains a membrane glycerol teichoic acid which is shorter in length and lacks D-alanine when compared with that from the coccal membrane [6]. This lack of D-alanine is due to a defect in the membrane of this L-form which prevents its incorporation into the polyglycerol phosphate polymer by enzymes in the cytoplasm [7]. This same L-form has now been rendered osmotically stable and found able to grow in physiologically isotonic growth media and to destroy human heart cells rapidly in tissue culture [8, 9].

The use of electron spin resonance (ESR) techniques for studying the structure and function of biological membranes and model systems has been extensively reviewed [10–14]. L-Forms derived from gram positive, pathogenic bacteria are ideal model systems for the study of membrane structure and function. However, because they are osmotically fragile, their survival in vivo is doubtful and their role in pathogenesis is only speculative. The recent adaption of an L-form from the group A streptococci to grow in isotonic media now presents us with a means of correlating changes in membrane structure with stability and can enhance our meager understanding of the capabilities of osmotically fragile bacterial aberrant forms to persist in vivo. The present study utilizes ESR to compare the lipid organization and changes in the physical state of the membrane of a human pathogen, *S. pyogenes*, and its osmotically fragile L-form with this same L-form adapted to grow under physiologically isotonic conditions.

MATERIALS AND METHODS

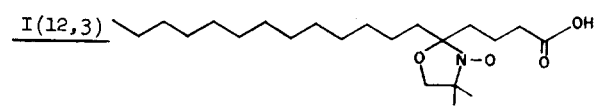
Streptococcus pyogenes, type 12, its stabilized but osmotically fragile L-form, and this L-form recently adapted to grow in physiologically isotonic media were used. The latter organism was derived from the osmotically fragile L-form by a multistep adaption procedure to an isotonic medium [8, 9]. The growth medium for each contained 2.8 % (w/v) *Albimi brucella* broth (Pfizer Diagnostics, Brooklyn, N.Y.) and was supplemented with 0.8 % (w/v) bovine serum albumin fraction V (Armour Pharmaceutical Co., Chicago, Ill.) [6, 7]. To the medium for the osmotically fragile and physiologically isotonic L-forms was added 3 % (w/v) and 0.35 % (w/v) sodium chloride, respectively in addition to the 0.5 % (w/v) sodium chloride already present as an ingredient. Cultures of each organism were incubated and harvested when in their late logarithmic phase of growth. The cells were washed (3 times) at 4 °C; the streptococcus and the physiologically isotonic L-form with saline (0.85 % w/v sodium chloride) and the osmotically fragile L-form with 3 % (w/v) sodium chloride solution.

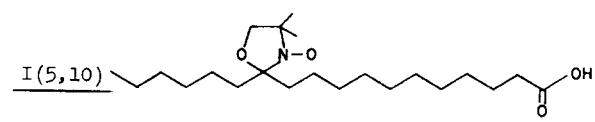
Cells of all three organisms were broken, after being suspended in 0.005 M Tris-HCl, pH 7.8 and 0.005 M $MgCl_2$, by shaking with plastic beads in a Bronwill

cell homogenizer at 4000 rev./min for 9.5 min as detailed elsewhere [7]. Whole cells, cell walls and debris were removed by differential centrifugation and the clean membrane fragments washed and suspended in 3 ml of this same buffer. Membrane preparations maintained their enzymatic activity for teichoic acid synthesis as measured by the D-alanine incorporation assay [7] and for membrane glycolipid synthesis as determined by UDPglucose consumption [15].

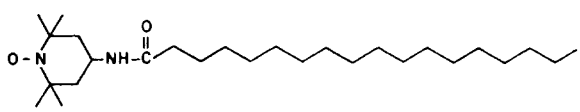
Two types of spin labels were used: type I(m, n) were N-oxyl-4′,4′-dimethyloxazolidine derivatives of 5-ketostearic acid where (m, n) is (1, 14), (5, 10) and (12, 3). These were purchased from SYVA, Palo Alto, Calif. and used without further treatment. The type II spin label was generously provided by Dr. Ian C. P. Smith, National Research Council, Ottawa, Canada and is the 2,2,6,6-tetramethylpiperidine-1-oxyl derivative of stearamide.

Type I (m, n)



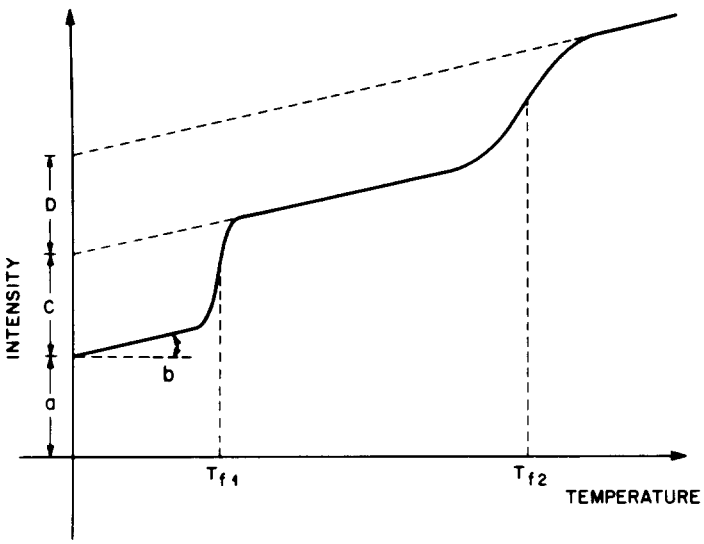


Type II



An identical labeling procedure was employed for all three organisms. 0.5 mg of the spin label was dissolved in a few drops of chloroform, the solvent evaporated to dryness and the film redissolved in 2 ml of fatty acid-free albumin (8 % w/v) [16]. A 3 ml membrane suspension (20 mg/ml) was added to the spin label and the probe allowed to diffuse into the membrane for 20 min at 10 °C. The labeled membranes were spun down, washed twice and suspended in 0.3 ml of the buffer indicated. The second wash contained only barely detectable amounts of the spin label. Experiments with ascorbate were conducted by mixing equal amounts of labeled membrane suspension and sodium ascorbate ($1 \cdot 10^{-2}$ M) and recording the peak height of the center magnetic field line ($m_I = 0$) as a function of time at 25 °C, 32 °C, and 40 °C. Electron microscopy failed to show any morphological changes between native and spin-labeled membrane fragments (Chevion, M. and Panos, C., unpublished results).

Electron spin resonance (ESR) spectra of the various spin labeled membranes were recorded on a Varian E-9, X-band spectrometer (center field 3310 gauss) using a thin flat quartz sample cell in a temperature range of 0–100 °C maintained by a Varian E-257 variable temperature control unit with an accuracy of ± 1 °C in the range investigated. Spectra were recorded from each membrane-labeled suspension at various temperatures (5–10 °C increments) with different gains and



$$y = a + bT + \frac{C}{1+\exp\left(n_1\left[(T_{f1}/T)-1\right]\right)} + \frac{D}{1+\exp\left(n_2\left[(T_{f2}/T)-1\right]\right)}$$

Fig. 1. The analysis of the temperature dependence of the signal intensity of the center magnetic field line as an n order reaction model describing two transitions (see text).

modulation amplitudes. Also, additional spectra from each sample were obtained after cooling to 30–35 °C.

The temperature dependence of the signal intensity of the center field line was analyzed using an n order reaction model [21] for the description of 2 transitions* in addition to the linear increase of this characteristic. The function used and the parameters measured are shown in Fig. 1, where:

y is the signal intensity of the center field line,

T is the absolute temperature,

T_{f1}, T_{f2} are the temperatures corresponding to the first and second transitions respectively.

n_1, n_2 are the cooperativities of the first and second transition respectively. The value of n determines the sharpness of a transition; higher cooperativity values indicating greater membrane rigidity. The temperature course of the transition is approximately that to be expected for an assemblage of independent units of the indicated size each one of which shows a strictly two state, all-or-none transition [22].

C, D are the vertical displacements occurring during the first and second transitions respectively, and serve as a measure for the extent of each transition.

Using least squares curve fitting procedures, it was possible to obtain the best value for each of these 8 parameters (a, b, C, D, n_1, n_2, T_{f1}, T_{f2}) for the three organisms under study, from which computer generated curves describing the signal intensity as a function of the temperature were derived (Fig. 7).

* Transition point where the fatty acid chains exhibit an increase in molecular motion.

RESULTS

A derivative of stearic acid containing a nitroxyl radical, I(12, 3), exhibits the 3 line-type spectrum in solution. When the spin label is free the spectrum is nearly isotropic (Fig. 2A). If it is bound to the membrane, the spectrum becomes anisotropic (Fig. 2B) as a result of a slower rate of tumbling of the spin label. The hyperfine splitting ($2T$) for such spectra is related to the freedom of rotation of the nitroxyl radical as measured between its low and high magnetic field lines; the greater the freedom of motion the smaller is $2T$ and the greater is the fluidity of the membrane. In this study, calculation of $2T$ was not reliable since the high magnetic field line was distorted by system noise and could not be amplified by increasing the concentration of the spin label bound to the membrane. Therefore, the comparison of the hyperfine

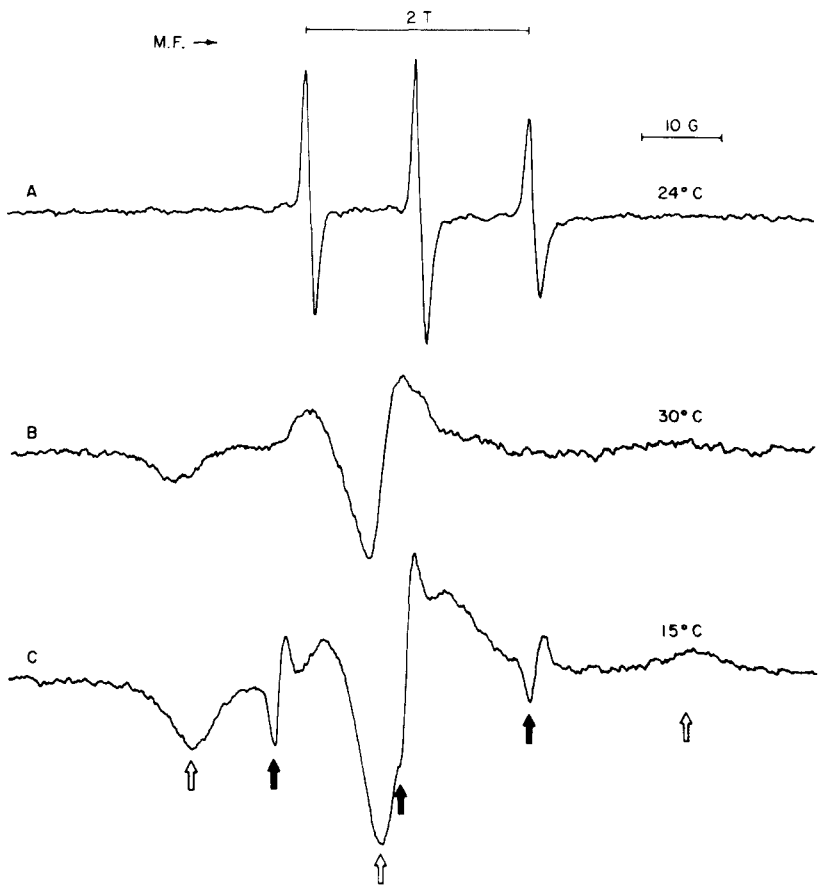


Fig. 2. The spectra of the *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid, I(12, 3). A, freely tumbling spin label in aqueous solution; B, bound to the membrane of the physiologically isotonic L-form; C, in the streptococcal or the osmotically fragile L-form membrane systems: 2 sets of lines one of which is due to the free-tumbling spin label (black arrow) and the other to its slower rotating membrane bound species (white arrow).

splittings from these organisms was based on the separation between the low and the center magnetic field lines of each spectrum.

With streptococcal membrane fragments and the I(12, 3) spin-label, a spectrum is obtained which is comprised of both a membrane bound and freely tumbling nitroxide spin label (Fig. 2C). A gradual decrease in the hyperfine splittings towards isotropy as a result of the decrease in lipid chain rigidity of the membrane is observed in the temperature range 0–100 °C, of which spectra obtained between 25 and 65 °C are shown in Fig. 3. As the temperature is raised to 85 °C the increasing freedom of molecular motion of the membrane bound spin label causes the membrane bound and the freely tumbling portions to coalesce over the entire spectrum. Approaching

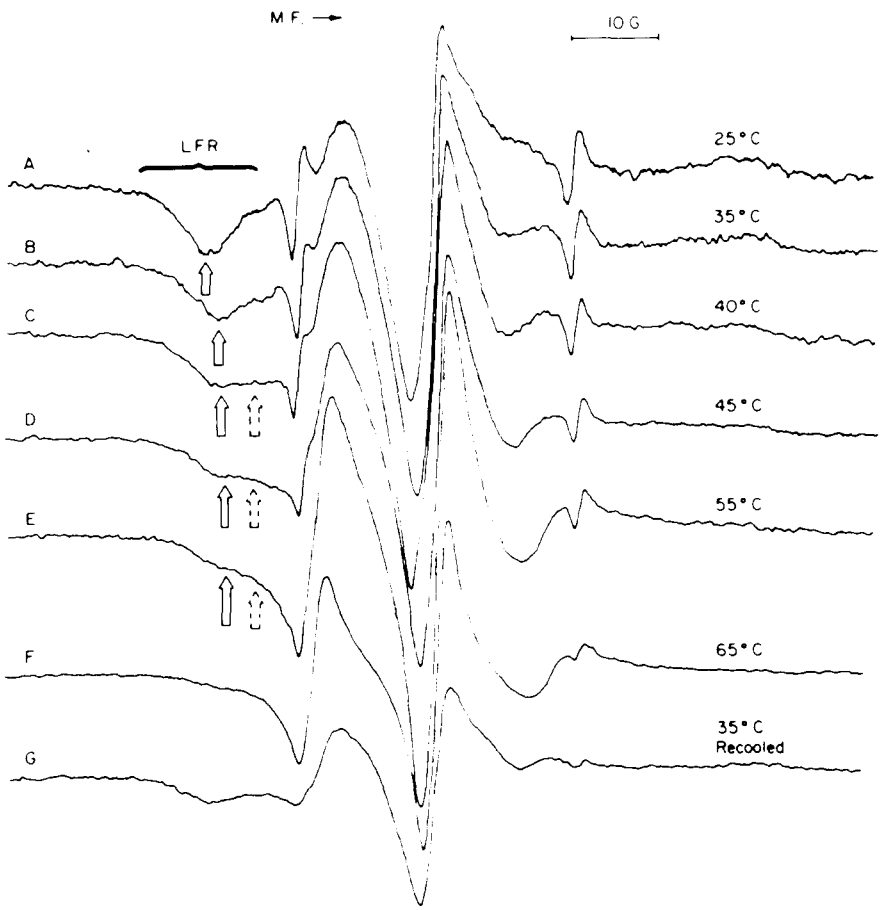


Fig. 3. Electron paramagnetic resonance first derivative spectra of *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid spin label I(12, 3) incorporated into membranes of *Streptococcus pyogenes* suspended in 0.005 M $MgCl_2$ and 0.005 M Tris-HCl, pH 6.8, at various temperatures. Spectra A–F were recorded from a sample at increasing temperatures while spectrum G was obtained from this same sample after cooling to 35 °C. Arrows in the low magnetic field region (LFR) indicate the two lines ascribed to membrane bound species.

35 °C (Fig. 3B) the line shape of the membrane bound spectrum begins to deviate from the expected pattern, arising from a single binding site whose freedom of motion is increasing with temperature, and suggests the presence of two or more binding sites. This phenomenon is revealed by observing the changes in the low magnetic field region of the spectrum (Fig. 3, B–E). The intensity and magnetic

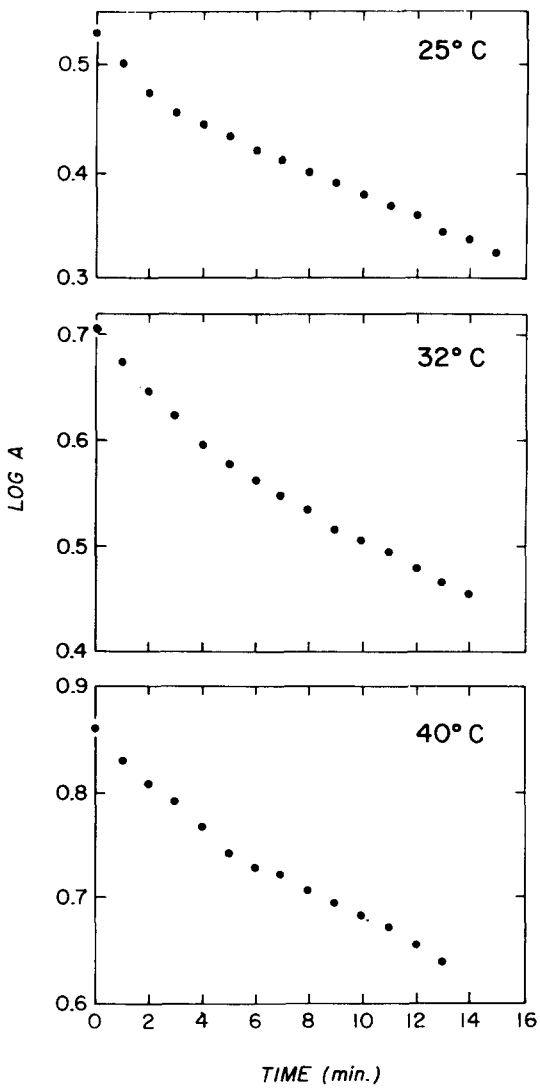


Fig. 4. Plots of the logarithms of the peak height of the center line, A, of *N*-oxyl-4′,4′-dimethyl-oxazolidine derivative of 5-ketostearic acid spin label, I(12, 3), incorporated into the streptococcal membrane with time at various temperatures. Note deviation from linearity. Experiments were done by mixing the membrane suspension with sodium ascorbate solution and locking the magnetic field on the peak after allowing 3–5 min for thermal equilibrium. Control experiments had shown that the free spin label is reduced instantaneously under these conditions.

field of one spectral species (dotted arrow) undergoes a more abrupt change than does the other species as the temperature is increased.

Spectral changes occurring over the range 10–50 °C were reversible. However, spectra recorded after the membrane was exposed to higher temperatures (up to 95 °C) and recooled showed discrepancies when compared to those taken at the same temperature before heat treatment; i.e. 35 °C (Fig. 3B and G). These deviations are between the values of the hyperfine splittings (relative to the center field line), between the values of the relative intensities of the free and bound spin label, and between the normalized intensities of each of the corresponding signals before and after heating, probably as a result of reduction of the spin label. While the loss in the low magnetic field line of the membrane bound portion of the spectrum is small, the decrease in intensity of the freely tumbling species is significantly larger. This implies that the incorporated (membrane bound) spin label is less vulnerable to heat effects as the membrane provides a partial protection against chemical changes, as may be expected.

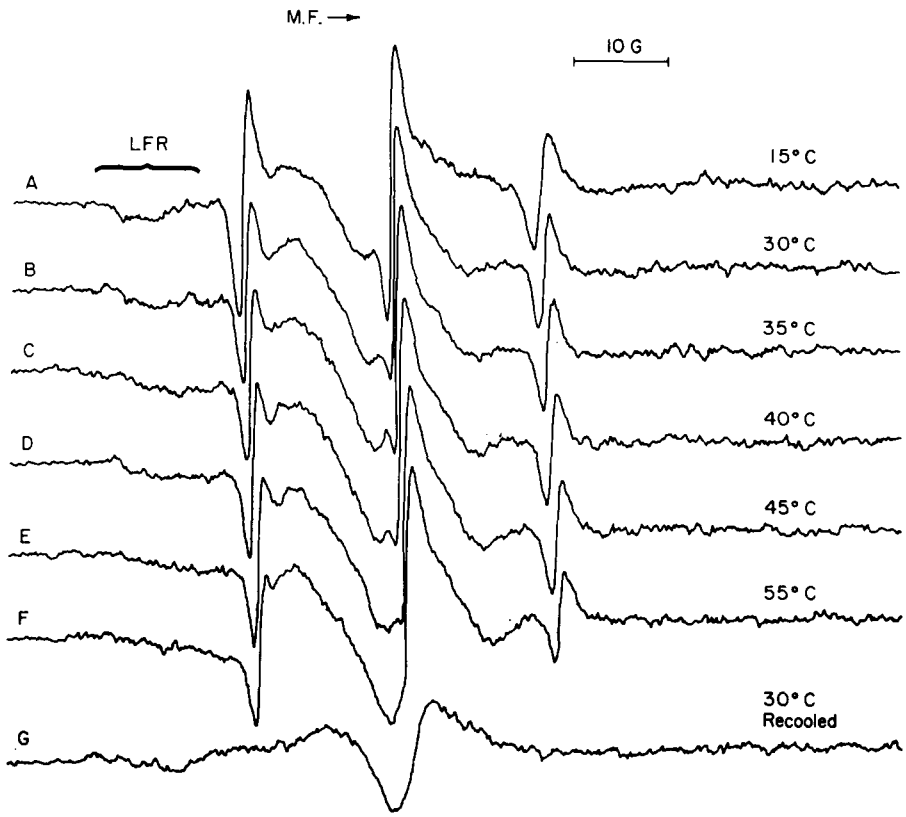


Fig. 5. Electron paramagnetic resonance first derivative spectra of *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid spin label, I(12, 3), incorporated into membranes of the osmotically fragile L-form suspended in 0.005 M $MgCl_2$ and 0.005 M Tris-HCl, pH 6.8, at various temperatures. Spectra A–F were recorded from a sample at different temperatures while spectrum G was obtained from this same sample after cooling to 30 °C.

The study of the kinetics of the decay of the center field line in the presence of excess ascorbate ($5 \cdot 10^{-3}$ M) showed curves that did not match exactly a first order expression but might be a superposition of two different first order exponential contributions (Fig. 4). As the rate of the disappearance is controlled by the rate of diffusion of the ascorbate, these results imply again the possible existence of two or more different sites for the membrane bound spin label in the streptococcal membrane.

Spectra of membrane fragments labeled with I(12, 3) from the osmotically fragile L-form (Fig. 5) are similar to those of the parental streptococcal system (Fig. 3). However, the hyperfine splittings are slightly larger in this system. As with the streptococcal membrane, the membrane bound portion of the L-form spectrum also suggests two or more possible binding sites for the fatty acid spin label (Fig. 5, B–F, note low magnetic field region). Although the signal-to-noise ratio, as a single criterion, is not sufficient to make this an unequivocal result, additional evidence (see Discussion) clearly substantiate this point. As before, one of the spectral species undergoes a transition at or below 30 °C (Fig. 5B), and the other changes over a wide temperature range (Fig. 5, C–F).

With membrane fragments from the physiologically isotonic L-form one observes the same general three line-type spectrum for the membrane bound species when labeled with I(12, 3) as with the parental coccus and its osmotically fragile L-form. However, no free label is apparent (Fig. 6) in this membrane. The membrane bound portion of this spectrum also indicates the presence of two or more binding sites for the spin label. The change in the membrane bound spin label was found to occur at a higher temperature than in the L-form and streptococcal systems tested earlier (Fig. 6D–G; note the low magnetic field region); a melting occurs at approximately 45 °C as compared with 35 and 30 °C in the other two systems tested. Finally, the hyperfine splittings for this organism are larger than those from both the coccus and its osmotically fragile L-form.

Preliminary studies with spin label I(1, 14) and I(5, 10) have shown spectra with identical hyperfine splittings for the coccal and L-form membrane systems. Also, membranes labeled with the stearamide probe (II) under identical conditions as with I(12, 3) showed exchange broadened spectra (indicating spin-spin interaction). This may be due to either exclusion of the spin label from the membrane or, to the greater rate of incorporation of the label, probably as a result of the unperturbed hydrocarbon chain.

In view of the temperature changes observed in the I(12, 3) spectra of these three organisms an attempt was made graphically to ascertain the membrane melting points. Generally, if τ_c, the rotational correlation time value, is not available then phase transitions are determined by plotting the order parameter S against temperature. However, in order to determine this order parameter it is necessary to be able to evaluate the effective hyperfine splittings $T'_{\parallel}$ (parallel) and $T'_{\perp}$ (perpendicular) introduced by Seelig [17] and Hubbell and McConnell [18]. In the spectra obtained with these organisms this is not a reliable measurement since the high magnetic field line is distorted by system noise. However, since τ_c is proportional to a function of the linewidth and any change in linewidth must have an associated change in intensity, the intensity at $m_I = 0$ transition (center line) of the membrane bound spectrum is taken as a measure of any change in motion of the spin label (Fig. 7). Using the mathematical expression introduced in Materials and Methods and

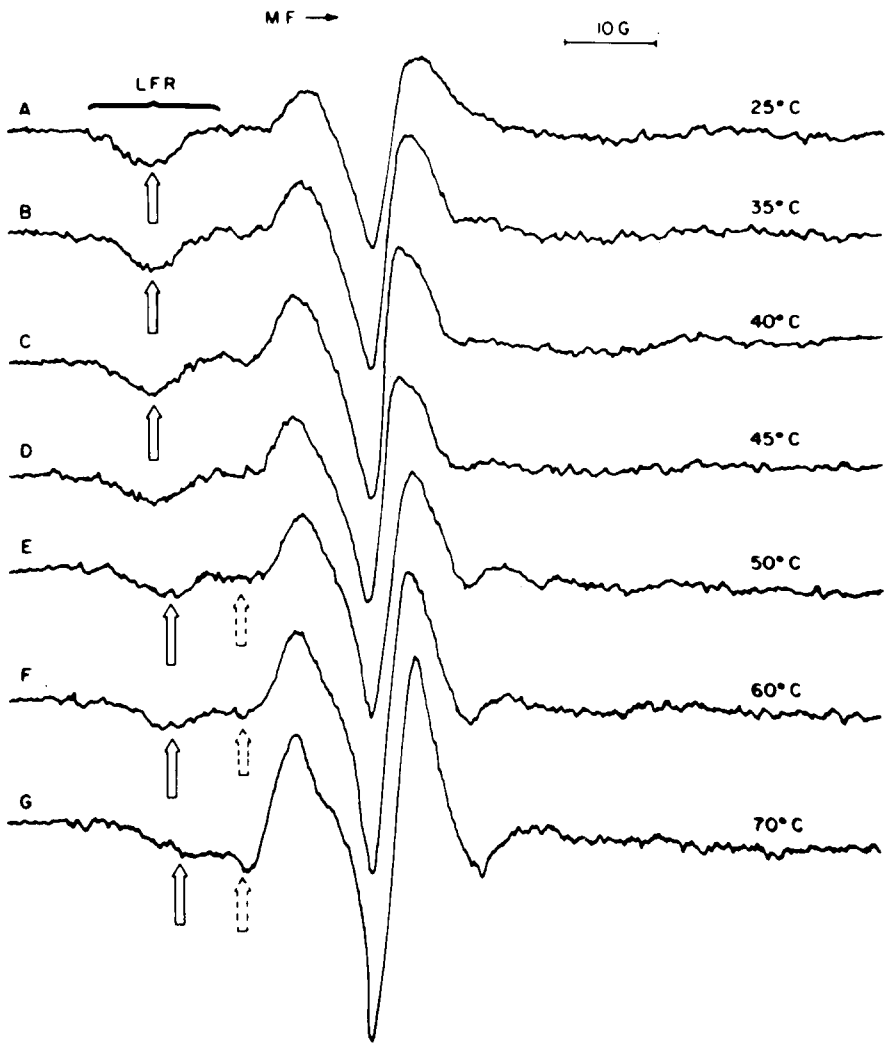


Fig. 6. Electron paramagnetic resonance first derivative spectra of *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid spin label, I(12, 3), incorporated into the membrane of the physiologically isotonic L-form suspended in 0.005 M $MgCl_2$ and 0.005 M Tris-HCl, pH 6.8, at various temperatures. Spectra A–G were recorded from the same sample at different temperatures. Arrows in the low magnetic field region (LFR) indicate the two lines ascribed to membrane bound species.

excluding the linear change of the signal intensity of the center field line (described by the two parameters a, b), the 6 remaining parameters were evaluated (Table I) by a least squares computer fitting program. As shown in Table I, the first transition takes place at 36, 26 and 45 °C for the streptococcus, the osmotically fragile L-form and the physiologically grown L-form respectively. During this transition however, only about 1/5 of the total increase in membrane fluidity is demonstrated, as determined from the vertical displacement at this transition compared to the total vertical

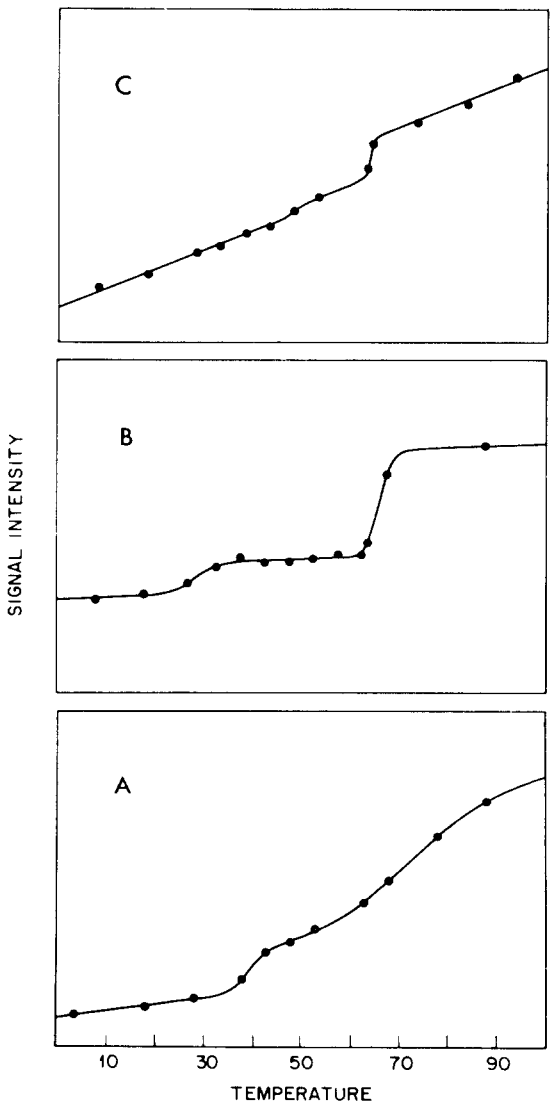


Fig. 7. Relative signal intensity of the center field line of *N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid spin label, I(12, 3), incorporated into the membranes of *Streptococcus pyogenes* (A) and its osmotically fragile (B) and physiologically isotonic L-forms (C) as a function of temperature. Each curve is computer generated using the parameters derived by least squares fit for two transitions summarized in Table I.

displacement of the 2 transitions. The first transition exhibits the cooperativity of 159, 120, 265 for the streptococcus, L-form and physiological-L form respectively.

The second transition represents about 4/5 of the total change in membrane fluidity taking place at between 60–70 °C. The degree of cooperativity of the membrane structure changes drastically from 35 for the streptococcus to 270 for the L,

TABLE I

THE TEMPERATURES (T_{f1}, T_{f2}), COOPERATIVITIES (n_1, n_2) AND THE DEGREE OF MEMBRANE DISASSEMBLAGE ($C\%$, $D\%$) FOR THE TWO TRANSITIONS OF THE SPIN-LABELED MEMBRANES FROM *STREPTOCOCCUS PYOGENES*, OSMOTICALLY FRAGILE L-FORM AND PHYSIOLOGICALLY GROWN L-FORM

Membrane system	First transition			Second transition		
	T_{f1}(°C)	n_1	C(%)	T_{f2}(°C)	n_2	D(%)
Streptococcus pyogenes	36.4	159	20	70.4	35	80
Osmotically fragile L-form	25.5	120	23	63.4	270	77
Physiologically grown L-form	44.8	265	19	59.4	910	81

and 910 for the physiological-L form (maximum factor of 26). For the streptoccocal membrane, the higher transition exhibited is very broad, with very low cooperativity, and starts at just above 50 °C. It is probable that this membrane possesses a high degree of inhomogeneity and the "apparent transition" is the overall of many consecutive transitions taking place at increasing temperatures. This would also explain the extremely low cooperativity observed at this transition. The cooperativity parameter of the second transition is related to lipid chain rigidity in these membranes and indicates the following order: physiological L-form > osmotically fragile L-form > streptococcus. The intensity of the center field line against temperature for the three organisms is plotted in Fig. 7A, B, C. The dots represent the experimental intensity of the center field line for the membrane bound species for the two L-forms, and the intensity ascribed to the sum of the membrane bound and the freely tumbling species of the streptococcus. The curves are computer generated using parameters summarized in Table I.

DISCUSSION

These results indicate that the lipid chain rigidity is greatest in the physiologically isotonic L-form followed by the osmotically fragile L-form. The membrane of the parental coccus permitted the greatest rate of tumbling of the spin label. Two lines of evidence substantiate this point: (1) differences between the corresponding hyperfine splittings of the fatty acid probe when bound to the membrane of each of these organisms; greater hyperfine splitting values indicating higher degrees of spatial hindrance on the tumbling of the spin label in the membrane, and (2) relative cooperativities during the higher transition of the membranes of these organisms, with a higher cooperativity suggesting greater membrane lipid rigidity.

The observation that the binding of the fatty acid probe in two (or more) different sites in each of the membranes examined was deduced from the (1) split of the membrane bound spectra into two sets of lines, (2) the signal intensity versus temperature analyses, indicating two transition points for each membrane, and (3) the kinetics of the ascorbate-induced reduction of the spin label, which does not obey simple first order kinetics.

With regard to the two transitions for each membrane examined, the first

probably relates to lipid phase transition [23–25] which, for the most part, span a temperature range in which these organisms can survive. The second transition signifies the collapse of the membrane structure which may be associated with protein denaturation. The presence of two or more binding sites for the spin label may be related to these two transitions; possibly implying a discrete, well defined and non-homogeneous distribution of the spin label between the lipid head region and the lipid core within the membrane bilayer.

It should be pointed out that the ESR spectra obtained from the streptococcus and its osmotically fragile L-form are similar but different from those of the physiologically isotonic L-form. Examination of these spectra show that in the streptococcal and osmotically fragile L-form systems the free spin label is always present. However, in the physiologically isotonic L-form this species is not observed. A possible explanation for this difference may be that the distribution coefficient for the fatty acid spin label between the membrane lipid bilayer and the aqueous buffer, in which the membrane is suspended, is greater for the physiologically isotonic L-form than for the coccus and its osmotically fragile L-form. This, again, emphasizes that a change in the physical structure of the membrane of this osmotically stable L-form has occurred resulting in its ability to grow or survive under physiologically isotonic conditions.

Studies with the fatty acid probes I(5, 10) and I(1, 14) showed identical membrane bound spectra regardless of the membranes being examined and were insensitive to the differences noted between these membranes when I(12, 3) was used. This may indicate that the differences between these membranes is in the environment closer to the lipid head group region, suggesting greater protein influence on this region, rather than in the hydrophobic lipid core.

The distribution of fatty acid probes in any membrane system is unknown and, therefore, one cannot draw conclusions on the extent or arrangement of the assumed lipid bilayers of a membrane. No qualitative differences were evident in the fatty acid analyses of the membrane from this streptococcus or its two L-forms. However quantitative differences in the ratio of saturated to unsaturated fatty acids contents were noted between the two L-forms [9]. Thus, it is possible that the spectral differences between these organisms might be related to quantitative changes in lipid composition or changes in the structure of the membrane associated protein. This would explain the increased osmotic stability for the membrane of the physiologically isotonic L-form. This may be similar to previous findings with *Mycoplasma hominis* and *Acholeplasma laidlawii*, labeled with the same probe used here, I(12, 3), indicating that membrane proteins, "... including those bound electrostatically to membrane lipids, influence the physical state of membrane lipids" [19].

M. hominis and *A. laidlawii* do not exhibit a membrane melting point below 40 °C indicating a relatively less fluid membrane. Thus, of the three organisms studied here, only the physiologically isotonic L-form, with a melting point of 45 °C, resembles these myocoplasmas in terms of membrane stability [19, 20].

As already indicated, we have recently shown that an osmotically fragile L-form from *Streptococcus pyogenes* can be adapted to survive and to grow in physiologically isotonic media for long periods of time. This L-form also destroys human heart cells in tissue culture. This report has now suggested an explanation to account for this survival by focusing on changes in the physical nature of the membrane of

this organism. The universality of such a change occurring in other L-forms remains to be established. Also, whether such a change occurs in vivo is still speculative. Nevertheless, the results of this study add to and reinforce the speculation of the potential survival in vivo and the involvement in pathogenesis of osmotically fragile aberrant forms of bacteria.

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