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Neisseria gonorrhoeae Membrane Microenvironment Studied by Spin-Label Electron Spin Resonance: Comparison of Colony Types

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Spin-label electron spin resonance was used to characterize the microenvironment around spin probes which localize (i) in membranes, (ii) at the membrane surface, or (iii) in the cytoplasm of living *Neisseria gonorrhoeae*. Four colony types (T1, T2, T3, and T4) of gonococci were compared on the basis of the electron spin resonance parameters $2T_{\parallel}$, S (order parameter), and τ_c (microviscosity). The concentration of spin label used had little or no effect on viability. T1 and T2 gonococci were found to have a more restricted environment for molecular motion of a membrane surface spin label than did T3 and T4. The membrane fluidity, as measured by a membrane lipid spin label, of T4 (S = 0.571) was significantly greater than that of T1 or T3 (S = 0.580). This difference was detected at 37°C, at 25°C, in agar-grown bacteria, and in exponential-phase cells. Studies using spin labels which probe different levels of the membrane indicated the presence of a membrane flexibility gradient. Cytoplasmic spin-label studies indicated that the cytoplasm of all gonococcal colony types was three to five times more viscous than water.

The outcome of the interaction between a bacterium and external components such as drugs and host cells is largely determined by events that occur at the microbial surface. Traditionally, identification of the bacterial factors that actively influence these sorts of interactions has involved comparative morphological and biochemical studies of the surface constituents. Although this approach is valuable in describing structure-function relationships, it is limited in defining mechanisms that may relate more to the organization of surface constituents than to either the gross chemical composition or the microscopic appearance.

The applications of spin-label (SL) electron spin resonance (ESR) methodology can provide information about molecular interactions. SL-ESR (for a review, see reference 1) involves the use of a free radical as a reporter group; the radical typically is an unpaired electron located on a nitroxide group of a membrane or cytoplasmic SL. The microwave absorption characteristics of the unpaired electron are altered by its interaction with the local electromagnetic fields present in its immediate surroundings. The resulting absorption spectrum is sensitive to relatively small changes in the microenvironment.

We exploited the SL-ESR approach as a part of studies aimed at defining the physical organization of the cell envelope and its influence on the biology of gonococci. We concentrated on the microstructure of the gonococcal outer membrane because of the role this membrane plays in the direct contact between the bacterium and the external environment. The system uses viable bacteria, since crucial spatial relationships in vivo could be altered during the preparation of isolated membranes and such alterations could compromise the relevance of observations to the biological interactions of interest. This report describes initial observations on the microstructure within and about membranes of living gonococci by using SL-ESR.

MATERIALS AND METHODS

Bacteria. *Neisseria gonorrhoeae*, strain 2686, types T1, T2, T3, and T4 were kindly supplied by Douglas Kellogg (Center for Disease Control, Atlanta, Ga.) and maintained by serial, selective subculture on solid medium (GCBIS [38]). Incubation was at 37°C in a candle jar. When later propagated on clear typing medium (35), the colonial types had the following color and opacity, as determined by the methods of Swanson (35, 36): T1—dark, opaque-transparent; T2—dark, opaque-transparent; T3—dark, opaque; T4—dark, opaque.

For experiments with cells grown on solid medium,

18-h cultures were harvested in warm (37°C) liquid medium (LGCBIS [38]). The suspensions were agitated vigorously on a Vortex mixer, centrifuged at 1,400 × *g* at 25°C for 20 min, and suspended in LGCBIS. The bacterial number was determined by direct count with a Petroff-Hausser chamber, and the concentration was adjusted with modified Hanks solution containing 0.01% bovine serum albumin and 0.1% glucose (HBG [21]) to approximately 5 × 10^8 bacteria/ml. Bacteria prepared in this manner were at least 90% viable, as measured by colony-forming ability.

For experiments in which cells grown in broth were used, 18-h cultures grown on GCBIS were harvested and inoculated at a starting density of 10^7 bacteria per ml into LGCBIS with $NaHCO_3$ added to a final concentration of 0.1 g/liter. After growth at 37°C to a density of about 2 × 10^8/ml, the exponential-phase cells were collected by centrifugation at 1,400 × *g* at 25°C for 20 min, washed with LGCBIS, and suspended as described above. Plating of these suspensions on LGCBIS indicated that the desired colony type predominated (>99%) after growth in liquid media.

Spin labeling. Figure 1 shows the chemical structures of the membrane SL used in these studies. The nitroxide-substituted analogs of stearic acid, 5-, 12-, and 16-doxylstearic acid (5DS, 12DS, and 16DS), were obtained from Syva, Palo Alto, Calif. James Magnuson, Washington State University, Pullman, kindly supplied myristyldimethylammoniumtetramethylpiperidine bromide (MDTAB). The amphophilic nature of these molecules determines their vertical orientation in the membrane bilayer (Fig. 1). Thus, one may selectively probe different membrane locations with this set of SL. The cytoplasmic SL 2,2,6,6-tetramethylpiperidine-*N*-oxyl (tempone) and 3-carboxyl-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl (PCA) were supplied by Alec Keith and Wallace Snipes, Pennsylvania State University, University Park.

A small amount, i.e., 15 μl for 5DS, of 5 mM SL in ethanolic solution was evaporated by a stream of dry nitrogen to a thin film on the bottom of a tube (13 by 100 mm). Approximately 2 ml of bacteria suspended in LGCBIS was then added to the tube and incubated at 25°C for 1 min. Bacteria were then collected by centrifugation (800 × *g* at 25°C for 1 min) into the sealed tip of either a Pasteur pipette or a 100-μl micropipette. The ESR spectrum was recorded at 25°C within 15 min. Since SL was not detected either in the supernatant or in tube washings, we assume complete incorporation. Slight modifications of this procedure were sometimes used and are detailed in Results.

Localization of 5DS. Localization experiments were performed by using T4 cells grown to mid-exponential phase in broth culture. The cell suspension was centrifuged and suspended in fresh broth at a

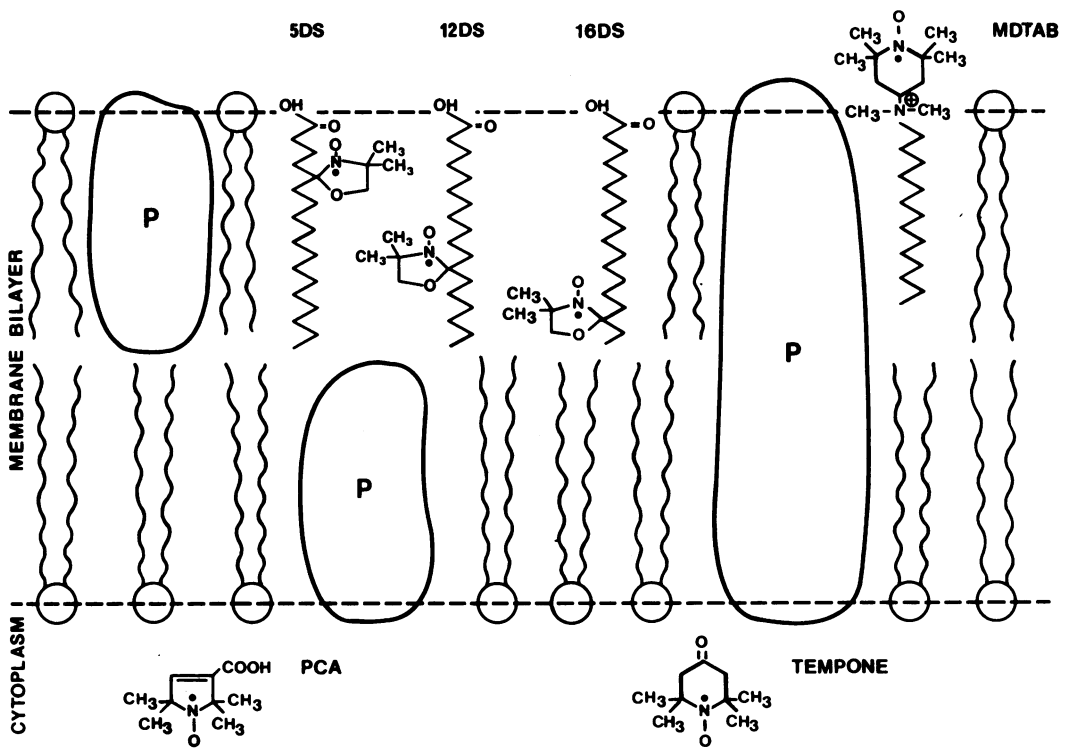

FIG. 1. *Diagrammatic representation of a generalized membrane with proteins (P) in the phospholipid bilayer. The structure and probable location (see text for localization data) of the SL used in this study are indicated. The stearic acid analogs 5DS, 12DS, and 16DS probe the membrane interior; MDTAB is a surface probe of the membrane-environment interface; PCA and tempone report from the cell cytoplasm.*

density of approximately 3×10^8 bacteria per ml. This suspension was then incubated for 3 min at 25°C with 5DS (final concentration, 40 μM) containing 0.4 μCi of [^{3}H]5DS (New England Nuclear Corp., Boston, Mass.) per ml. Crude cytoplasmic-membrane (CM) and outer-membrane (OM) fractions were isolated from the labeled cells by using the procedure of Walstad et al. (39). Enriched OM and CM were obtained from the crude fractions by sucrose gradient centrifugation (14). All membrane fractions were assayed for succinate dehydrogenase (SDH; EC 1.3.99.1) activity, the content of the 34×10^3-dalton major OM protein (MOMP), and ^{3}H radioactivity.

Enzyme assay. SDH was used as a marker for CM and was measured as described by Johnston and Gotschlich (13). Enzyme activities were expressed as the change in absorbance (at 550 nm) per minute per microliter of sample. The total enzyme activity for a particular fraction was obtained by multiplying by the total volume of the fraction.

Determination of MOMP content. The 34×10^3-dalton MOMP was used as a marker for OM. Membrane fractions were solubilized in sodium dodecyl sulfate and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (40). Gels were stained with Coomassie brilliant blue and destained by the method of Fairbanks et al. (6). Absorbance scans were obtained at 580 nm, using an ISCO gel-scanning device. The area under the peak corresponding to MOMP was measured by weighing the cut-out peak area. MOMP content was expressed as the mass of the cut-out peak per volume of sample applied to the gel (milligrams per microliter). The total MOMP in a fraction was determined by multiplying by the total volume of the fraction.

^{3}H determination. Radioactivity was determined in a model 3255 Packard Liquid Scintillation Spectrometer. Portions of enriched OM and CM fractions were dried, solubilized in 90% NCS tissue solubilizer (Amersham/Searle) for 2 h at 56°C, and counted in a toluene-base scintillation cocktail (28). ^{3}H disintegrations per minute (dpm) were determined by a quench calibration curve, using external standardization.

Distribution of 5DS between OM and CM. Each of the isolated membrane fractions is potentially composed of a mixture of OM and CM in varying proportions. If we assume a constant number of 5DS molecules per arbitrary unit of OM, define this parameter as h, and assume a constant number of 5DS molecules per arbitrary unit of CM and define this parameter as f, then for any fraction i which contains both OM and CM the total number of 5DS molecules in that fraction (N_i) will be given by

$$N_i = (\text{OM units})_i \cdot h + (\text{CM units})_i \cdot f \quad (1)$$

By expressing OM units in terms of MOMP units, CM units in terms of SDH units, and molecules of 5DS in terms of ^{3}H dpm, this equation may be rewritten as

$$(^3\text{H dpm})_i = (\text{MOMP units})_i \cdot h' + (\text{SDH units})_i \cdot f' \quad (2)$$

where h' = ^{3}H dpm/MOMP units, and f' = ^{3}H dpm/SDH units, which are directly proportional to h and f, respectively. The values of h' and f' can be determined by simultaneously solving the equations for two different membrane fractions, e.g., enriched OM and enriched CM. Once f' and h' are known and SDH content and total ^{3}H dpm are experimentally measured for the original cell suspension, then the percent ^{3}H dpm associated with the OM in the original cell suspension can be calculated as follows

$$\%^3\text{H dpm in OM} = \frac{\text{total } ^3\text{H dpm} - (\text{SDH units}) \cdot f'}{\text{total } ^3\text{H dpm}} \cdot 100\% \quad (3)$$

ESR data acquisition and analysis. ESR spectra were obtained on a standard balanced-bridge spectrometer with diode detection operating at 9.1 GHz. Phase-sensitive detection with a 50-kHz magnetic field modulation frequency was used. Sample heating and broadening of spectral lines were avoided by recording all spectra at low microwave power (12 mW) incident on the Varian V4535 large-sample access cavity. A peak-to-peak modulation amplitude of 1.3 gauss (G) was used after it was determined that comparisons between samples were independent of amplitude up to this value. Other instrument settings were identical for a particular set of samples to be compared. An ESR tube containing the sample was carefully positioned in a quartz Dewar tube. The temperature was monitored with a thermocouple at the sample and maintained to within 0.5°C of the desired temperature by using a chilled or heated N_2 gas flow system. First-derivative absorption spectra were recorded by using a 100-G field sweep and a scan time of 5 min. The magnetic field sweep was calibrated by using a standard marker of Mn^{2+} in MgO (a = 86.6 G at 290°K; 20).

Two spectra from each sample were recorded, and the resultant parameters were averaged. All samples were coded and read blind. For experiments in which several samples were measured and data for several days were available, a P value was calculated by a two-way analysis of variance by utilizing the null hypothesis that the ESR parameters were identical (expected if the physical state of the membrane was the same in each case) and an alternate hypothesis that these parameters were different.

The order parameter (S), a measure of SL order and motion, was calculated (1, 7, 11).

$$S = \frac{T_\parallel - T_\perp' - C}{T_\parallel - 2T_\perp' + 2C} \cdot 1.66 \quad (4)$$

where $C = 1.4 - 0.053(T_\parallel - T_\perp')$.

The measurement of the hyperfine splitting parameters $T_\parallel$ (T parallel) and $T_\perp'$ (T' perpendicular) from a typical ESR spectrum of 5DS-labeled bacteria is shown in Fig. 2. The distance between the outer extrema corresponds to $2T_\parallel$ and between inner extrema to $2T_\perp'$. As the chain flexibility decreases, the order parameter increases. This implies that as the environment surrounding the nitroxide group becomes more ordered (less fluid), the order parameter increases. The theoretical limits of S are $S = 0$ for a completely fluid, isotropic system and $S = 1$ for a completely rigid

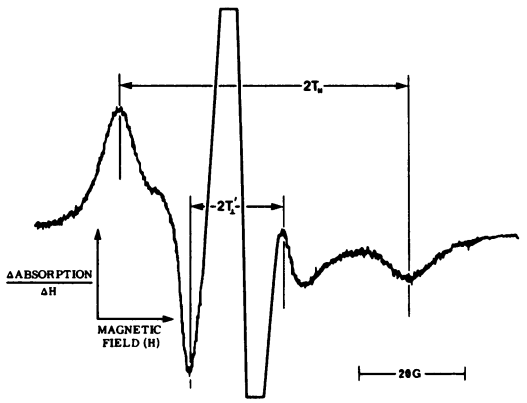

FIG. 2. *First-derivative ESR spectrum of 5DS-labeled N. gonnorrhoeae strain 2686 type 4 taken at 25°C. The hyperfine splitting parameters $2T_{\parallel}$ and $2T_{\perp}'$ are the separation between outer and inner extrema, respectively. Labeling conditions and instrument settings are described in the text.*

or ordered environment. Typical values for SL membranes range from $S = 0.5$ to $S = 0.7$. Changes in S as small as 0.5% can be statistically significant at a 95% confidence level (18).

The hyperfine coupling constant (a') can be calculated from $T_{\parallel}$ and $T_{\perp}'$ as follows

$$a' = 1/3(T_{\parallel} + 2T_{\perp}' + 2C) \quad (5)$$

As the polarity of the medium surrounding the nitroxide is decreased, a' decreases. For example, for 5DS in water, $a' = 16.1$ G; in hexane, $a' = 14.5$ G. This decrease arises from a destabilization of the dipole character of the nitroxide. Thus, this parameter provides information about the location of SL; e.g., SL located within a membrane have values of a' typical for a nonpolar environment.

No theory has yet been reported relating ESR spectral parameters to molecular order and motion for an SL with the structure of MDTAB. To quantify the changes observed in the spectra of MDTAB-labeled bacteria, we have measured the distance between the outer extrema ($2T_{\parallel}$) as shown in Fig. 3. As the temperature of either bacteria or SL in water is lowered, $2T_{\parallel}$ increases to a maximum of approximately 72 G at −125°C. Thus, differences in $2T_{\parallel}$ among MDTAB-labeled colony types recorded at the same temperature are suggestive of altered SL molecular motion.

Mason et al. (22) have recently developed a more sensitive measure of changes in molecular motion of spin probes. This spectral parameter, $\Delta\ell$, is the half width at half height of the low field extrema (Fig. 3). The rationale for its determination is based on the theory of Mason and Freed (23), whereas the theoretical basis of the calculation of S (equation 4) stems from slightly different assumptions (1, 7, 11). The details of these theoretical considerations are beyond the scope of this paper, but it is of practical significance that S, $2T_{\parallel}$, and $\Delta\ell$ values can serve as a valid measure of SL motional changes.

Tempone and PCA have a greater degree of rotational freedom in the bacterial cytoplasm than does 5DS in the membrane. This class of spectra (rapid motion) is most amenable to a quantitative assessment of τ_c, the rotational correlation time. For tempone and PCA-labeled bacteria, this parameter was calculated as previously described (9).

RESULTS

Effect of SL on gonococcal viability. Stearic acid has little or no effect on growth of gonococci (24). To examine the effect of 5DS, the doxyl derivative of stearic acid, T4 gonococci were treated with 5DS for various times, the SL concentration was quickly lowered by a 10^{-4} dilution, and survival of the bacteria was measured as colony-forming units on GCBIS (Fig. 4). At the concentrations used herein, about 3×10^6 molecules per cell, 5DS did not affect viability or cause detectable spin-broadening of the ESR spectra. Similar results were obtained in surveys with the other colony types. This concentration of SL corresponds to a label-to-lipid molecular ratio of approximately 1:25, considerably less than the 1:10 ratio at which ESR parameters begin to fail to reflect the basic properties of erythrocyte membranes (4). For this calculation, we used the lipid composition data of Guymon et al. (8) and estimated that gonococci are 10% lipid (2). Considerably greater concentrations of 5DS caused discernible spin-broadening of the ESR spectra. Such concentrations, e.g., about 2.5×10^8 molecules per cell, killed the majority of the cells within 15 min (Fig. 4). Therefore, to reflect as accurately as possible the living cell, all ESR measurements were made on cells labeled with about 3×10^6 molecules of SL per cell and were completed within 15 min.

The methods for labeling bacteria with

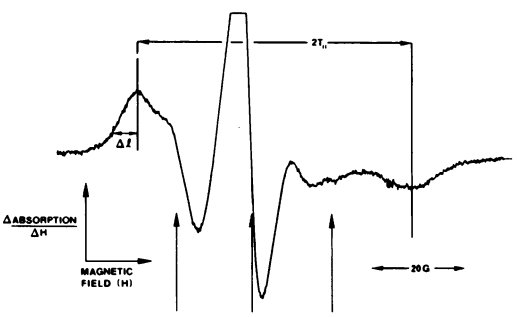

FIG. 3. *First-derivative ESR spectrum of MDTAB-labeled N. gonorrhoeae strain 2686 type 4 taken at 18°C. The hyperfine splitting parameter $2T_{\parallel}$ is the separation between outer extrema. The half width at half height of the low field extrema is $\Delta\ell$. The arrows indicate a small amount of SL tumbling rapidly in solution. Labeling conditions and instrument settings are described in the text.*

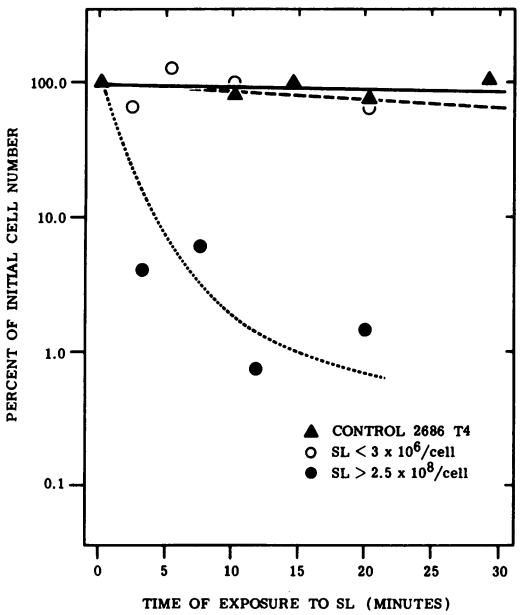

FIG. 4. *Effect of 5DS on colony-forming ability of N. gonorrhoeae strain 2686 type 4.*

MDTAB differed in that incubation was at 4°C to minimize cellular reduction of the SL, the concentration was 10^6 MDTAB molecules per cell, and the ESR measurements at 18°C were completed within 10 min. Under these conditions, essentially all T1 and approximately 50% T4 gonococci were viable at the end of the experiment.

Localization of membrane labels. In contrast to model bilayer systems and to the cytoplasmic membrane of mammalian cells, the cell envelope of gram-negative bacteria contains two membrane bilayers, the OM and CM. Addition of a membrane SL to a cell could result in the labeling of both membrane structures. To determine the distribution of 5DS between the CM and OM, 2686 T4 cells were grown and labeled with [^{3}H]5DS, and fractions enriched in CM and OM were obtained. The ^{3}H dpm associated with the CM and OM in the original cell suspension was calculated from equation 3 and taken as an indication of the location of the 5DS in the ESR studies. In each experiment, several parallel calculations between the various fractions were possible; results of these calculations seldom varied by more than 5% and were used to calculate a mean value. In three experiments 55, 52, and 53% of the [^{3}H]5DS associated with the OM.

Jones and Osborn have demonstrated that intact cells of *Salmonella typhimurium* are capable of translocating exogenously added phospholipids from the OM to the CM on a time scale of tens of minutes (14). If a similar phospholipid-translocating mechanism is present in the gonococcus which does not discriminate against stearic acid and its 5-doxyl derivative, 50% of the 5DS would be expected to associate with the OM at equilibrium. The time scale of the experiments described above (tens of minutes) was somewhat longer than that necessary for the ESR measurements (complete within 15 min). If either the 50 to 55% value represented an equilibrium state or significant mixing of 5DS occurred during membrane isolation, the percent 5DS in the outer membrane during the ESR experiments may have been somewhat higher.

A membrane surface location is expected for MDTAB (Fig. 1) with the quaternary charge at the polar interface of the membrane and the hydrocarbon chain inserted in the hydrophobic region of the bilayer. This conclusion was reported for several analogous SL with hydrocarbon tails of different lengths (5, 10, 12, 19). Data supporting this location in phospholipid vesicles were obtained by the rapid reduction of the nitroxide group of this label by aqueous reducing agents (5, 10). In addition, the spectrum of MDTAB-labeled gonococci (Fig. 3) was very similar to that of MDTAB in water at −50°C. The matching of these spectra indicated that the polarity of the environment around the SL in the bacteria is hydrophilic, again consistent with a membrane surface location.

Comparison of T4 gonococci harvested from solid and liquid culture. The membrane fluidities of 5DS-labeled T4 gonococci grown in liquid and on solid media were compared. Seven samples from four experiments gave a mean value of $S = 0.619$ for cells harvested from solid media. The corresponding means for broth-grown cells harvested at mid-exponential phase and at early stationary phase were $S = 0.647$ and $S = 0.643$, respectively. The difference in S between cells harvested from solid media and cells harvested from mid-exponential-phase broth culture, $\Delta S = 0.028$, was significant at the $P = 0.001$ level. Similarly, the difference in S between cells harvested from solid media and cells harvested from stationary-phase liquid culture, $\Delta S = 0.024$, was significant at the $P = 0.001$ level. There was not, however, a significant difference between the S values for cells harvested from stationary- and mid-exponential-phase liquid cultures.

Colony type comparison. Four colony types of strain 2686 were labeled with MDTAB and the ESR spectra were recorded at 18°C (Table 1). $2T_{\parallel}$ was measured to compare the colony types. T1 and T2 had greater $2T_{\parallel}$ (59.7 and 59.6 G), suggestive of a more restricted environment for the SL, than did T3 and T4 (59.0 and 58.8

TABLE 1. *$2T_{\parallel}$ and $\Delta\ell'$ for N. gonorrhoeae strain 2686 colonial types spin-labeled with MDTAB at 4°C*[a]

Colony type	$2T_{\parallel}$ (G)	P[b]	$\Delta\ell'$ (G)	P[b]	n
T1	59.7	0.480	4.4	0.11	5
T2	59.6	—	4.2	—	5
T3	59.0	0.003	4.7	0.01	5
T4	58.8	0.004	4.6	0.02	5

[a] Data were taken at 18°C. n, Number of samples; —, base for comparison.
[b] P value for equality with T2.

G). The P value for the null hypothesis was <0.004. This result was collaborated by direct spectral measurement of $\Delta\ell'$. In contrast to $2T_{\parallel}$, as the SL environment becomes more restrictive, $\Delta\ell'$ decreases (22). The $\Delta\ell'$ values for T1 and T2 (4.4 and 4.2 G) were significantly lower than those for T3 and T4 (4.7 and 4.6 G).

The membrane fluidity of the four colonial types was measured at 37°C with the 5DS SL. Cellular reduction of the SL at this temperature was so rapid that *N*-ethylmaleimide (NEM) was used to prevent the loss of SL signal. Agar-grown cells were harvested, incubated with 4 mM NEM for 40 min, and labeled as before (Table 2). The membrane fluidity of T1 and T3 was similar, but lower (S value was higher), than that of T4. Although the difference was small (1.5%), statistical calculation showed high significance (P for the null hypothesis <0.006). The a' values were between those found for 5DS in methyl oleate (a' = 14.5 G) and in water (a' = 16.1 G). This indicated that the reporter group of 5DS was below the surface of the membrane bilayer (Fig. 1). Although the SL concentration in these experiments caused minimal perturbation of the membrane, the NEM treatment rapidly killed the cells. The difference in membrane fluidity was not an artifact of NEM treatment. T3 that were heat-killed at 65°C for 45 min had a higher $2T_{\parallel}$ (56.7 G) than did T4 (55.1 G). This difference, which is compatible with a less-fluid membrane in T3, corresponds with the differential observed with the corresponding NEM-treated bacteria.

Measurements were also made at 25°C, where data could be obtained from viable cells. At 25°C, the cellular reduction of 5DS was significantly lower than at 37°C, and NEM treatment was not necessary (Table 3). T1, T2, and T3 have similar S values, and all are higher than T4. Thus the environment surrounding the reporter group of 5DS was more restrictive in T1, T2, and T3 membranes than in T4 membranes. Although the difference was again small (1.1%), the results were statistically significant (P value for null hypothesis < 0.003). The a' values were again consistent with a membrane location for 5DS.

To ensure that the colony type difference between the T1-T2-T3 set and T4 was not related to possible heterogeneity of the population of cells grown on solid media, broth-grown T3 and T4 cells were labeled with 5DS and compared at 25°C. The order parameter for T3 was again higher than T4; ΔS = 1.6%.

Flexibility gradient. T4 cells were labeled with either 5DS, 12DS, or 16DS; S was calculated for each label and related to the molecular position of the nitroxide in the SL compounds (Fig. 5). S decreased as the nitroxide group was moved down the fatty acid chain away from the carboxyl terminus. A similar increase in molecular motion in the bilayer center has been reported in several model systems (7, 31) and in *Acholeplasma laidlawii* membranes (29).

Cytoplasmic microviscosity. The microviscosity of the cytoplasm was evaluated by using tempone and PCA, small water-soluble SL (Fig. 1). These SL were added to a bacterial suspension to a final concentration of 1 mM. SL signal from outside the cells was eliminated by the addition of 0.1 M $NiCl_2$ as previously described (9, 16). Under the conditions and time scale of the ESR measurements, this procedure was slightly toxic to the gonococci. The paramagnetic nickel ion, which is impermeable to lipid bilayers, greatly broadens the signal from nearby

TABLE 2. *S and a' for N. gonorrhoeae strain 2686 colony types treated with 4 mM NEM and spin-labeled with 5DS*[a]

Colony type	S	P[b]	a' (G)	n
T1	0.580	0.004	15.3	7
T2	0.575	0.200	15.2	8
T3	0.580	0.006	15.2	9
T4	0.571	—	15.3	15

[a] Data were taken at 37°C. n, Number of samples; —, base for comparison.
[b] P value for equality with T4.

TABLE 3. *S and a' for N. gonorrhoeae strain 2686 colonial types spin-labeled with 5DS*[a]

Colony type	S	P[b]	a' (G)	n
T1	0.656	0.003	15.5	17
T2	0.658	0.001	15.5	11
T3	0.657	0.001	15.5	16
T4	0.650	—	15.4	22

[a] Data were taken at 25°C. n, Number of samples; —, base for comparison.
[b] P values for equality with T4.

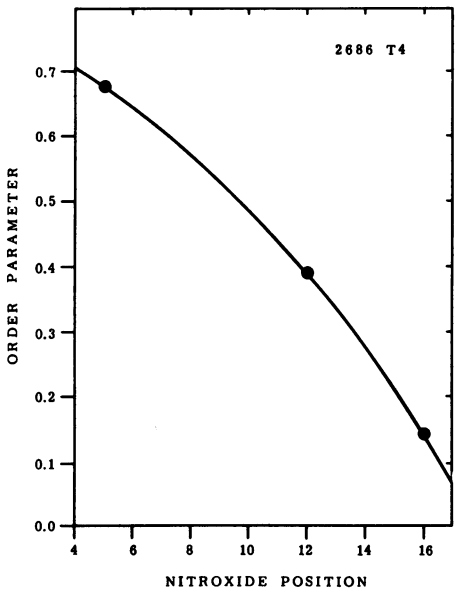

FIG. 5. *The order parameter (S) as a function of nitroxide position along the stearic acid hydrocarbon chain in spin-labeled intact gonococci. Data points are for 5DS, 12DS, and 16DS.*

SL through magnetic dipole interactions, thereby effectively eliminating that signal from the absorption spectrum (25). The cytoplasmic microviscosity was measured as an R value, the ratio: τ_c(SL in cytoplasm)/τ_c(SL in water). Taking the viscosity of water as 1 centipoise, R is a relative measure of the microviscosity of the cytoplasm for that particular SL size. Intercomparison of samples by this method is relatively accurate (9), although absolute values may be in error by 50%. For the different colonial types, the mean values for R ranged from 4.5 to 4.9 with tempone and from 3.3 to 3.4 with PCA. These results indicate (i) that the gonococcal cytoplasm is three to five times more viscous than water, and (ii) that there are no significant differences between the cytoplasmic microviscosities of T1, T3, and T4 gonococci.

DISCUSSION

These experiments demonstrate that intact *N. gonorrhoeae* can be studied by ESR, using SL concentrations that have little effect on viability. These applications of SL-ESR appear to offer a very sensitive means of examining phenotypic characteristics of gonococci that may depend on the in vivo organization or arrangement of cellular components for their expression. Valid interpretation of the results required the identification of the cellular site probed by the SL. The ESR signal of cells labeled with the positively charged MDTAB SL was similar to that of MDTAB in water at −50°C, evidence consistent with the expected membrane surface location. Membrane isolation experiments with [^{3}H]5DS showed that over 50% of this SL is in the outer membrane. It is expected that the other stearic acid analogs, 12DS and 16DS, localized in a similar manner. PCA and tempone have τ_c and a' consistent with a cytoplasmic location.

The cytoplasmic microviscosity and membrane flexibility gradient found for intact gonococci compares favorably with the results measured for other cells and isolated membrane systems. The cytoplasmic microviscosities of T1, T3, and T4 are similar and are approximately 3 to 5 times more viscous than water. Similar values have been reported for the protoplasm of *E. coli* and *Pseudomonas* BAL-31 (16), for barnacle muscle cytoplasm (30), and for nerve axoplasm (9). The gonococcal membrane flexibility gradient reported here, together with the results found for model systems (7, 31) and bacteria (29), using SL-ESR and deuterium nuclear magnetic resonance (33), suggest that such a gradient may be a general feature of membrane bilayers.

S for 5DS-labeled T4 gonococci is approximately 0.65 at 25°C. This is significantly lower than the S = 0.70 reported for intact human erythrocytes (4, 15), presumably due to the protein spectrin which rigidifies the erythrocyte membrane. Intact rabbit polymorphonuclear leukocytes have similar membrane fluidities (S = 0.65), whereas mouse polymorphonuclear leukocytes are more fluid (S = 0.62) (R. A. Haak et al., J. Cell Biol. **75**:222a, 1977). Although the cell systems described above are functionally diverse, their membranes have all been modeled after the "fluid mosaic" Singer model (32). Within such a model, many variables can affect membrane fluidity, including temperature, water content, amounts and kinds of proteins and lipids, and factors such as ions which contribute to the interactions of the constituents (32). Within a single cell system, an alteration in one or more of these variables affecting membrane fluidity can have significant effects on the biological potential of the cell. Although such alterations are difficult to detect by biochemical methods, SL-ESR can detect the effect on membrane fluidity with high sensitivity. For example, the erythrocyte membranes of patients suffering from congenital mytonia or mytonic muscular dystrophy have been extensively compared to normal subjects for constitutive differences of protein, lipids, and carbohydrates (3), and few differences have been reported. SL-ESR studies of intact erythrocytes have clearly shown, however, the involvement of abnormal membrane fluidity in these two diseases (3).

Comparison of the different colonial types of *N. gonorrhoeae* showed that T1 and T2 have a more restricted environment for motion of the MDTAB SL than does T3 and T4. Evidently, in the gonococcus the surface location probed by MDTAB is not sensitive to differences in membrane fluidity as probed by 5DS. The greater restriction of MDTAB at the membrane surface of T1 and T2 correlates with piliation and with the disease potential of the organisms, since T1 and T2 possess pili (37) and are more virulent than nonpiliated T3 and T4 (17). The restricted membrane surface could reflect piliation or be a part of a bacterial mechanism for proper presentation of its pili, or both.

The present study found that the membrane fluidity of T4 is greater than that of T1, T2, or T3. This difference was detected with 5DS in both agar- and broth-grown cells, in intact, viable cells at 25°C, and in cells treated at 37°C with NEM. The greater fluidity may be due to (i) an altered membrane composition or (ii) an altered interaction among the membrane constituents.

Data concerned with the chemical composition of the gonococcal envelope are accumulating but do not yet offer a complete description. Colony type-dependent differences in lipopolysaccharide composition have been reported by Perry et al. (27), who found that the lipopolysaccharide of T1, but not T4, possessed O-side chains. However, these observations were not confirmed by others (33). Differences in the amount of an outer membrane protein in dark- and light-colored colonial variants of strains FA19 and FA140 have been detected by sodium dodecyl sulfate-polyacrylamide gels (39). Similar gels run in our laboratory (data not shown) with dark colonial variants of 2686 have not detected any differences in amounts or molecular weights of outer membrane proteins.

In the present study, the membrane fluidity measured by 5DS was not markedly different in exponential- and early stationary-phase cells grown in liquid medium. Substantial difference was noted, however, between cells grown on agar and in liquid medium. Beebe and Wlodkowski (2) reported that lysophosphatidylethanolamine accumulated with autolysis accompanying late cell culture growth. A similar change was noted for a phospholipid component in 24-h stationary-phase T3 gonococci (34). In addition, the proportion of unsaturated fatty acids in this component increased markedly in the late-stationary-phase gonococci used (34). There is sufficient precedent (1) to expect that a similar change in lysophosphatidylethanolamine content or composition in agar-grown gonococci could contribute to the observed increase in membrane fluidity as compared to liquid-grown bacteria. However, the relative differences among the colony types were found in both liquid- and agar-grown gonococci and thus appear to be independent of environmental factors.

Rosenthal et al. (28) have indicated that T1 and T3, but not T4, have an antiphagocytic activity that is sensitive to the action of EDTA. In many gram-negative bacteria, EDTA causes the release of cell wall macromolecules, notably lipopolysaccharide (26). However, under the conditions used, the effect of EDTA on phagocytosis of gonococci was independent of the release of lipopolysaccharide and other cell envelope constituents (28). These observations suggested that the effect of EDTA resided in an alteration of the microstructure of the gonococcal outer membrane. The data are consistent with the fundamental difference observed herein in surface structure between the T1-T2-T3 set and T4. The relationship between membrane fluidity, cation binding sites, and antiphagocytic activity in T3 and T4 gonococci merits further investigation.

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