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^{1}H-^{31}P NUCLEAR MAGNETIC DOUBLE RESONANCE STUDY OF *ESCHERICHIA COLI* LIPOPOLYSACCHARIDE MOBILITY

WILLIAM EGAN [a] and LORETTA LEIVE [b]

[a] *Division of Biochemistry and Biophysics, Bureau of Biologics, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20205 and* [b] *Laboratory of Biochemical Pharmacology, National Istitute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Building 4, Room 116, Bethesda, MD 20205 (U.S.A.)*

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Purified lipopolysaccharide preparations from *Escherichia coli* K12 were studied by $^{1}H-^{31}P$ nuclear magnetic double resonance methods (cross-polarization and dipolar decoupling). At each of the temperatures studied (24, 37 and 46°C), the overall rotational mobility of a sizeable fraction of the phosphorus nuclei was restricted on the time scale of the NMR measurements, approx. 10^{-4} s. The fraction of motionally restricted material, as well as the extent of restriction, decreased with increasing temperature. These studies provide the first numerical estimate of the percentage of lipopolysaccharide that is motionally restricted at a given temperature.

The outer membrane of Gram-negative bacteria has a unique lipid composition in which the lipid in the outer leaflet of the membrane consists almost exclusively of lipopolysaccharide (LPS), and the inner leaflet of phosphotidylethanolamine. The unusual physiological properties of this membrane have prompted measurements of its transition temperature, mobility, and fluidity, and comparisons with the inner membrane. Early X-ray and fluorescence data suggested that the transition temperatures of the two membranes were the same, but that only 25–40% of the phospholipids participated in the transition for the outer membrane as opposed to 60–80% for the inner membrane (and for most other biological membranes) [1]. ESR studies by Rottem and Leive [2] showed a decreased mobility of lipid in the outer, as opposed to the inner, membrane. In contrast, Nikaido et al. [3] found no difference in ESR spectra between inner and outer membranes and postulated no difference in mobility of the lipid available to spin-labeled probe; however, later work of Davis et al. [4] and Nichol et al. [5], using deuterium NMR, indicated that the acyl chains in the fluid phase of the outer membrane are less mobile than those of the inner membrane, providing support for the conclusion of Rottem and Leive [2]. Nichol et al. [5] also found a 7°C difference in transition range for the two membranes.

To understand the effects of LPS, protein and phosphatidylethanolamine on the behavior of the outer membrane, it is desirable to study the mobility of these moieties alone and in combination. The mobility of LPS molecules, which contain several chemically distinct phosphorus atoms, can be estimated with ^{31}P-NMR, in which line broadening is related to restricted mobility [6,7]. In the most complete study of this sort, Van Alphen et al. [7] compared the mobility of normal LPS with LPS after some of the counter-ions were removed by electrodialysis and others substituted. Somewhat greater mobility was observed for the LPS

Abbreviations: LPS, lipopolysaccharide.

subsituted primarily by Na^+ and less for LPS substituted primarily by Ca^{2+}. Unfortunately, these studies were not able to provide estimates (or limits) of the reorientation correlation times or define what fraction of the molecules was motionally restricted.

In this current work, we have measured the ^{31}P-NMR spectrum of *E. coli* LPS dispersions using high-power decoupling and cross-polarization methods [8] to define further the motional characteristics of the LPS molecules.

E. coli PL2 (from K12: HfrH; *galE28 thi-1 relA1*) was grown at 37°C in a fermentor in LB broth [9] in the presence of 0.25% each of glucose and galactose to approx. $5 \cdot 10^8$ cells per ml, harvested, washed with distilled water, and lyophilized. LPS was prepared by the method of Galanos et al. [10]. Prior to use, approximately 7 mg LPS was rehydrated by sonicating in an Ultramet water bath sonic cleaner (Buehler, Ltd., Evanston, IL) for approx. 15 min in approx. 0.6 ml deionized water. ^{31}P-NMR spectra were recorded on a home-built spectrometer using described procedures [11,12]. The unique phosphorus spectral parameters are provided in the figure legends.

Non-decoupled (ND), ^{1}H dipolar-decoupled (DD), and cross-polarization (CP) spectra of sonicated samples of LPS from *E. coli* PL2 were recorded at 24, 37 and 46°C, as well as their difference spectra, are shown in Fig. 1. Comparison of these spectra revealed the following:

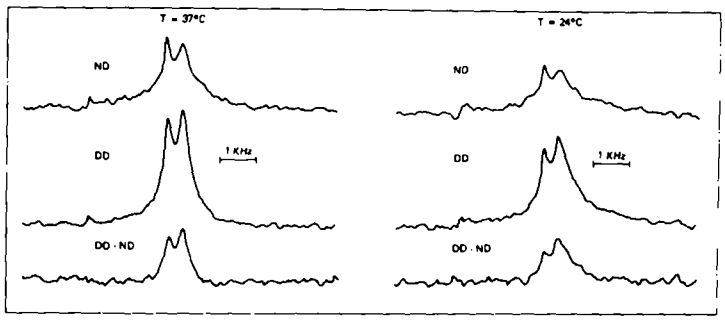
First, at each of the temperatures studied, the integrated absorption intesity of the dipolar-decoupled spectrum was greater than that of the non-decoupled spectrum. The difference between the two spectra (DD − ND) was greater the lower the temperature.

Second, as the temperature was increased, the ^{31}P-NMR signals in both the non-decoupled and dipolar-decoupled spectra sharpened. The CP spectra (Fig. 2) showed a similar resonance narrowing with increasing temperature; additionally, the integrated absorption intensity of the spectra above 20°C was considerably less than that at −40°C.

These spectra permit several new conclusions regarding the mobility of aqueous dispersions of LPS. Previous studies on LPS (for example, Refs. 6 and 7) were done under the usual, low-power decoupling conditions, in which dipolar interactions broaden the ^{31}P signal sufficiently that a portion of it is lost in the baseline noise and thus not detected. High-power ^{1}H decoupling removes the effects of the dipolar interaction, and the lost signal is recovered. The difference in integrated absorption intensity (DD − ND) is therefore a measure of the fraction of phosphorus atoms in the LPS sample that is motionally restricted. For the *E. coli* LPS sample at 24°C, using the just-mentioned definition for restriction, approx. 34% of the phosphates are restricted in their motion, while at 37 and 47°C approx. 22 and 18%, respectively, of the phosphates are restricted. Motional restriction refers to the time scale of the ^{1}H-^{1}H dipolar interaction, approx. $2.1 \cdot 10^4$ Hz [8]. With increasing temperature, the phosphorus resonance narrows, indicating that the degree of spatial restriction, as well as the amount of material that is restricted, has decreased.

The CP-NMR technique detects only those atoms whose motions are restricted on the time scale of the relevant dipolar interaction. Since CP spectra are obtained with samples of LPS, motional restriction is demonstrated. Moreover, since the line width of the CP spectrum is governed by the extent to which the chemical shift anisotropy of the phosphate resonances (approx. 200 ppm [13]) is averaged to zero by molecular motions, the measured line width furnishes another, albeit similar, time scale for assaying motional rigidity. Nearly the full chemical shift anisotropy is observed at −40°C, while at 23 and 37°C less than half this value is observed. Hence, at these latter temperatures, large-amplitude motions, with frequencies on the order of 10^3 Hz, are occurring. Additionally, the line width of the CP spectrum at 37°C is less than that at 23°C, suggesting an increased mobility at this latter temperature. The integrated absorption intensity of the CP spectrum at 37°C was approx. 50% that of the −40°C spectrum, indicating that less of the LPS sample is rigid at the higher temperature.

These results confirm the previous findings [6,7] that the phosphorus groups of LPS are motionally restricted at physiological temperatures and, moreover, provide the first numerical estimates for the extent and time scale of the restriction. However, since phosphorus appears at several positions on



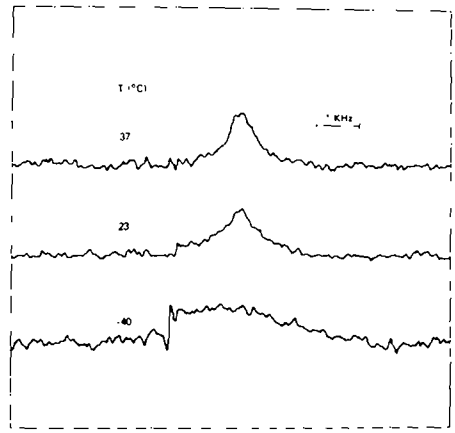


Fig. 1. (Left) ^{31}P-NMR spectra (25 MHz) of sonicated samples of LPS (7 mg) in deionized water (0.6 ml) obtained under non-decoupling (ND) and high-power, ^{1}H-decoupling (DD) conditions at 24°C and 37°C. Spectra were collected using a 90° – t pulse cycle, $t = 2$ s; 32768 transients were accumulated. Other spectral parameters include: 40 kHz spectral window, 2048 data points (0.025 s acquisition time). Prior to Fourier transformation, the spectra were zero-filled and then exponentially multiplied so as to result in an additional 25 Hz broadening in the frequency domain spectrum.

Fig. 2. (Right) Proton-enhanced ^{31}P-NMR spectra (25 MHz) of LPS samples at different temperatures. At 23 and 37°C, 32768 transients were accumulated, while at −40°C, 16384 transients were accumulated; the spectra in the figure were normalized to account for this difference in the number of acquisitions. Spectral parameters include: 1 ms Hartmann-Hahn matching time, 2.0 s pulse repetition time, 40 kHz spectral window, 2048 data points (0.025 s acquisition time). Spectra were acquired using high-power ^{1}H decoupling. Prior to Fourier transformation, the spectra were zero-filled and then exponentially multiplied so as to result in an additional 50 Hz line broadening in the frequency domain spectrum.

LPS, further studies will be required to determine whether certain phosphorus atoms are completely restricted in motion, or all phosphorus atoms are partially restricted. Using these magnetic resonance methods, it should, in the future, be possible to determine the effect of different counterions, or added outer membrane components, such as specific proteins or lipid, on LPS mobility.

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