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# Orientation measurements on membrane systems made from lipopolysaccharides and free lipid A by FT-IR spectroscopy

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**Abstract.** Here we report on investigations into the orientational behaviour of hydrated membrane systems made from lipopolysaccharide and its lipid component, free lipid A, using Fourier-transform infrared spectroscopy and applying attenuated total reflectance. For the investigated lipopolysaccharides – extracted from mutants of *Salmonella minnesota* and *Escherichia coli*, and differing in the length of the polysaccharide moiety – the dependence of dichroic ratios on temperature for several vibrations of the hydrophilic and hydrophobic portions was measured, from which the order parameter for the lipid assembly can be calculated. In the lower temperature range ($<40\,°C$) for all lipopolysaccharide preparations the evaluation of the dichroic ratios clearly shows the existence of a highly ordered phase, i.e. the gel state of the hydrocarbon chains within lamellar structures. For this phase an order parameter $S=0.70\pm0.05$ could be calculated which is lower than that of typical phospholipids in the same phase state ($S=0.80\pm0.05$ for e.g. phosphatidylethanolamines). For deep rough mutant lipopolysaccharides at higher temperatures ($>40\,°C$) a transition into a disordered, isotropic phase can usually be observed for which an order parameter $S=0.25\pm0.05$ could be approximated. The other rough mutant lipopolysaccharides at higher temperatures predominantly form lamellar structures. Only in special cases, under the influence of divalent cations like $Mg^{2+}$, could isotropic phases also be observed. Free lipid A preparations over the whole temperature range exhibited no unequivocal orientational behaviour. However, the existence of a pure $L_\beta$-phase even at lower temperatures may be excluded for these compounds. The observed structural preferences might be of great importance with respect to the expression of biological activities of lipopolysaccharide and free lipid A systems in vivo and in vitro.

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*Abbreviations*: LPS = lipopolysaccharide(s), IR = infrared, ATR = attenuated total reflectance

**Key words:** Lipopolysaccharide, lipid A, Fourier-transform infrared spectroscopy, attenuated total reflectance, dichroic ratio, phase transition

## Introduction

Lipopolysaccharides (LPS) – the major amphiphatic constituents of the outer membrane of Gram-negative bacteria – are assumed to be responsible for the high permeability barrier of the bacterial cell, especially against larger hydrophobic drugs (Schlecht and Schmidt 1969). Furthermore, LPS express various biological activities such as pyrogenicity, lethal toxicity, induction of arachidonic metabolism, and induction of tumor necrosis which are thought to originate from the lipid moiety of LPS, lipid A, its 'endotoxic principle' (Rietschel et al. 1984, 1987). For the expression of biological activity it can be assumed that the ordered membranous LPS assembly in an aqueous environment plays a major role beyond the properties of the single molecules. It is well known that lipid bilayers made from LPS generally exhibit similar characteristics to phospholipids. This was shown e.g. by calorimetry, fluorescence spectroscopy, and film balance measurements (Brandenburg and Seydel 1984). However, from some data, behaviour quite different from that of phospholipids could be deduced and this might be important for the unique function of this class of amphiphatic molecules: The values of the phase transition enthalpies are considerably lower than for phospholipids with comparable lengths of the hydrocarbon chains, e.g. 14:0 phosphatidylethanolamines. A detailed investigation of free lipid A by IR spectroscopy (Naumann et al. 1987) gave very complex phase behaviour. Investigation of LPS and free lipid A systems via 90°-light scattering showed transitions into non-lamellar states with simultaneous chain-melting, especially at high $Mg^{2+}$ concentrations, at low pH and partially at higher temperatures (Seydel and Brandenburg 1986 and unpublished results).

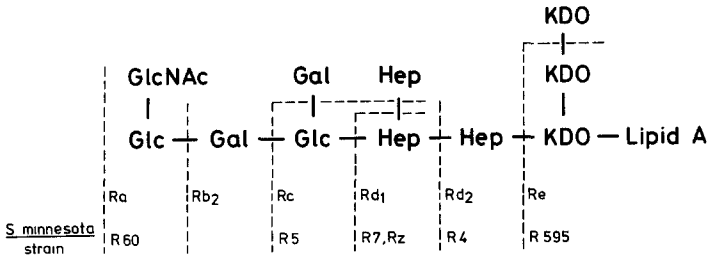
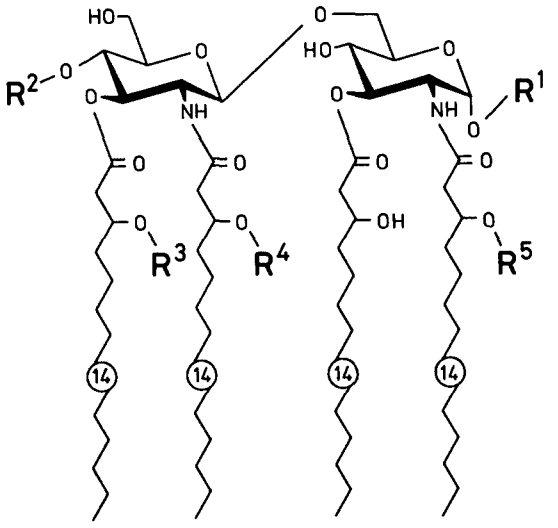



**Fig. 1.** Schematic structure of the core region of various lipopolysaccharides from rough mutants of *Salmonella minnesota* according to present knowledge (without phosphate groups). The wild-type (S-form) LPS additionally contains the covalently-linked O-chain. Abbreviations: *KDO* = 2-keto-3-deoxyoctonate, *Hep* = heptose, *Glc* = glucose, *Gal* = galactose, *GlcNAc* = glucose-amine-acetyl




| Preparation | Nature of | | | | |
|---|---|---|---|---|---|
| | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ |
| 503 | H | H | 14:0 | 12:0 | H |
| 504 | H | P | 14:0 | 12:0 | H |
| 505 | P | H | 14:0 | 12:0 | H |
| 506 ≙ E.coli Lipid A | P | P | 14:0 | 12:0 | H |
| 516 ≙ S. minnesota heptaacyl Lipid A | P | P | 14:0 | 12:0 | 16:0 |

**Fig. 2.** Chemical structure of synthetic lipid A's and lipid A part structures (synthesis see Imoto et al. 1984). For natural lipid A's, a microheterogeneity is observed leading to varying amounts of substituents at $R^1$, $R^2$, and $R^5$ (see Rietschel et al. 1984)

The aim of this paper is to contribute to a deeper understanding of the complex membrane system of LPS and free lipid A by characterizing the orientational behaviour of the different phase states using Fourier-transform infrared (FT-IR) spectroscopy applying attenuated total reflectance (ATR). Results from ATR measurements with hydrated samples (i.e. water content 30% to 50%) are presented which show that for LPS from rough mutant strains of *Salmonella minnesota* or *Escherichia coli* at lower temperatures the dichroic ratios of several vibrations of the hydrophilic and hydrophobic moiety are comparable to those measured for phospho- and sphingolipids (Brandenburg and Seydel 1986). From this it can be concluded that at lower temperatures an ordered aligned phase exists, for which an order parameter of $S = 0.70$ can be calculated. At higher temperatures, however, from the detailed analysis of deep rough mutant LPS it can be concluded that this type of LPS may rearrange into a more isotropic phase ($S = 0.25$) than that known for many phospholipids in the liquid crystalline phase. In contrast to LPS, free lipid A even at lower temperatures does not exhibit a unique behaviour which is in agreement with statements deduced from results from other investigations (Naumann et al. 1987; Seydel and Brandenburg 1986) postulating the existence of mixed phase states, e.g. lamellar/non-lamellar.

## Materials and methods

Figure 1 shows the different core structures of various rough mutant LPS of *S. minnesota*. Of these, the mutant LPS, Re (strain R595), Rd2 (strain R4), Rd1 (strains R7 and Rz) and Ra (strain R60) were investigated in more detail. The LPS were isolated from bacteria by the phenol/chloroform/light petroleum ether procedure (Galanos et al. 1969) and were analyzed in their natural salt form. Lipid A (chemical structure see Fig. 2) was isolated from LPS either by acid hydrolysis or by acetate buffer treatment, purified, and converted to the triethylamine (Ten) salt form (Brade et al. 1983). Both hydrolysis techniques lead to a quantitative cleavage of 2-keto-3-deoxyoctonate but differ in the degree of cleavage of organic phosphate, the acid treatment being more effective and leading to complete dephosphorylation of the 1-phosphate. Besides free lipid A, the compound lipid A (OH), in which the ester-bound fatty acid residues were cleaved by NaOH treatment, was investigated.

For comparative purposes, some synthetic mono- and bisphosphoryl lipid A analogues (compounds 503, 504, 505, 506, and 516, chemical structure see Fig. 2) kindly provided by T. Shiba and S. Kusumoto (Osaka University, Japan) were used. Similarly, the spectra of the compounds α-D-glucose-1-phosphate, α-D-glucosamine-1-phosphate, and glucosamine-N-acetyl purchased from Sigma (München, FRG), were investi-

gated. The lipids were prepared for attenuated total reflectance (ATR) measurements as oriented multilayers at a water content of 5% to 20% (free lipid A) or 30% to 50% (various LPS) in a similar way to that described by Brandenburg and Seydel (1986) by spreading a $10^{-3}$ $M$ lipid suspension in double distilled water on the ATR plate and evaporating the excess water by slow periodic movement with a nitrogen stream at room temperature. Water-free (i.e. water content $<1\%$) multilayers of LPS R595 were prepared from a chloroform : methanol (1 : 1 molar) dispersion on the crystal. In some cases, the thermotropic phase behaviour of LPS R595 and free lipid A samples – prepared by vortexing the respective dispersions – was also measured at high water concentrations ($>80\%$). The water content was estimated from the ratio of the peak intensities of the OH-stretch around 3,400 $cm^{-1}$ to the antisymmetric stretch of the methylene groups around 2,920 $cm^{-1}$. The crystal plate within the ATR assembly (Harrick, Ossining, NY, USA) was made from ZnSe (K. Korth Monokristalle, Kiel, FRG), the polarization of infrared light was done by a grid polarizer from Barnes (Stamford, CT, USA). The internal reflection plates were enclosed in a brass cell sealed by a 100 µm mylar foil and were thermostatted by a water-bath with a precision of 0.5 °C measured by a thermocouple.

The infrared measurements were performed on a Fourier-transform infrared (FT-IR) spectrometer '5-DX' (Nicolet Instruments, Madison, WI, USA). For each measurement, 200 scans were accumulated, and the interferogram was apodized with a Happ-Genzel function (Gronholz and Herres 1985). After Fourier transformation, the absorbance spectra were smoothed according to a least-square procedure (Savitzky and Golay 1964) and evaluated by determining the position of the maximum (peak position) and the area of the absorption band (band intensity). The peak positions of weaker bands, appearing as a shoulder within stronger bands, were determined at the points of intersection of the tangent – obtained by parallel shift of the base line – with the respective band. The precision in determining the peak position was as high as 0.1 $cm^{-1}$, whereas the error in calculating the band intensity (area) could be estimated to be 2% from the uncertainty of choosing the proper band limits. In the case of very weak absorption bands second derivative spectroscopy was applied. To get minimal distortion of the original spectrum by the smoothing procedure, the original emittance spectrum was converted into the transmittance spectrum. This was smoothed, and then the second derivative was calculated. In this way, absorption bands analogous to the usual absorbance bands are obtained.

Orientation measurements were evaluated as described by Fringeli (1977). Briefly, by measuring the dichroic ratio – defined as the ratio of absorption coefficients $R = A_{II}/A_{\perp}$ for parallel and vertical polarized light, which are approximated by the band areas – of various absorption bands the molecular orientation of the lipid assembly, i.e. the order parameter $S$ ($S=0$ for isotropic and $S=1$ for perfect alignment) can be obtained if the directions $\theta$ of the respective oscillating dipole moments with respect to the molecular axis are known. The relation between $R$, $S$, and $\theta$ is given by the expression (Brandenburg and Seydel 1986)

$$S = \frac{k-1}{3 \cdot \cos^2\theta - 1 + k(1-3/2) \cdot \sin^2\theta} \tag{1}$$

with $k = (R - E_x^2/E_y^2)/(E_z^2/E_y^2)$.

The electric field amplitudes in the rarer medium (lipid) can be approximated in the case of weak absorption bands for thin layers to

$$E_x = \frac{2 \cdot \cos\vartheta \cdot (\sin^2\vartheta - n_{31}^2)^{1/2}}{(1-n_{31}^2)^{1/2} \cdot [(1+n_{31}^2) \cdot \sin^2\vartheta - n_{31}^2]^{1/2}}$$

$$E_y = \frac{2 \cdot \cos\vartheta}{(1-n_{31}^2)^{1/2}}$$

$$E_z = \frac{2 \cdot \cos\vartheta \cdot n_{32}^2 \cdot \sin\vartheta}{(1-n_{31}^2)^{1/2}\,[(1+n_{31}^2) \cdot \sin^2\vartheta - n_{31}^2]^{1/2}}$$

with $\vartheta$ = angle of incidence (usually 45°), $n_{ik} = n_i/n_k$ representing the refractive indices of medium $i$ and $k$, $n_1$ refractive index of the crystal, $n_2$ of the lipid, and $n_3$ of the surrounding medium. With $n_1 = 2.40$ (ZnSe), $n_3 = 1.00$ (air), and $n_2$ varying between 1.50 (pure lipid) and 1.35 (lipids with high, i.e. 90% water content), the numerical values for the field amplitudes are $E_x = 1.383$, $E_y = 1.556$, and $E_z = 0.712$ (pure lipid), $= 0.750$ (lipid in 20% water), $= 0.801$ (lipid in 40% water), or $= 0.939$ (lipid in 90% water), respectively.

## Results and discussion

### *Assignment of vibrations*

The assignments of the absorption bands resulting from the hydrocarbon moiety are given in the legend of Fig. 3 (see also Naumann et al. 1987), in which the IR spectra of biphosphoryl lipid A, rough mutant LPS from R595 (Re) and Rz (Rd1) – all from *Salmonella minnesota* – are presented. As can be seen from the ratio of the OH-stretch of the water (around 3,400 $cm^{-1}$) to the symmetric or antisymmetric stretch of the lipid methylene groups (at 2,920 and 2,850 $cm^{-1}$, respectively), there is an increase in the degree of hydration in the order: lipid A → lipopolysaccharide which depends on the increase in polysaccharide content. The higher amount of sugar also leads to an increase in the peak heights of the typical

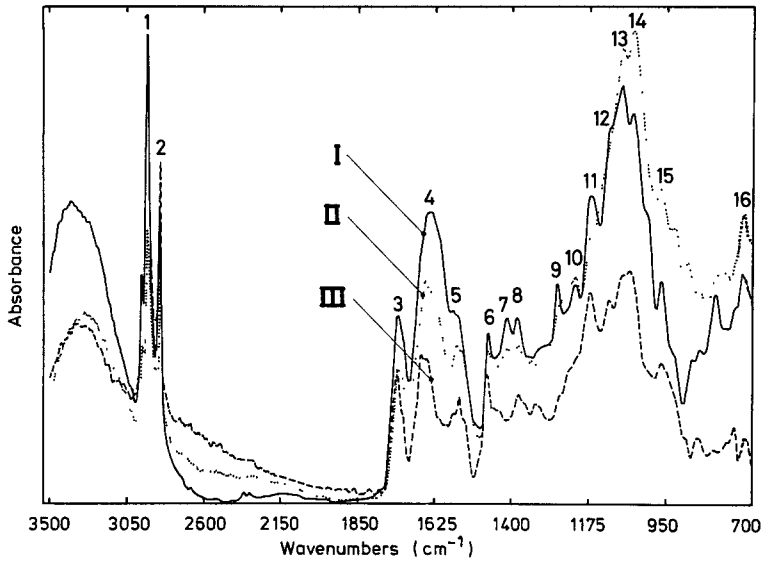



**Fig. 3.** 90°-polarized infrared ATR spectra of bisphosphoryl lipid A from *Escherichia coli* (III) and lipopolysaccharides from mutant strains R595 (I) and Rz (II). Assignment of main vibrations (explanation see text): (1) $\nu_{as}(CH_2)$, (2) $\nu_s(CH_2)$, (3) $\nu(C=O)$, (4) amide I, (5) amide II, (6) $\delta(CH_2)$, (7) $\delta(\alpha\text{-}CH_2)$, (8) $\delta_s(CH_3)$, (9) $\nu_{as}(PO_2)$, (10) Wagging progression vibrations $\geq CH_2$, (11) $\nu(C-O)_{Ester}$, (12) $\nu_s(PO_2)$, (13)–(14) Sugar vibrations, (15) Phosphate vibration, (16) Rocking progression vibration $\geq CH_2$

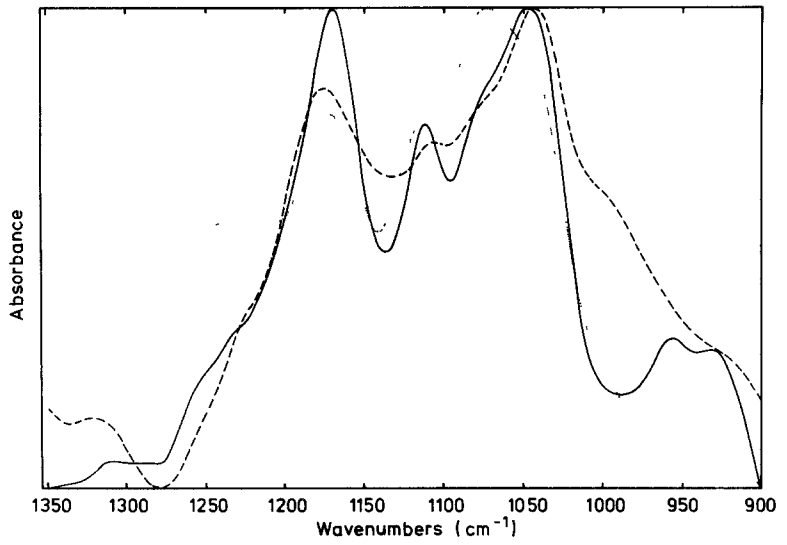



**Fig. 4.** Infrared ATR spectra (unpolarized light) of hydrated preparations of monophosphoryl free lipid A from *S. minnesota* (*dotted line*) and of synthetic lipid A analogues 503 (phosphate-free, *dashed line*) and 506 (bisphosphorylated, *full line*)

vibrations of the polysaccharide moiety in the spectral range 1,200 to 900 $cm^{-1}$. Furthermore, it can clearly be seen that the vibrations at wavenumbers around 1,730 $cm^{-1}$ (ester double bound stretch $\nu$ (C=O)), 1,650 $cm^{-1}$ (amide I), and 1,550 $cm^{-1}$ (amide II) show a complex splitting which not only indicates a different binding of the hydrocarbon chains to the reducing and non-reducing parts of the diglucosamine backbone, but also a different binding to the respective monoglucosamines.

For a further band assignment of some vibrations of the polar backbone a detailed analysis was performed by comparing the spectra of lipid A and LPS with some selected lipid A analogues and with the compounds α-D-glucosamine-1-phosphate, α-D-glucose-1-phosphate, and glucosamine-N-acetyl. Figure 4 shows examples of FT-infrared spectra for the synthetic lipid A analogues 503 (phosphate-free) and 506 (bisphosphorylated) as well as for monophosphoryl free lipid A from *S. minnesota* in the wavenumber range 1,350 to 900 $cm^{-1}$.

*Band at 1,170 $cm^{-1}$*: The mono- and bisphosphoryl lipid A analogues (chemical structures: see Fig. 2) all show a sharp band around 1,169 to 1,171 $cm^{-1}$ which is shifted to higher wavenumbers for free lipid A's, especially for the monophosphoryl compound (1,171 to 1,173 $cm^{-1}$). For the phosphate-free preparation (compound 503) this band is shifted to values in the range 1,175 to 1,178 $cm^{-1}$ (Fig. 4). From this behaviour it can be concluded that isolated natural lipid A (Acb or HCl) is different from the respective pure substances (compounds 504 or 506), or a mixture of these. Furthermore, the natural lipid A assembly may also

contain phosphate-free molecules. The band around 1,170 $cm^{-1}$ does not differ significantly for LPS Re and LPS S-form, therefore it must originate from the lipid A portion of LPS. Moreover, as lipid A(OH) samples, in which all ester-bound fatty acid residues are cleaved, show a drastic reduction of the band intensity of the 1,170 $cm^{-1}$ vibration, it must result mainly from the stretching vibration of the ester single bond, as described for phospholipids (Fringeli and Günthard 1981; Brandenburg and Seydel 1986).

*Band around 1,110 $cm^{-1}$*: The synthetic bisphosphoryl compounds have a relatively strong vibration at 1,112 $cm^{-1}$. For natural lipid A, the band intensities are reduced in the sequence lipid A (Acb) → lipid A (HCl) and are similar – when compared to the latter – for the synthetic monophosphoryl compounds. For the phosphate-free compound this vibration is very weak and has shifted to lower wavenumbers (Fig. 4). Therefore, the assignment of Fringeli (1977) – stated for phospholipids – seems reasonable and this vibration should mainly arise from the symmetric stretch of the negatively charged phosphate group $\nu_s(PO_2)$. Furthermore, from the behaviour described it may be concluded that natural bisphosphoryl lipid A, on the average, contains less than two phosphate groups per molecule.

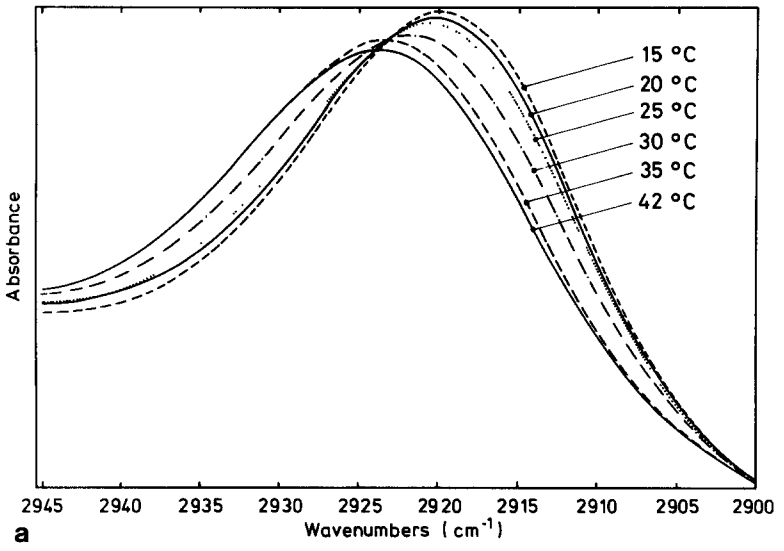
*Bands between 1,100 and 900 $cm^{-1}$*: The band lying around 1,070 to 1,085 $cm^{-1}$ seems to be a rather unspecific vibration resulting from the sugar moiety (see Figs. 3 and 4) which is taken from the comparison of LPS and free lipid A with the synthetic compounds and the compounds α-D-glucosamine-1-phosphate and glucosamine-N-acetyl. From the fact that it appears significantly stronger for the natural lipid A samples than for the synthetic compounds (Fig. 4) it may be concluded that a sugar vibration outside the diglucosamine backbone is involved. As 2-keto-3-deoxyoctaonate (KDO) and other monosaccharides such as glucose and galactose also have a strong absorption band in this region, it may be possible that the isolation procedure for lipid A does not lead to complete cleavage of the core oligosaccharide, especially KDO. From this argument an assignment of this band to the symmetric stretch $\nu_s(PO_2)$ (for phospholipids, see e.g. Lee and Chapman 1986) is not very likely.

The analysis of the band at approximately 1,045 $cm^{-1}$ showed that it should originate exclusively from the sugar moiety as an unspecific superposition of different single vibrations.

The single stretch vibration $\nu(P-O)$ found for phospholipids in the range 900 to 800 $cm^{-1}$ (Fringeli and Günthard 1981; Casal and Mantsch 1984) seems to lie at distinctly higher values for LPS, it is in the range 920 to 970 $cm^{-1}$ and is split in most cases (see Fig. 4). Regarding the synthetic compounds, this vibration is most distinct for the bisphosphoryl and less distinct for the monophosphoryl compounds and nearly vanishes for the phosphate-free compound. This correlates with the evaluation of the glucosamine derivatives, which only show a remarkable absorption band around 935 $cm^{-1}$ for the phosphate-containing samples. Additionally, the expression of this vibration seems to be conformation-dependent, as the phosphate groups bound to the non-reducing end of the diglucosamine cause a much smaller peak than that bound to the reducing end.

*Orientational data for LPS from Re-mutant*

For the investigation of the orientational behaviour a most detailed analysis was performed with lipopolysaccharides from deep rough mutants from *S. minnesota* R595 and *E. coli* F515, which also includes the investigation of a number of different batches of the same LPS chemotype. Figure 5a shows the variation of the absorption band around 2,920 $cm^{-1}$ (antisymmetric stretch $\nu_{as}(CH_2)$) with temperature for a LPS R595 preparation at approximately 95% water content. The data shows a shift of the position of the peak maximum to higher wavenumbers typical for melting of the hydrocarbon chains. To test whether and how the phase behaviour of lipopolysaccharides depends on the water content (lyotropism), in Fig. 5b for LPS R595 the temperature dependence of the peak position of $\nu_{as}(CH_2)$ is plotted for different water concentrations. From Fig. 5b it can clearly be seen that the distinctness of the phase transition and the transition temperature depend strongly on the water content and shows constant behaviour only at a water concentration of above 80% w/w. This observation, that beyond the complete hydration of the lipid sample (approximately 40% to 55% for LPS Re) the presence of excess water still influences the thermotropic behaviour, is similar to the situation for phospholipids (Brandenburg and Seydel 1986) and has to be taken into account when comparing results from techniques differing in the degree of practicable water content. In the present paper, sample preparation was done to get fully hydrated but oriented samples (see Methods). Measurements of the orientational parameters in excess water, corresponding to highly physiological conditions, are inadequate because the strong water bands – overlapping several vibrations important for diagnostic purposes – prevent an exact quantitative evaluation and, more crucial, the oriented lipids tend to decay in forming vesicles or other 3-dimensional aggregates under the influence of the free water so that no orientational data can be obtained by the ATR technique. Thus, the results presented in this paper are



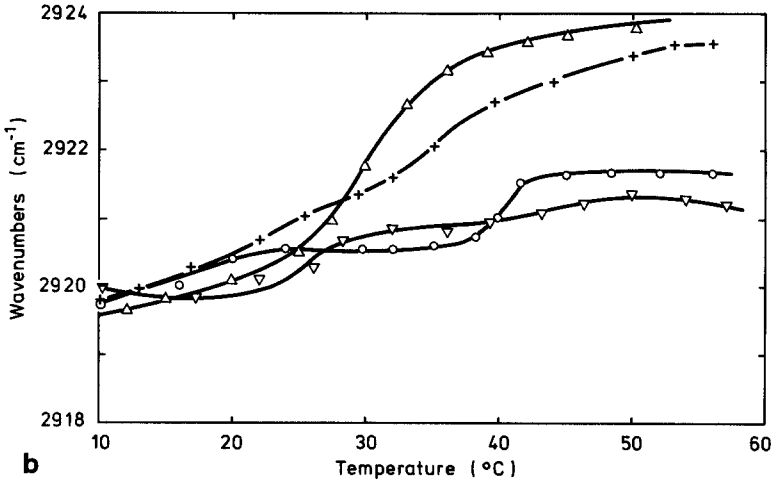




**Fig. 5 a.** Absorbance versus temperature of the antisymmetric stretching vibration around 2,920 $cm^{-1}$ with unpolarized IR light for a $1.5 \cdot 10^{-2}$ *M* preparation from *S. minnesota* deep rough mutant R595-LPS. **b** Peak position of the antisymmetric stretching vibration $\nu_{as}(CH_2)$ versus temperature for LPS preparations from strain R595 for different water contents. ▽ < 1%, ○ app. 50%, + app. 80%, △ app. 95% water content by weight

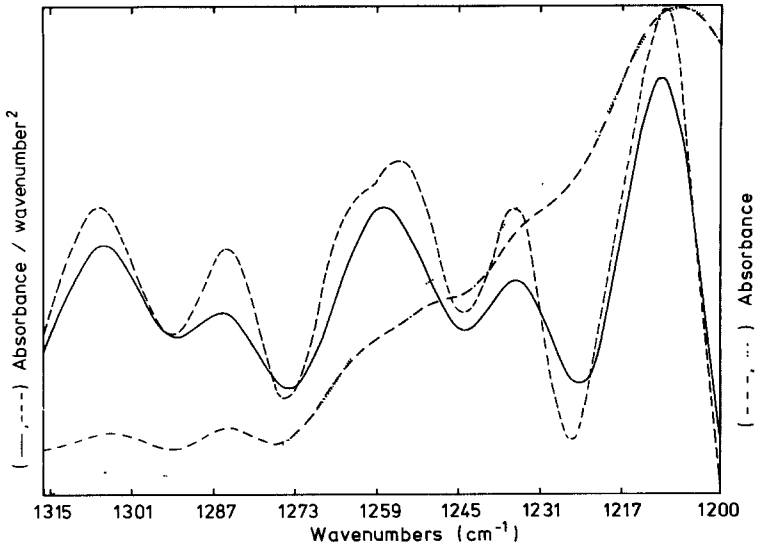


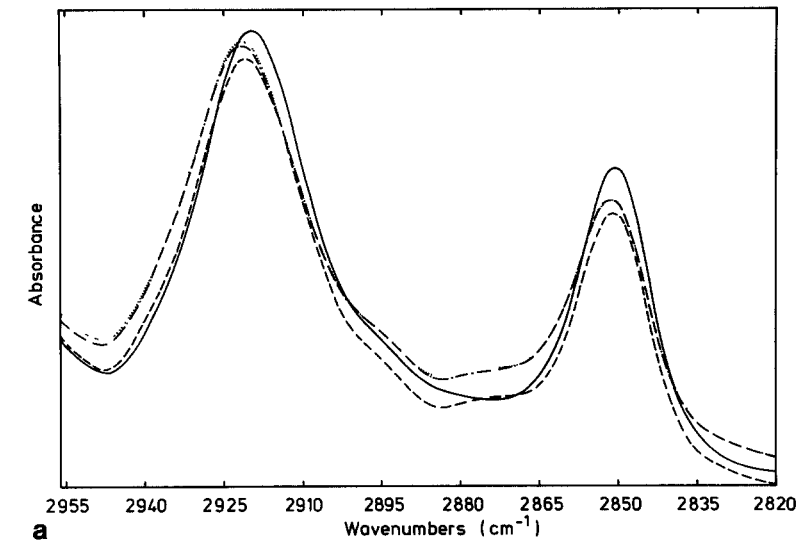

**Fig. 6.** Polarized infrared spectra and the corresponding inverted second derivatives for a hydrated (20 µl of $10^{-3}$ *M*) LPS preparation from *S. minnesota* R595 in the spectral range 1,315 to 1,200 $cm^{-1}$ (wagging progression bands and antisymmetric stretch of the negatively charged phosphate group) at 15 °C. Original spectrum, 90° polarization (*dashed/dotted line*), original spectrum. 0° polarization (*dotted line*), inverted second derivative spectrum, 90° polarization (*dashed line*), inverted second derivative spectrum, 0° polarization (*full line*)

**Table 1.** Mean values of the dichroic ratios $R$ with standard deviations $\Delta R$ within the given temperature ranges for various infrared vibrations of hydrated lipopolysaccharide preparations from *Salmonella minnesota* strain R595 spread on a ZnSe ATR plate

| Absorption band at [$cm^{-1}$] | Temperature range [°C] | Dichroic ratio $R \pm \Delta R$ | Remarks/Tentative assignment |
|---|---|---|---|
| 2,920 | 5–37 | $0.86 \pm 0.03$ | Antisymmetric stretch of $\geq CH_2$ $\nu_{as}(CH_2)$ |
| 2.920 | 40–55 | $1.01 \pm 0.03$ | |
| 2,850 | 5–37 | $0.82 \pm 0.05$ | Symmetric stretch of $\geq CH_2$ $\nu_s(CH_2)$ |
| 2,850 | 40–55 | $0.99 \pm 0.03$ | |
| 1,730 | 5–55 | $0.98 \pm 0.03$ | Stretch of the ester double bond C=O $\nu$(C=O) |
| Around 1.645 | 5–55 | 0.78 to 0.90 | Amid-I-vibrations |
| Around 1,550 | 5–55 | 0.78 to 0.90 | Amid-II-vibrations |
| 1,466 | 5–55 | $0.91 \pm 0.04$ | Bending vibration of $\geq CH_2$ $\delta(CH_2)$ |
| 1,377 | 5–55 | $1.27 \pm 0.09$ | Symmetric bending vibration of the end methyl group $\delta_s(CH_3)$ |
| 1,307 | 5–35 | $1.67 \pm 0.30$ | Wagging progression band |
| | 40–55 | $1.00 \pm 0.05$ | $\geq CH_2$ |
| 1,307 | 8–55 | $1.20 \pm 0.15$ | Wagging progression band $\geq CH_2$, other batch |
| 1,285 | 5–30 | $2.12 \pm 0.30$ | Wagging progression band |
| | 32–50 | (1.05) | Weak, disappearing |
| 1,285 | 5–50 | $1.76 \pm 0.29$ | other batch |
| 1,263 | 5–30 | $1.15 \pm 0.07$ | Wagging progression band, phosphate vibration $\nu_{as}(PO_2^-)$ |
| | > 32 | – | disappearing |
| 1,234 | 5–55 | $1.33 \pm 0.10$ | Wagging progression band |
| 1,234 | 5–55 | $0.98 \pm 0.13$ | Wagging progression band, other batch |
| 1,207 | 5–35 | $1.22 \pm 0.03$ | Wagging progression band $\geq CH_2$ |
| | 37–55 | $1.03 \pm 0.04$ | |
| 1,167 | 5–55 | $1.10 \pm 0.05$ | Stretch of the ester single bond C–O $\nu$(C–O) |
| 1,112 | 5–50 | $1.00 \pm 0.03$ | Symmetric stretch of phosphate ($PO_2^-$) |
| 1,070 | 5–35 | $1,06 \pm 0.03$ | Sugar vibration |
| | 37–50 | $0.97 \pm 0.04$ | |
| 1,070 | 5–50 | $1.09 \pm 0.08$ | Other batch |
| 1,045 | 5–55 | $1.05 \pm 0.03$ | Sugar vibration |
| 950 | 5–55 | $1.20 \pm 0.08$ | Phosphate vibration |
| | 5–55 | $0.85 \pm 0.05$ | Other batch |
| 850 | 5–55 | $1.40 \pm 0.11$ | Sugar vibration |
| 722 | 5–55 | $0.78 \pm 0.07$ | Rocking progression band |

strictly valid only under these restrictions. Regarding the nearly planar geometry of the LPS in the outer membrane, however, a transference of the results should be appropriate with respect to the directions of the vibrations of the molecular groups.

Figure 6 shows typical polarized ATR spectra for a LPS R595 preparation at 15 °C in the spectral range 1,315 to 1,200 $cm^{-1}$. To emphasize the differences between vertical and horizontal polarization, inverted second derivative spectra are also shown. It can clearly be seen that most of the bands in this spectral range – the wagging progression bands – express a significantly higher absorption of the parallel than that of the vertically polarized light (high dichroic ratio) indicating a high order of the hydrocarbon chains. The broadness of the band around 1,260 $cm^{-1}$ for the vertical and the splitting for the parallel polarized light in Fig. 6 indicate a superposition of two vibrations, a wagging progression band at 1,263 $cm^{-1}$ and the antisymmetric stretch of the negatively charged phosphate group around 1,255 $cm^{-1}$, which was confirmed in several independent measurements with LPS from other mutants. The dichroic behaviour of these and other important absorption bands are summarized in Table 1, in which for various molecular vibrations the measured dichroic ratios $R \pm \Delta R$ for the given temperature ranges are listed. Obviously, in the lower temperature range all preparations show essentially the same behaviour. Furthermore, for many but not for all LPS Re preparations a jump in the values of $R$ around 37 to 40 °C appears for several vibrations. Lyotropism could be excluded as the reason for these variations between different batches, because between some of the samples with deviating phase behaviour the degree of hydration was practically identical. When calculating the 'critical packing parameter' (Israelachvili et al. 1980) $x = v/al$ ($v$ volume of the hydrophobic part, $a$ = area of the hydrophilic head group, $l$ length of the acyl chain) – which is a measure of the tendency of an amphiphile to form either lamellar or non-lamellar



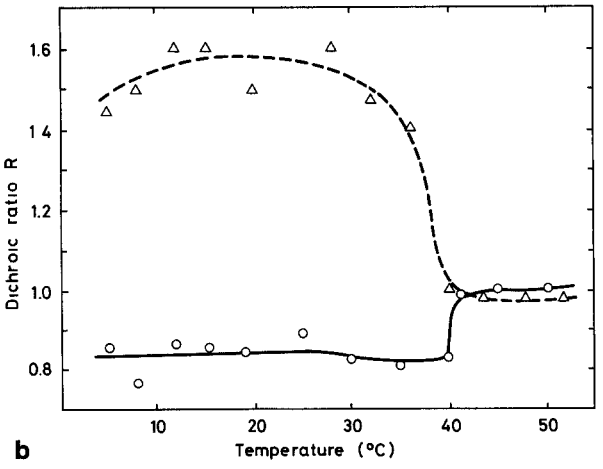




**Fig. 7a.** Polarized infrared spectra in the wavenumber range 2,955 to 2,820 $cm^{-1}$ (symmetric and antisymmetric stretch of $>CH_2$) for a hydrated LPS preparation from *S. minnesota* mutant R595 at 15 °C ($< T_c$) and at 42 °C ($> T_c$). 90° polarization, 15 °C (*dashed line*), 0° polarization, 15 °C (*full line*), 90° polarization, 42 °C (*dotted line*), 0° polarization, 42 °C (*dashed/dotted line*). **b** Dichroic ratio versus temperature for a LPS preparation from strain R595. △ Wagging progression band at approx. 1,307 $cm^{-1}$, ○ antisymmetric stretching $\nu_{as}(CH_2)$ at approx. 2,920 $cm^{-1}$

phases – by taking data from X-ray diffraction experiments of Labischinski et al. (1985) and from monolayer investigations of Brandenburg and Seydel (1984), LPS Re seems to be a border case with values of $x$ around 1. Thus, the ambivalent behaviour of the deep rough mutant LPS are understandable because minor variations in the isolation and purification procedures can never be excluded.

Figure 7a shows the polarized infrared spectra for LPS from mutant R595 in the spectral range 2,955 to 2,820 $cm^{-1}$ (symmetric and antisymmetric stretch of $CH_2$) exhibiting a clear vertical polarization at the lower temperature ($< T_c$) especially for $\nu_s(CH_2)$ but hardly any polarization ($R$ around 1) at the higher temperature ($> T_c$). The behaviour of the dichroic ratio for LPS Re (from strain R595) over the entire temperature range is presented in Fig. 7b for the antisymmetric stretching vibration of the methyline groups around 2,920 $cm^{-1}$ and the wagging progression band at 1,307 $cm^{-1}$. The values for the angles between the oscillating dipole moments and the molecular axis are expected to lie around 20° for the wagging progression vibrations and 65° for $\nu_{as}(CH_2)$ at $T < T_c$ as measured for phospholipids (Brandenburg and Seydel 1986). A calculation of the order parameter by applying Eq. (1), however, gave diverging values for $S$ (above 1 and around 0.5, respectively). Therefore, the angles $\theta$ were varied to get consistent values for $S$, and as best fit an order parameter of $S = 0.70 \pm 0.05$ was found with values $\theta = 20°$ for the wagging vibration and $\theta = 80°$ for $\nu_{as}(CH_2)$. From this, the existence of a highly ordered phase can be concluded. From the approximation of $S$ and the measured values of $R$ listed in Table 1, the angles $\theta$ for the individual vibrations can now be calculated. Thus, for example, for the sugar vibration at 850 $cm^{-1}$ an angle of 38° for the oscillating dipole moment with respect to the molecular axis can be determined.

In a similar way, the value of $S$ at $T > T_c$ can be approximated to 0.20–0.30 corresponding to a highly

disordered phase. It should be noted, however, that the angles $\theta$ for the wagging progression bands (e.g. that at 1,307 $cm^{-1}$) now adopt significantly higher values than in the ordered phase, i.e. values around 70°, apparently due to the introduction of an increasing number of gauche conformers.

A transition from a lamellar (gel state or mixed gel/fluid) to an isotropic phase is in accordance with the observed phase behaviour. The existence of a lamellar phase below $T_c$ is further confirmed by the low $R$-values measured for the symmetric stretch of the methylene groups at 2,850 $cm^{-1}$ $\nu_s(CH_2)$, the bending vibration of the methylene groups at 1,467 $cm^{-1}$ $\delta(CH_2)$, the most intense rocking progression band of the methylene groups at 722 $cm^{-1}$, and by the high $R$-values for several other wagging progression bands and the bending vibration of the end methyl group ('scissoring') $\delta_s(CH_3)$. Regarding the stretch of the ester single bond $\nu(C-O)$ at 1,167 $cm^{-1}$, the values for phospholipids lie significantly higher, in the range 1.4 to 2.2 (Brandenburg and Seydel 1986). The relatively low values of $R$ for LPS R595 may be explained by the different orientations of the oscillating dipole moments within the hydrophilic/hydrophobic interface (double esters). Regarding the values of the dichroic ratio for the wagging progression bands, the vibrations at 1,307, 1,284, 1,263, 1,234 and 1,207 $cm^{-1}$ in most cases have $R$-values significantly above 1 (compare Table 1). The band at approximately 1,340 $cm^{-1}$ (not listed in Table 1) which is often assigned to a $CH_2$ wagging vibration (e.g. Mantsch et al. 1981), either does not show a clear behaviour or has an $R$ value around 1. Thus, as already stated for phospholipids, the assignment of this band to a wagging progression is probably not correct.

From the orientational data a temperature-governed drastic change of the supramolecular structure at $T > T_c$ can be deduced. This is connected with a simultaneous melting – at least partially – of the hydrocarbon chains, which can be seen from the abrupt change of the peak position of several vibration bands, and with a drastic decrease of the band intensities of some wagging progression bands.

As shown recently (Seydel and Brandenburg 1986), divalent cations such as $Mg^{2+}$ should promote a similar transformation such that after heating above $T_c$ the original lamellar structure below $T_c$ disappears in all further scans. An IR measurement performed with a preparation LPS Re: $Mg^{2+}$ 2:1 molar shows, in the first scan, for the wagging progression bands (in the spectral range 1,350 to 1,180 $cm^{-1}$) at $T < T_c$ high $R$-values typical for lamellar structures. At temperatures above $T_c$, however, most of these bands show a drastic reduction of the dichroic ratio, which now approaches values around 1. Recooling of the sample did not immediately yield the old spectrum. The initial lamellar state could only be restored partially, but never completely after prolonged cooling at −20 °C. These results indicate that a very stable non-lamellar phase is triggered by the divalent cations and with that support the interpretation presented recently (Seydel and Brandenburg 1986 and unpublished results).

*Orientational data for natural and synthetic lipid A*

To study the influence of the sugar moiety on the 3-dimensional conformation of the LPS molecules, free lipid A preparations lacking the whole core region of the LPS were investigated (see Figs. 1 and 2). Prior to the orientation measurements, the lyotropism of free lipid A preparations was determined in a similar way as for LPS Re. It turned out that when decreasing the water concentration the shift of $T_c$ towards higher temperatures is much less distinct than for LPS Re samples, e.g. the phase transition temperature of hydrated free lipid A preparations is only approximately 5 °C higher than that in excess water (>80%). In Table 2 the dichroic ratios for some selected vibrations in the given temperature ranges are given for two batches of monophosphoryl lipid A from *S. minnesota* R595. It may be stated generally that – as for LPS Re

**Table 2.** Mean values of the dichroic ratios with standard deviations $\Delta R$ for some selected infrared vibrations within the given temperature ranges for a hydrated monophosphoryl free lipid A preparation from *Salmonella minnesota* R595 spread on a ZnSe ATR plate

| Absorption band at [$cm^{-1}$] | Temperature range [°C] | Dichroic ratio $R \pm \Delta R$ | Remarks/Tentative assignment |
|---|---|---|---|
| 2,920 | 5–42 | 0.95±0.03 | Antisymmetric stretch $>CH_2$ |
| | 45–60 | 1.02±0.03 | |
| 2,920 | 5–40 | 1.25±0.06 | Other batch |
| | 42–60 | 1.37±0.04 | |
| 1,377 | 5–55 | 1.26±0.09 | Symmetric bending of $>CH_3$ |
| 1,377 | 5–60 | 0.52±0.15 | Other batch |
| 1,285[a] | 5–45 | 1.20±0.18 | Wagging progression band $>CH_2$ |
| | 47–60 | 0.85±0.04 | |
| 1,262 | 5–55 | 1.35±0.50 | Wagging progression band antisymmetric stretch of $PO_2^-$ |
| 1,235 | 5–60 | 1.60±0.20 | Wagging progression band |
| 722 | 5–55 | 1.47±0.13 | Rocking progression band |
| | 5–60 | 0.98±0.15 | Other batch |

[a] Not observable or evaluable for all samples

– only for certain vibrations does a change of the dichroic ratio at $T_c$ (42 to 47 °C) take place (e.g. $\nu_{as}(CH_2)$ and the wagging progression band at 1,285 $cm^{-1}$ listed in Table 2) which thus may be regarded as conformation-sensitive. Moreover, from several vibrations, e.g. $\nu_{as}(CH_2)$ at 2,920 $cm^{-1}$, $\delta_s(CH_3)$ at 1,367 $cm^{-1}$, and the rocking progression band at 722 $cm^{-1}$, large variations of the *R*-values not only between different batches, but also in different experiments with the same batch were observed. This holds true not only for this particular lipid A, but also for bisphosphoryl lipid A's. The *R*-values presented indicate that for some batches more or less highly ordered structures similar to that of LPS Re are adopted, while in other cases the lipid A molecules assemble into less uniform structures. In some measurements, the values for $\nu_{as}(CH_2)$ and the ester and amide vibrations (with higher *R*-values, data not shown) and for $\delta_s(CH_3)$ (low *R*) are consistent with a supramolecular structure, in which the acyl chains are uniformly distributed in the *x*-*y* direction, i.e. parallel to the crystal surface, and have no or only a small component in the *z*-direction (normal to the crystal). From this, as a possible supramolecular structure an inverted hexagonal state ($H_{II}$) could be deduced with the axis of the aqueous cylinders oriented normal to the crystal and with the acyl chains on the cylindrical surface directed outwards. In some cases free lipid A apparently already has a tendency to form non-lamellar structures at lower temperatures.

To investigate the possible influence of microheterogeneity or chemical pretreatment on the 3-dimensional structure, the temperature dependence of the dichroic ratio was also studied for the synthetic bisphosphoryl lipid A's (compounds 506 and 516 corresponding to *E. coli* and *S. minnesota* free lipid A, respectively). The analysis shows a behaviour similar to that of free lipid A's, e.g. significant variations of the *R*-values for some characteristic bands (antisymmetric stretch $\nu_{as}(CH_2)$, wagging progression bands) in different experiments, and usually small *R*-values for the bending vibration $\delta_s(CH_3)$ and the ester stretching vibration $\nu(C-O)$. Thus, as already found for the complex phase behaviour of free lipid A preparations (Naumann et al. 1987), no significant difference between the natural and synthetic products can be seen, thus excluding the possibility of a decisive influence of variations in the chemical structure of the natural isolate on its 3-dimensional supramolecular assembly.

*Orientational data for other rough mutant LPS*

Our investigations were extended to LPS from *S. minnesota* rough mutants Rd2 (strain R4), Rd1 ($P^-$) (strain R7), Rd1 ($P^+$) (strain Rz), and Ra (strain R60). All these *R*-form LPS show an orientational behaviour very similar to that observed for LPS Re below its phase transition temperature (data not shown). However, within the temperature range of 5° to 55 °C, no drastic changes of the dichroic ratios could be measured. This may be due to the distinct lyotropism of all *R*-form LPS (compare Fig. 5 b) which leads to a drastic increase in $T_c$ with decreasing water content. Preliminary results from transmission measurements at higher water content (app. 90%), however, show (especially from the evaluation in the spectral ranges of the wagging progression bands and some phosphate vibrations) the tendency of these LPS also to form lamellar structures above their phase transition temperatures.

Differences in the *R*-values as compared to LPS Re and free lipid A could be observed for Rd-LPS for some vibrations resulting from the hydrophilic hydrophobic interface. Particularly for the amide vibrations, e.g. the amide II vibrations around 1,550 $cm^{-1}$ a distinct tendency towards a change of the *R*-values can be seen. These are lowest for free lipid A ($R = 0.60$ to 0.80), higher for LPS Re ($R = 0.80$ to 0.90) and still increasing with increasing length of the polysaccharide moiety ($R = 1.2$ to 1.6 for the different *R*-form LPS). As the amide II vibration was found to be a superposition of the C–N stretching and the N–H deformation mode (Bellamy 1975), it can – on the average – be assumed to oscillate predominantly in the direction of the acyl chains. Thus, the described behaviour would be in accordance with the interpreatation that the lipopolysaccharides with an increasing length of the polysaccharide chain tend increasingly to adopt parallel oriented hydrocarbon chains.

As for Re-LPS, the influence of divalent cations on the orientational behaviour was studied. From these and other measurements published by Brandenburg and Blume (1987) it can generally be stated that with increasing length of the polysaccharide chain the influence of divalent cations on the supramolecular structure decreases, i.e. the tendency of the lipid assembly to adopt more isotropic states at higher temperatures vanishes. This is in accordance with light microscopic observations that for LPS:$Mg^+$ ratios in the molar range 10:1 to 1:1 for more rough mutant LPS (like Rd- and Re-LPS), but not for Ra-LPS and wild-type forms an agglomeration and eventually precipitation takes place.

## Conclusions

The results from the ATR measurements using polarized IR light clearly show that this technique not only allows the determination of important orientational parameters for systems such as phospho- and

sphingolipids (Brandenburg and Seydel 1986) but also for the more complex LPS assemblies. From the measurements of the vibrations resulting from the hydrophobic moiety similar data were found for all rough mutant LPS with respect to the dichroic ratios and the directions of the oscillating dipole moments, especially for the vibrations $\nu_{as}(CH_2)$, $\nu_s(CH_2)$, $\delta(CH_2)$, $\delta_s(CH_3)$, and the rocking and wagging progression bands, all indicating planar, i.e. lamellar structures. Regarding the vibrations from the polar backbone, the band at 1,170 $cm^{-1}$ can now be assigned unequivocally to a stretching vibration of the ester single bond C–O, the oscillating dipole moment of which, however, is aligned more isotropically in contrast to phospholipids according to the different orientations within the double esters. Also, the assignment of the bands around 1,110 and 940 $cm^{-1}$ mainly to phosphate vibrations could be assured.

Regarding the orientational behaviour, as important conclusion the strong dependence of the orientations of the LPS and lipid A assemblies on the length of the carbohydrate moiety is noteworthy. For the lipid samples with very low content of saccharides (free lipid A, LPS Re) or for other rough mutant LPS under the influence of sufficiently high concentrations of divalent cations, the ratios of the cross-sections of the hydrophobic to the hydrophilic moiety are in favour of the former and thus promote the occurrence of non-lamellar structures. This correlates with the 'critical packing parameter' mentioned above assuming values significantly above 1 for free lipid A, around 1 for LPS Re, and lower than 1 for other rough mutant LPS. At this point, however, it should be emphazised that the relatively low water content (30% to 50%) necessitated by the requirements of the ATR technique does not necessarily allow the extrapolation to more physiological conditions.

A comparison of the order parameters below their respective phase transition temperatures shows a lower state of order for LPS ($S = 0.70 \pm 0.05$) than for phospholipids ($S = 0.80 \pm 0.05$) in the same phase state. This is in full agreement with the statements – concluded from calorimetric investigations – that the hydrophobic moiety of LPS is already partially fluid below $T_c$ (Brandenburg and Seydel 1985) or that the cooperative binding of the LPS acyl chains is lower than that of comparable phospholipids (14:0 PC and 14:0 PE, Coughlin et al. 1985). Thus, the assumption of a particularly high state of order of the LPS assembly (Nikaido 1986) is only valid when it is compared – at 37 °C – with the highly fluid phospholipids from the inner leaflet of the outer membrane.

The orientational behaviour of free lipid A and LPS could be of relevance for the expression of biological activities at 37 °C. In many test systems, LPS Re expresses the highest activity, free lipid A usually shows a similar behaviour (Rietschel et al. 1986) except for some biological systems in which it is considerably less active than LPS Re (Kirikae et al. 1986; Vukajlovich et al. 1986). All other rough mutant LPS as well as smooth forms usually are less active than LPS Re. Thus, for example, we found recently in collaboration with Th. Lüderitz (Forschungsinstitut Borstel) that the temperature of the main phase transition of LPS assemblies from different mutants from *Salmonella minnesota* directly correlate with the ability of these LPS to induce the release of leukotriene C4 from mouse peritoneal macrophages (unpublished results). Considering the described ability of $Mg^{2+}$ – which is present in all biological test systems – to influence the orientational behaviour, the partial or total presence of non-lamellar structures at 37 °C beside a minimum degree of fluidity (chain melting) might be the prerequisite for the expression of certain biological activities. The presented data show that this prerequisite might be accomplished most readily for deep rough mutant LPS.

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