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INVESTIGATIONS INTO THE THERMOTROFIC PHASE BEHAVIOUR OF NATURAL MEMBRANES EXTRACTED FROM GRAM-NEGATIVE BACTERIA AND ARTIFICIAL MEMBRANE SYSTEMS MADE FROM LIPOFOLYSACCHARIDES AND FREE LIPID A

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ABSTRACT

It is reported on investigations into the thermotropic phase behaviour of outer, cytoplasmic, and cell envelope preparations of Gram-negative bacteria, and their amphiphatic compounds - lipopolysaccharides and phospholipids - applying calorimetric, infrared spectroscopic, and 90°-light scattering techniques. Except for free lipid A, the lipid moiety of lipopolysaccharide, all investigated compounds exhibited gel-liquid crystalline phase transitions of the hydrocarbon chains in the temperature range 10 to 42 °C. The transition regions of the outer membrane and lipopolysaccharide preparations are narrower and appear at significantly higher temperatures (around 37 °C) as those from the cytoplasmic membrane and phospholipid preparations (around 26 °C). For both amphiphatic compounds the phase transitions are shifted to lower values at reduced growth temperature of the cells.

INTRODUCTION

Gram-negative bacteria contain beside the cytoplasmic membrane and the murein layer - which are common to most bacterial cells - an additional barrier, the outer membrane. Regarding its lipid moiety, the outer membrane was shown to be extremely asymmetric (ref.1), i.e. the inner leaflet is composed mainly of phospholipids (PL), essentially phosphatidylethanolamine, and the outer leaflet of lipopolysaccharides (LPS). LPS consists of a poly- or an oligosaccharide portion being covalently linked to its lipid moiety called lipid A which anchors the LPS in the outer membrane and which is assumed to represent the "endotoxic principle" of LPS. Lipid A is composed of a bisphosphoryl diglucosamine backbone which is acylated by up to 7 fatty acid residues (ref. 2). The whole cell envelope of the Gram-negative bacterial cell is illustrated schematically in fig. 1.

Beside the expression of various biological activities

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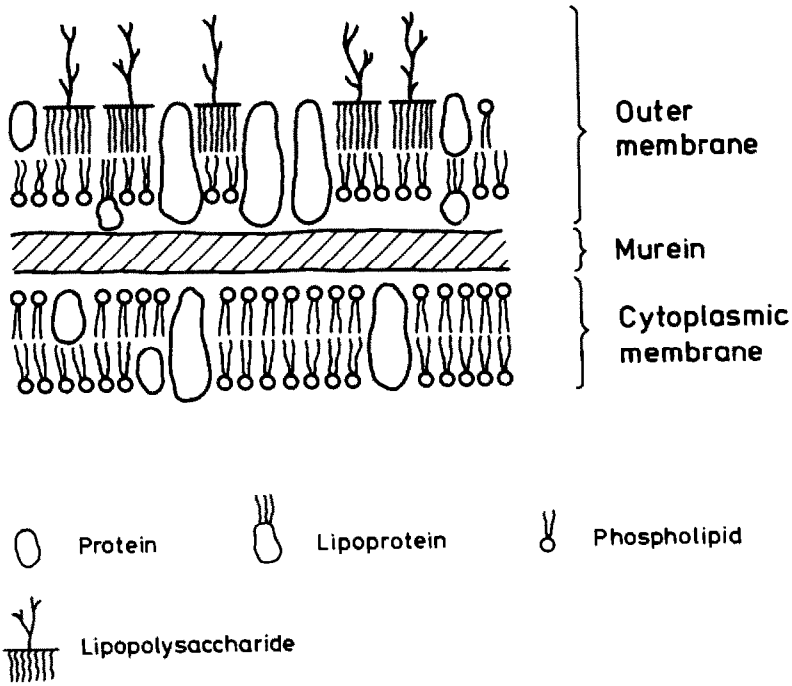

Fig. 1. Schematic structure of the cell envelope of Gram-negative bacteria.

(ref. 3) the lipopolysaccharide molecular assembly may be made responsible for the barrier function of the outer membrane especially against hydrophobic drugs due to the ability of LPS to form highly ordered structures at physiological temperature which was measured with artificial LPS membrane systems (ref. 4). This high state of order of the LPS aggregates - as compared to the phospholipid assembly from the inner leaflet - correlates with a relatively high temperature of the gel-liquid crystalline phase transition of the hydrocarbon chains lying between Tc = 30 to 36 °C for LPS from rough mutants - deficient in the biosynthesis of certain sugars (e.g. deep rough mutants are not able to synthesize heptose) - and around 37 °C for LPS from wild-type strains, all from Salmonella minnesota strains.

However, as the investigations into the physical structure of LPS and free lipid A preparations were performed exclusively with artificial LPS/lipid A membrane systems after extraction from the bacterial cells, the structural preferences and phase states might be different within the biological membrane from that in

the pure LPS:water system, for example because of the known ability of outer membrane proteins to bind to LPS molecules. Thus, this paper comprises beside the measurement of isolated LPS/lipid A membrane systems also that of the phospholipid extract, the whole outer and cytoplasmic membrane as well as the cell envelope (outer and cytoplasmic membrane inclusive the murein layer) approaching more and more the pure biological system. Moreover, to study the influence on ambient conditions on membrane fluidity, investigations were performed also with membranes from cells grown at temperatures other than 37 °C.

An investigation into the thermotropic behaviour of natural membranes seems also important, because in the literature contradictory results have been reported. This refers to the values of the phase transition temperature Tc for cytoplasmic and outer membranes as well as for the Tc's at different growth temperatures. For example, Nakayama et al (ref. 5) specify values of 28.5 and 31.5 °C for the cytoplasmic and outer membrane, respectively, of E. coli bacteria, while by Janoff et al (ref. 6) the corresponding values are 21.5 and 43.5 °C.

MATERIALS AND METHODS

Lipids and membranes

Phospholipids from the inner leaflet of the outer membrane and the cytoplasmic membrane were extracted from Salmonella minnesota rough mutant strains R595 and R60 and Escherichia coli mutaflor at the end of the exponential growth phase according to the methanol/chloroform (1:2 molar) procedure (ref. 7-8). The cells were cultivated at 37 °C, the strain R595 also at 22 °C. Similar to the results of Ames (ref. 8), the composition of the phospholipid extract measured by thin-layer chromatography turned out tc be app. 70 %phosphatidylethanolamine, 18 % phosphatidylglycerol, and a few % cardiolipin. The outer and cytoplasmic membrane fractions were extracted from Salmonella minnesota strains R595 and R60 (rough mutant Ra) by the lysozyme/EDTA treatment and sucrose gradient centrifugation according to the technique of Osborn et al (ref. 9) giving a so-called H-fraction with a buoyant density of 1.22 corresponding to the outer membrane and two further, L1 and L2, fractions corresponding to the cytoplasmic membrane (probably a protein-rich and a protein-poor fraction) with respective values of 1.14 and 1.16. The preparation of the cell envelope was performed similar as described by VanAlphen

et al (ref. 10) by ultrasonic treatment of whole cells with subsequent removal of intact cells by low speed centrifugation and extraction of cell envelopes by untracentrifugation of the supernatant.

Lipopolysaccharide from rough mutant strains R595, R4, Rz, and R60 of Salmonella minnesota (chemical structure see fig. 2) was extracted in the late exponential phase from cells grown at 37 °C, in one case (LPS R595) also at 22 °C, according to the phenol:chloroform:light petrol ether procedure (ref. 11) and was further purified by HCl precipitation (ref. 12). Various rough mutant LPS as well as wild-type forms were purchased from Sigma Chemicals (Deisenhofen F.R.G.). All LPS preparations were analysed in their natural salt forms. Free lipid A - a generous gift of H. Brade (Forschungsinstitut Borstel) - was isolated from LPS by acetate buffer treatment, purified, and converted to the triethylamine form (ref. 13). The synthetic E. coli lipid A analogue LA-15-PP was purchased from Daiichi Pure Chemicals (Tokyo, Japan).

Sample preparation

All samples were prepared by vigorously vortexing a 10^{-3} to 10^{-2} M lipid dispersion in distilled water at temperatures below the gel-liquid crystalline phase transition. In several experiments also higher lipid concentrations were used, and in some cases Mg^{2+} cations in varying ratios were added to the lipid dispersions.

Instrumentation

90°-light scattering measurements at 400 nm were performed with a Kontron SFM-25 spectrophotofluorometer similar as described in ref. 14. The samples were placed in a thermostated cuvette and scanned at a rate of 1 °C/min. Temperature was controlled by a programmable temperature bath. Fourier-transform infrared transmission measurements were done on a Nicolet "5-DX" in a thermostated temperature-programmable cell with windows from CaF_2 at a scan-rate of 0.3 °C/min. For each temperature, 200 scans were accumulated and apodized with a Happ-Genzel function (ref. 15). For a precise description of measuring and evaluation details see ref. 16. Calorimetric measurements usually were performed as described in ref. 17 on the DASM-1 (Privalov calorimeter) at a scanning-rate of 0.8 °C/min. In some experiments,

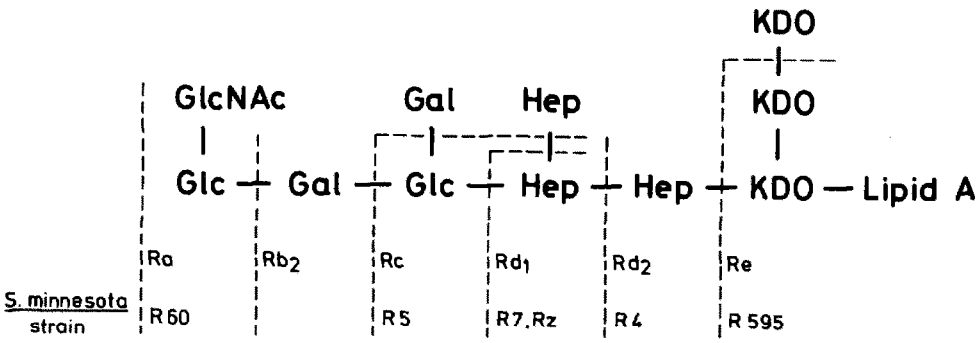

Fig. 2. Schematic structure of the core region of various lipopolysaccharides of rough mutants from Salmonella minnesota (without phosphate groups). The wild-type strain (S-form) additionally contains the covalently-linked O-chain consisting of repeating units of a pentasaccharide. Abbreviations: KDO 2-keto-3-deoxyoctonate, Hep heptose, Glc glucose, Gal galactose, GlcNAc Glucoseamineacetyl.

measurements were also done on a heat-flux device described earlier (ref. 14), and on a DSC-2 (Perkin-Elmer) with a low-temperature supplement. The latter measurements, which were started at -30 °C, were kindly performed by Mr. E. Emde.

RESULTS AND DISCUSSION

In fig. 3 the peak position of the symmetric stretch of the methylen groups $\nu_s(CH_2)$ is plotted versus temperature and compared with the respective calorimetric endotherm for a cell envelope, the outer (H-fraction) and cytoplasmic (L-fractions) membranes, and a LPS preparation all from Salmonella minnesota mutant R60. It can be seen clearly that a gel-liquid crystalline phase transition centered around 33 to 35 °C takes place for all samples except for the cytoplasmic membrane. Thus the melting of the hydrocarbon chains of the LPS moiety is expressed also within the outer membrane and cell envelope preparations indicating that the behaviour of LPS located in the outer membrane seems to be largely independent of the presence of proteins and should therefore run parallel to the behaviour of the artificial LPS membrane system.

A comparison of the phase behaviour of the cytoplasmic membrane (see fig. 3) and that of the phospholipid extract (not shown) - exhibiting a broad transition in the temperature range 21 to 34 °C - shows a correspondence of their transition ranges. Thus, it may be concluded that the proteins - similar as in the case of the outer membrane - have, if at all, only a slight influence on the thermotropic phase behaviour of the cytoplasmic membrane.

In fig. 4 the thermotropic phase behaviour of two LPS preparations from Salmonella minnesota mutant R595 from cells grown at 22 and 37 °C, respectively, is demonstrated for 3 different physical techniques, i.e. 90°-light scattering at 400 nm, infrared spectroscopy (peak position of the antisymmetric stretching vibration $\nu_{as}(CH_2)$ of the methylen groups), and DSC (specific molar heat). It can be taken from fig. 4 that all techniques show phase transitions with temperatures being 5 °C higher for the LPS from cells grown at 37 °C as compared to the preparation from cells grown at 22 °C. This indicates that when lowering the growth temperature, the bacterial cell attempts to maintain a sufficient fluidity of the outer membrane. An analysis of the fatty acid moieties of the respective lipopolysaccharides - kindly performed by H. Moll, Forschungsinstitut Borstel - shows that this increase in fluidity for the 22°C-preparation runs parallel to the biosynthesis of a 16:1 acyl chain at the expense of the amount of synthesis of 3-OH-fatty acid residues. In this way, also an explanation for the reduced enthalpy change of the 22 °C-preparation (see fig. 4) seems possible. The different fatty acid pattern between the two preparations, however, cannot account exclusively for the large enthalpy difference, rather fluctuations observed especially for Re-LPS seem to be the main origin (compare fig. 6). To test whether the phospholipids show a similar change - with respect to the phase behaviour and the biosynthesis of the acyl moiety - these were extracted also from cells grown at 22 °C. Again, a broad transition can be observed as measured IR-spectroscopically which, however, shifts to a significantly lower range, i.e. 12 to 24 °C. A determination of the fatty acid pattern was performed with the phospholipid extracts from 22°C-E. coli cells - exhibiting a similar phase behaviour as S. minnesota. The analysis performed with laser desorption mass spectrometry published in ref. 18, shows a significant increase in the biosynthesis of 16:1 and 18:1 fatty acid residues for the 22°C-

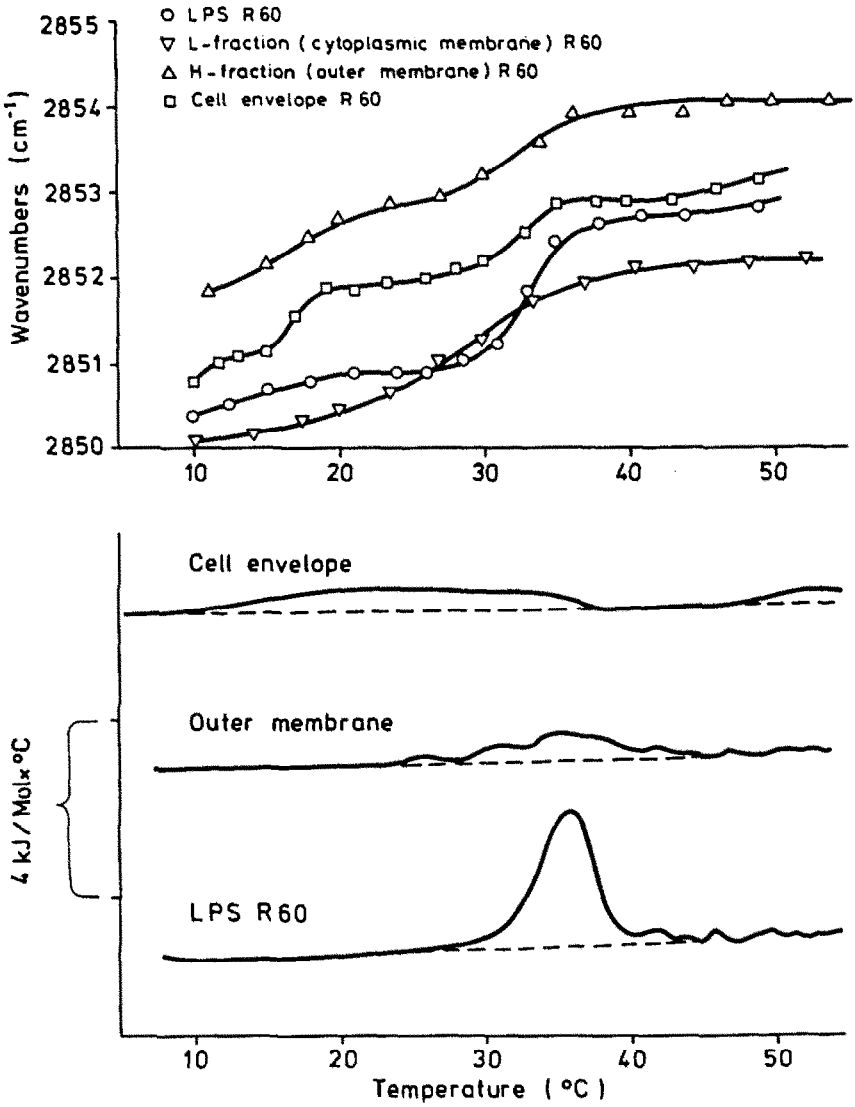

Fig. 3. Peak position of the symmetric stretch of the methylen groups $\nu_s(CH_2)$ and specific molar heat versus temperature for natural membranes and lipopolysaccharide preparations (approximately 10^{-3} M) from Salmonella minnesota from mutant strain R60. The measured enthalpy changes are: Cell envelope 0.89 J/g, outer membrane 0.67 J/g, and LPS 7.1 J/g.

preparation thus again increasing the membrane fluidity.

Unusual is the behaviour of the 90°-light scattering measurement for the 22°C-preparation as illustrated in fig. 4 showing a

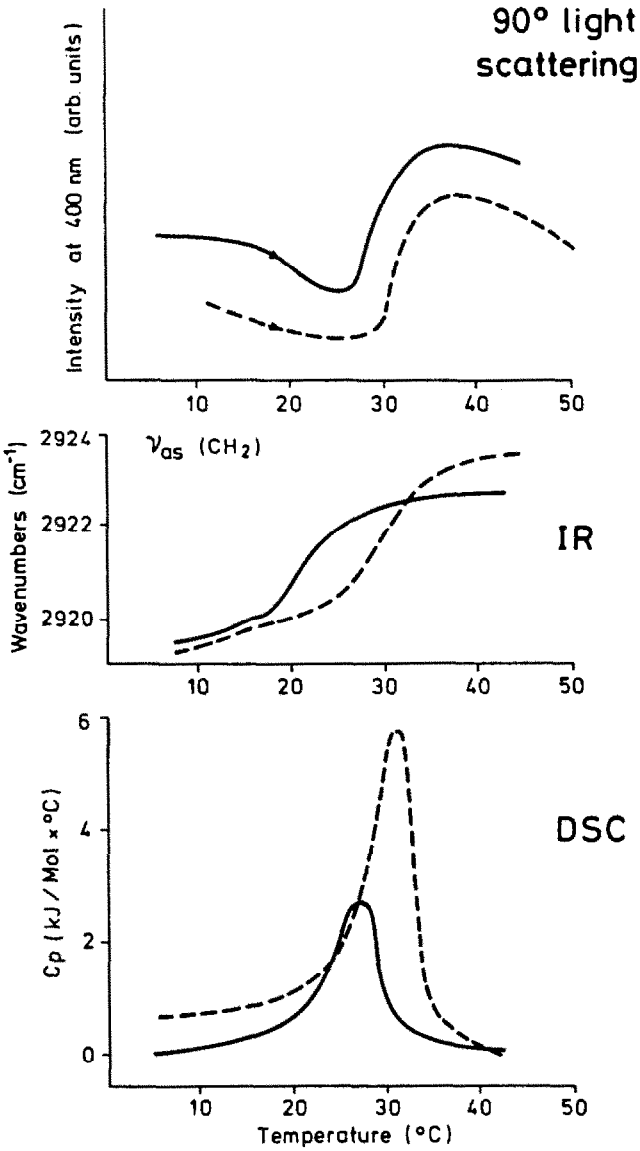

Fig. 4. 90°-light scattering intensities at 400 nm, peak position of the antisymmetric stretching vibration of the methylen groups $\nu as(CH_2)$, and specific molar heat C_p versus temperature for a 10^{-3} M LPS R595 preparation grown at 22 (solid line) and 37 °C (dashed line), respectively. The enthalpy values are ΔH (22 °C) = 25.1 kJ/mol, ΔH (37 °C) = 39.3 kJ/mol.

decrease in light intensity at the beginning of the transition and later a drastic increase. As discussed in ref. 19, the light

intensity decrease may be interpreted as a pure melting of the hydrobarbon chains, and the increase as a fusion and/or aggregation of the LPS vesicles. The latter process superimposes the intensity decrease caused by the chain-melting process partially for the 22 °C- and completely for the 37 °C-preparation. Whether the vesicle fusion or aggregation process is connected with a simultaneous transition into a non-lamellar (e.g. inverted hexagonal) structure, cannot be answered unequivocally at present.

As divalent cations are known to play an important role for the stability of the LPS in the outer membrane (ref. 1), investigations were performed with LPS preparations under the influence of Mg^{2+} (data not shown). From DSC measurements it can be taken that for a 1:1 molar LPS R595 : Mg^{2+} sample - prepared below Tc - in the first scan no change of the endotherm was visible as compared to Mg^{2+}-free preparations, while in the second scan a broadening to lower temperatures (but only a slight reduction of Tc) connected with a higher enthalpy value could be observed (1st scan: 27.2, 2nd scan: 33.9 kJ/mol). When repeating the measurement after cooling to 15 °C, the broadening was maintained, after cooling to 0 °C or lower, however, the original endotherm of the 1st scan was retained. This behaviour runs parallel to characteristic changes in the light scattering behaviour from which Mg^{2+}-induced structural changes of the supramolecular assembly may be deduced. In this respect, further investigations are in progress. It can be stated generally - parallel to IR-spectroscopic investigations (ref. 20) - that divalent cations (especially Mg^{2+} and Ca^{2+}) have a stronger influence on LPS with a lower saccharide content (e.g. from Re- and Rd-mutants) as on LPS for example from Ra-mutants and S-forms. Thus, the calorimetric endotherms of a $1.4 \cdot 10^{-3}$ M preparation of LPS R60 with added Mg^{2+} up to a concentration of 10^{-2} M are identical in all scans (not shown). This should be due to the fact that the anionic character of LPS increases in the presence of a higher amount of saccharides which usually runs parallel with an increasing number of phosphate groups. Therefore, divalent cations may be taken up in molecular regions far from the lipid A moiety which reduces their influence on the 3-dimensional structure of the lipid assembly.

In fig. 5, the thermotropic phase behaviour of free bisphosphoryl lipid A from E. coli is illustrated with IR-spectroscopy

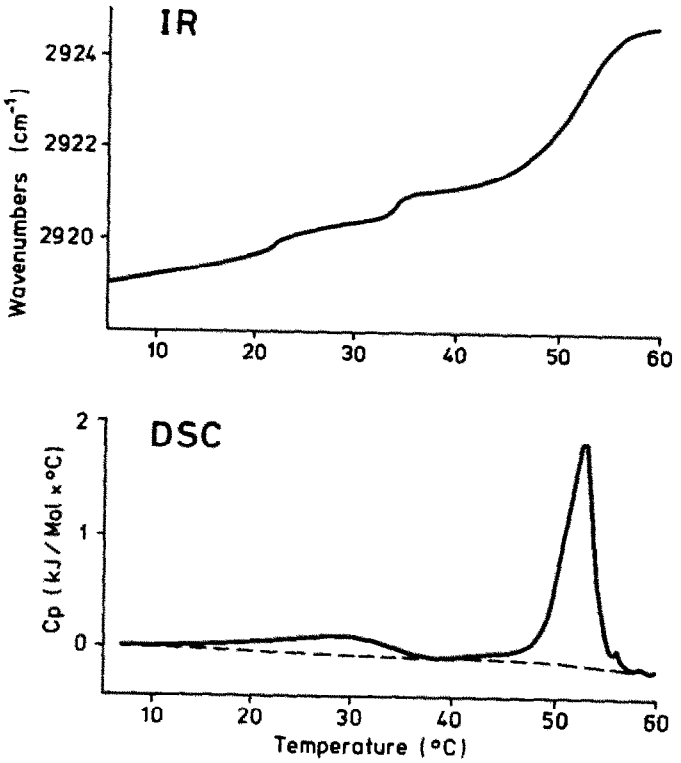

Fig. 5. Peak position of the antisymmetric stretch of the methylen groups $\nu as(CH_2)$ and specific molar heat versus temperature for a $2 \cdot 10^{-3}$ M bisphosphoryl free lipid A preparation from E. coli. In further scans, the "pretransitions" in the temperature range 18 to 36 °C tend to disappear.

(peak position of the antisymmetric stretch $\nu as(CH_2)$ of the methylen groups) and DSC (specific molar heat). The small transitions around 23 and 34 °C, which were already analysed by Naumann et al (ref. 21), correspond to a broad and slow enthalpy change in the temperature range 17 to 36 °C disappearing in further scans. It is noteworthy on the one hand that the main phase transition temperature Tc for this batch was significantly higher as measured earlier for other batches (Tc 37 to 47 °C, see ref. 4,19,21 and unpublished results) which is true also for the values of the enthalpy change (25 to 29 kJ/mol as compared to 10 to 15 kJ/mol measured earlier, ref. 4 and 21). On the other hand, the enthalpy change for synthetic E. coli-lipid A with a molar value of app. 54 kJ is even much higher than for natural lipid A, and the analysis of different batches of the synthetic compound,

for which differences in the chemical structure can be excluded, shows also variations in Tc (in the range 42 to 52 °C). As various physical techniques like X-ray diffraction, 90°-light scattering, IR spectroscopy, and calorimetry presented here and elsewhere (ref. 20-22) unequivocally show that natural and synthetic lipid A behave identically, and this is true also with respect to the expression of biological activity (ref. 23), it can be excluded that the heterogeneity of the natural compound (ref. 24) or its chemical pretreatment are responsible for the variations of the above mentioned parameters. Rather it may be concluded that the geometry within the lipid A molecule, e.g. the cross-sections of the hydrophilic and hydrophobic portions, allow strong variations and thus permit the formation of quite different supramolecular structures which could be induced by even minor variations in the preparation procedure. This is in agreement with newer results from IR orientational data from which the existence of ordered (i.e. lamellar) as well as unordered (isotropic) structures for the same batch were deduced (ref. 20).

In fig. 6 calorimetric endotherms are plotted for several LPS from rough mutants and wild-type strains. Similar as observed elsewhere for the behaviour of R- and S-form LPS from S. minnesota (ref. 4), the phase transition temperatures Tc for R-form LPS are usually lower than those for S-form LPS. Regarding the ΔH-values, the molar enthalpy of the phase transition of LPS are approximately equal or slightly higher than comparable phospholipids like dimyristoyl-phosphatidylcholine or -phosphatidylethanolamine. Since the fatty acid content of the LPS molecule on the average is more than 3-times higher than that of the corresponding phospholipid molecule, at least a factor of 3 for the heat of melting is lacking. To prove the hypothesis that LPS is partially molten already below Tc (ref. 25), DSC-measurements with LPS from S. minnesota mutant strains R595 and R4 were done in the low temperature range beginning at -30 °C to detect a possible 2nd transition. However, unless it was masked by the strong water-ice transition between -3 and 8 °C, no further transition could be observed. From the evaluation of the peak positions of some IR active bands, e.g. νs and $\nu as(CH_2)$, which are assumed to be a measure for the membrane fluidity, also no definite decision is possible: A preliminary analysis including also lipid A analogues (kindly provided by Dr. T. Shiba, University of Osaka,

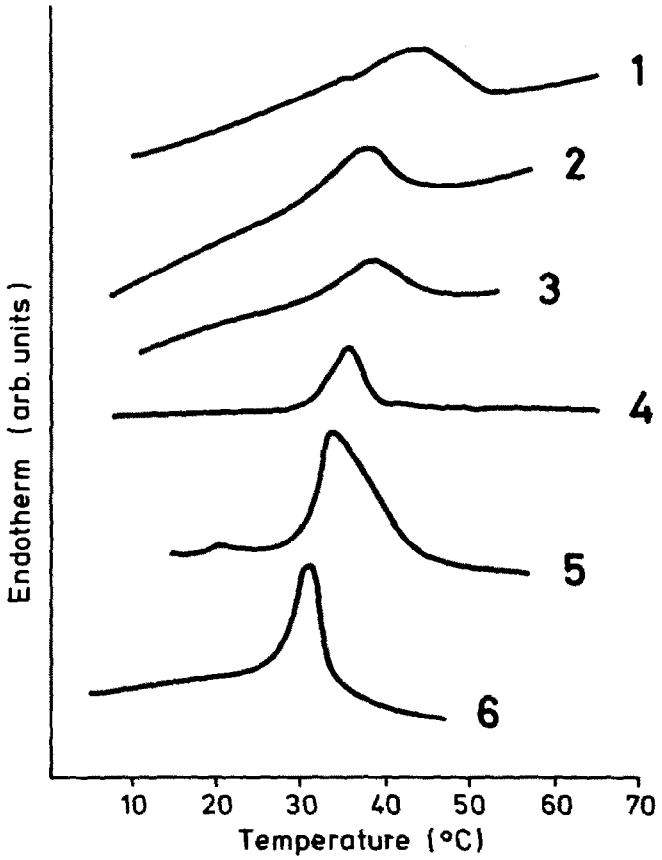

Fig. 6. Calorimetric endotherms for various R- and S-form lipopolysaccharides. For the calculation of the enthalpy changes, the molecular weight of S-form LPS is assumed to be 20000. (1) LPS S-form from Salmonella minnesota, ΔH = 46.2 kJ/mol, (2) LPS S-form from Serratia marcescens, ΔH = 28.5 kJ/mol, (3) LPS S-form from Salmonella abortus equi, ΔH = 27.6 kJ/mol, (4) LPS Ra-mutant R60 from Salmonella minnesota, ΔH = 24.1 kJ/mol, (5) LPS Re-mutant from Proteus mirabilis, ΔH = 52.7 kJ/mol, (6) LPS Re-mutant R595 from Salmonella minnesota, ΔH = 39.3 kJ/mol. For this particular LPS, different batches were investigated showing fluctuations of the ΔH-values in the range 25 to 40 kJ/mol.

Japan) shows that the compounds with pure acyl chains (only C:14) have significantly lower frequencies than the other - natural and synthetic - compounds containing C14-OH substituents or even double esters. From this fact a higher fluidity of free lipid A and LPS below Tc as compared to the gel state of phospholipids could be concluded confirming the above hypothesis. However, it

cannot be excluded that merely the presence of 3-OH fatty acids might lead to the observed shift in the frequencies despite unchanged fluidity of the molecular assembly. Therefore, further experiments are in progress with fluorescence and IR polarisation to elucidate this question.

From fig. 6 and other data (U. Seydel and K. Brandenburg, unpublished results) it can be taken that the respective Tc-values for the R-form and S-form LPS from different species lie close together, whereas the enthalpy changes ΔH fluctuate considerably. This might be explained on the one hand by the fact that biological samples have varying chemical structures and on the other hand - similar as for free lipid A - by variations of the supramolecular structure especially for Re-LPS - also of the same batch - in agreement with observations made with IR orientation measurements. Furthermore, the ΔH-values are dependent on the lipid:water ratio (lyotropism), at higher lipid concentration the enthalpy change decreases, e.g. for LPS from Rd1 at a concentration of 30 % per weight in excess water the ΔH-value is reduced by 50 % as compared to highly physiological conditions (>90 % water). This shows that beyond the complete hydration (30 to 50 % water content) of the LPS assembly the presence of excess water contributes to the observed enthalpy change, which correlates with the fact, that also Tc is concentration-dependent, i.e. Tc decreases with increasing water content (ref. 20). From this, a significant dependence of the physical structure on the water concentration can be concluded which is important for the interpretation of data resulting from different techniques, but also with respect to the observed biological activity of these molecules.

CONCLUSIONS

Summarizing the results of the measurements for natural membranes, lipopolysaccharides, and phospholipid extracts, it can be stated that the outer membrane and LPS preparations have the highest transition ranges with the endpoint of the transition around the physiological temperature of 37 °C, while that of the cytoplasmic membrane and phospholipid preparations are broader and lie significantly lower. Thus, the high state of order and with that, the high permeability barrier (ref. 1)of the outer membrane at 37 °C due to the presence of the relatively homo-

geneous - with respect to its acyl moiety - compound LPS becomes understandable. Lowering the growth temperature of bacteria, in both natural membranes the transition ranges shift to lower temperatures - apparently to ensure those processes necessary for cell metabolism - as a result of changed biosynthesis of the acyl region of LPS and phospholipids. Again, the melting of the phospholipids takes place at significantly lower temperatures and in a broader range as that of the lipopolysaccharides.

The phase behaviour of lipopolysaccharides and free lipid A seems to be more complex than that of "normal" phospholipids like phosphatidylethanolamines. This can be deduced not only from the unusual shape of the 90°-light scattering measurements (fig. 4) indicating the appearance of processes other than pure chain-melting, but also from the small molar enthalpy changes from which a higher fluidity at temperatures below the phase transition temperature as compared to gel state phospholipids can be derived. The measurements clearly demonstrate that with decreasing chain-length of the polysaccharide portion (more rough mutant LPS) lipopolysaccharide and even stronger free lipid A preparations - especially under the influence of divalent cations - tend to show variations when forming supramolecular structures. This should be of utmost importance because the more rough mutant LPS - usually under physiological concentrations of Mg^{2+}, i.e. at a 10^{-3} M concentration as used in the present paper - exhibit the strongest activities in many biological test systems.

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