Biochimica et Biophysica Acta, 775 (1984) 225–238
Elsevier

BBA 72228

PHYSICAL ASPECTS OF STRUCTURE AND FUNCTION OF MEMBRANES MADE FROM LIPOPOLYSACCHARIDES AND FREE LIPID A

KLAUS BRANDENBURG and ULRICH SEYDEL

Forschungsinstitut Borstel, D-2061 Borstel (F.R.G.)

(Received February 22nd, 1984)

Key words: Membrane structure; Outer membrane; Lipopolysaccharide; Phase transition; Fluorescence; Differential thermal analysis

The physical structure of the lipid component of the outer membrane of Gram-negative bacteria, here mutants of *Salmonella minnesota*, was studied with different optical and calorimetric techniques on the bilayer and with film balance measurements on the monolayer system. Special emphasis was laid on the elucidation of the phase behaviour of lipopolysaccharides and its isolated lipid component, free lipid A, differing in the length of the polysaccharide moiety. All samples exhibit the phase transition gel-liquid crystalline of the hydrocarbon chains with T_c for free lipid A lying well above the growth temperature of the bacteria (37°C), while the mutant lipopolysaccharides show values in the range 30–37°C. The state of order of the hydrocarbon chains at 37°C is lowest for the deep rough mutant lipopolysaccharides. Thus, an explanation of the high sensitivity of the mutants Re and Rd against hydrophobic drugs is possible. The behaviour of several physical quantities, e.g. enthalpy and cooperativity of the phase transition indicates that free lipid A and, to a lower extent, also the deep rough mutant lipopolysaccharide (mR595) form inverted structures. From this a model is derived for the mechanism of incorporation of lipopolysaccharide into the outer leaflet of the outer membrane after its lipid and saccharide parts have been translocated separately from the cytoplasmic membrane.

Introduction

In contrast to the cell envelope of Gram-positive bacteria, Gram-negatives contain a second membrane, the outer membrane, located at the cell surface. Both membranes act as particular physical and functional barriers to protect the cell from or adapt it to environmental changes. The two membrane differ considerably in their lipid composition: while the inner membrane is symmetric and contains only phospholipids the outer membrane is asymmetric. Its inner leaflet is built up from phospholipids, the outer leaflet from lipopolysaccharides. The architecture of the outer membrane of Gram-negative bacteria has been the subject of recent reviews [1,2]. The objective of this paper was to investigate the special role of lipopolysaccharides in its barrier function.

Briefly, these lipopolysaccharide molecules consist of a covalently bound lipid component, called lipid A, which anchors the lipopolysaccharide in the membrane, and a polysaccharide moiety, the core and the O-chain (responsible for the O-antigen property). Polysaccharide-free, i.e. precipitated (or isolated) lipid A is termed free lipid A [3,4]. Phospholipids take care of their part in this barrier function as constituents of as well the cytoplasmic as of the outer membrane by regulating the fluidity, i.e. the mobility of the hydrocarbon chains, via

Abbreviations: PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PE, phosphatidylethanolamine.

the synthesis of different fatty acids with respect to length and degree of saturation [5,6]. In a normal physiological state most of the membrane phospholipids are in a liquid-crystalline state. Upon a change in, for instance, temperature, pH value, or upon the action of some drugs they may be transfered to a gel state which leads to a reduction in their permeability. Since a participation in the barrier function of the outer membrane can be assigned to the lipopolysaccharide, it is to be expected, that it shows similar phase behaviour (thermotropic phases with a gel-liquid crystalline phase transition).

However, although this assumption is somehow stringent and the respective behaviour of phospholipids was and is still subject of a great number of investigations, astonishingly little effort was spent on the elucidation of this topic. Furthermore, the few available data on lipopolysaccharides do not render a uniform description but are even contradictory. This refers mainly to differences in the observation of the gel-liquid crystalline transition and of the fluidity of the hydrocarbon chains of lipopolysaccharides [7–12].

With the present investigation (abstract in Ref. 13) an attempt was made to elucidate the sometimes contradictory statements by combining the results obtained from the application of independent techniques which should allow to comprehend information on the basic structure and the phase behaviour, especially on the phase transitions, of lipopolysaccharides under physiological conditions. Furthermore, the influence of variations in the length of the polysaccharide moiety (using different mutant-lipopolysaccharides) on the various physical parameters was studied. These results may be helpful for an understanding of the correlation between the different sensitivities against hydrophobic substances, the different biological activities, and the physical structures.

From the results a proposal for the 3-dimensional structure of lipopolysaccharide and free lipid A is made which implies a modification of existing models for the mechanism of the incorporation of lipopolysaccharide in the outer leaflet of the outer membrane.

The methods applied comprise various fluorometric techniques and differential thermal analysis with the bilayer system and film balance measurements with the monolayer system.

Materials and Methods

The optical and calorimetric techniques have been described in detail elsewhere [14]. Briefly, fluorescence measurements were performed on an Aminco-Bowman fluorescence spectrometer using *N*-phenylnaphthylamine as a probe to determine the temperature range of the phase transition and its midpoint, i.e. the phase transition temperature T_c, and the van't Hoff enthalpy ΔH_{vH}. Fluorescence polarisation measurements were done with diphenylhexatriene as a probe to get information on microviscosities and the state of order of the lipid molecules. The formation of closed lamellar structures (vesicles) was checked via the release of the fluorescent dye 6-carboxyfluorescein at the phase transition.

For the calorimetric measurements a heat-flow device described earlier [15] was used. The lipid samples were placed in the measuring cell and the measured (downward) endotherms were calibrated by direct comparison with fatty acid samples placed in the reference cell (upward endotherm) [14].

Monolayer measurements at the air-water interphase were performed with a film-balance from Krüss (Hamburg, F.R.G.) scanning the pressure-area curves at constant temperatures (isotherms). The monolayers were prepared by spreading a 10^{-3} M lipid solution in chloroform on a 0.1 M NaCl subphase. In some cases the pH of the subphase was varied by addition of suitable amounts of hydrochloric acid. Lipopolysaccharides with a higher amount of polysaccharide, being not soluble in chloroform, were dissolved in a mixture of phenol/chloroform/petroleum ether (2 : 5 : 8, by vol.). After spreading the sample (20 to 30 μl) on the interphase, the solvent was allowed to evaporate during 30 min before measurements. Control experiments with the latter solvent and with pure chloroform showed identical isotherms for phospholipids indicating that no solute was lost in the subphase.

The various lipopolysaccharides analysed originated from different mutants of the strain *Salmonella minnesota* (structures see Fig. 1) and were taken in their natural salt form [16]. The wild-type lipopolysaccharide (S form), the structure of which is not shown in Fig. 1, contains an additional saccharide moiety, the O-side chain,

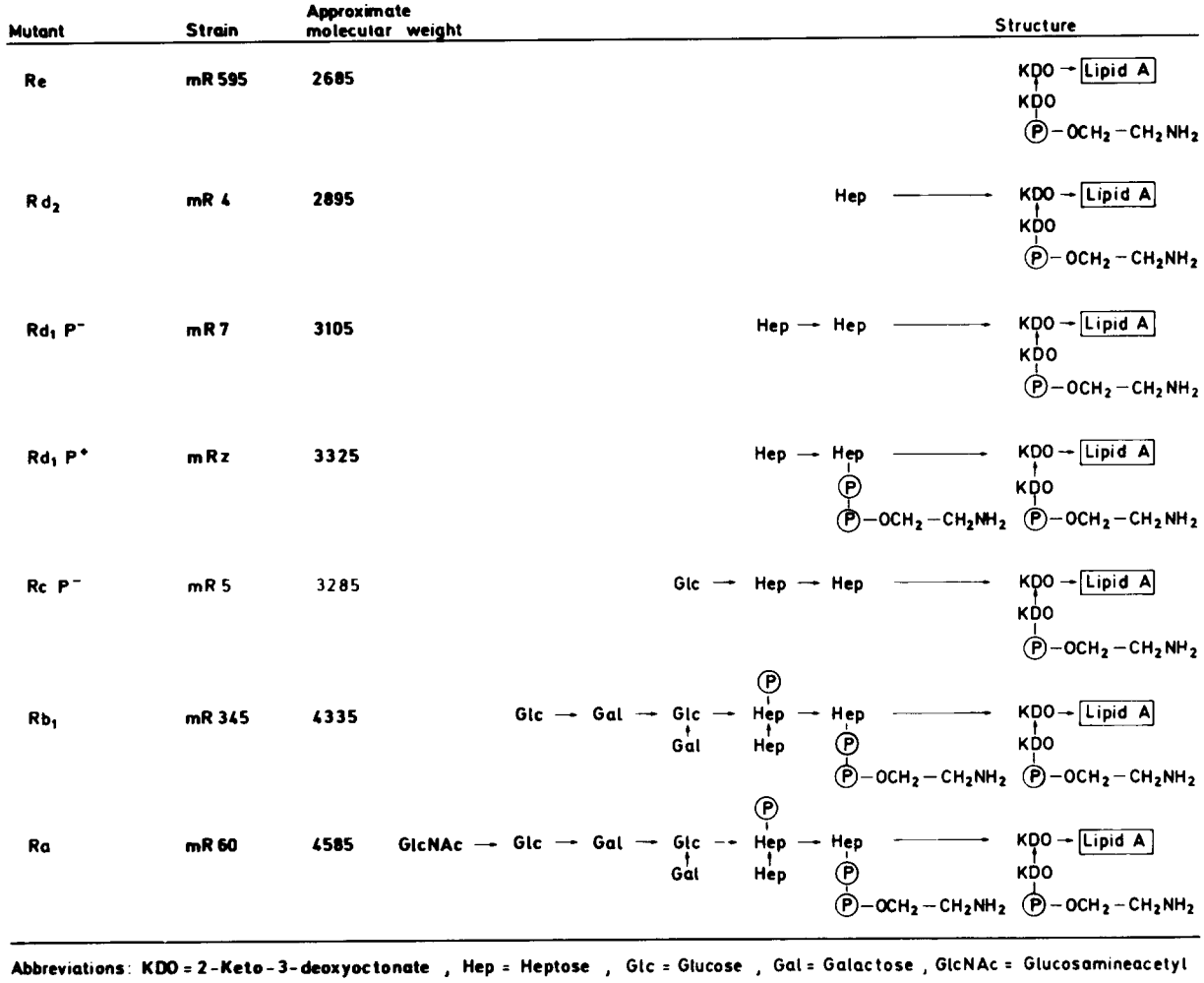

Fig. 1. Chemical structures of the defective lipopolysaccharides from the mutants Ra through Re of *S. minnesota* after [21]. The wild-type lipopolysaccharide additionally contains the covalently-linked O-chain essentially consisting of a varying number of repeating units of a pentasaccharide [17].

which is specific for each bacterial serotype. For *S. minnesota*, the composition of the O-side chain was determined by Lüderitz et al. [17].

The strains and the corresponding lipopolysaccharides have been described previously [18]. The smooth and the rough form lipopolysaccharides were isolated from bacteria either by the phenol-water method [19] or by the phenol/chloroform/light petroleum procedure [16,20].

Also, free lipid A's of *S. minnesota* were investigated (structure Fig. 2). It should be pointed out that lipid A's from different mutants of the same strain are identical in structure. However, the structure of lipid A as illustrated in Fig. 2 is not homogeneous in respect to fatty acid composition and phosphate content (dashed lines in Fig. 2). For example, in approx. 50% of the lipid A molecules of *Salmonella*, the phosphate group linked to the glucosamine I (on the right side in Fig. 2) is substituted by a phosphorylethanolamine residue [21].

Lipid A was isolated from lipopolysaccharide usually by acid hydrolysis, purified, and converted to the triethylamine-(TEN), in some cases also to

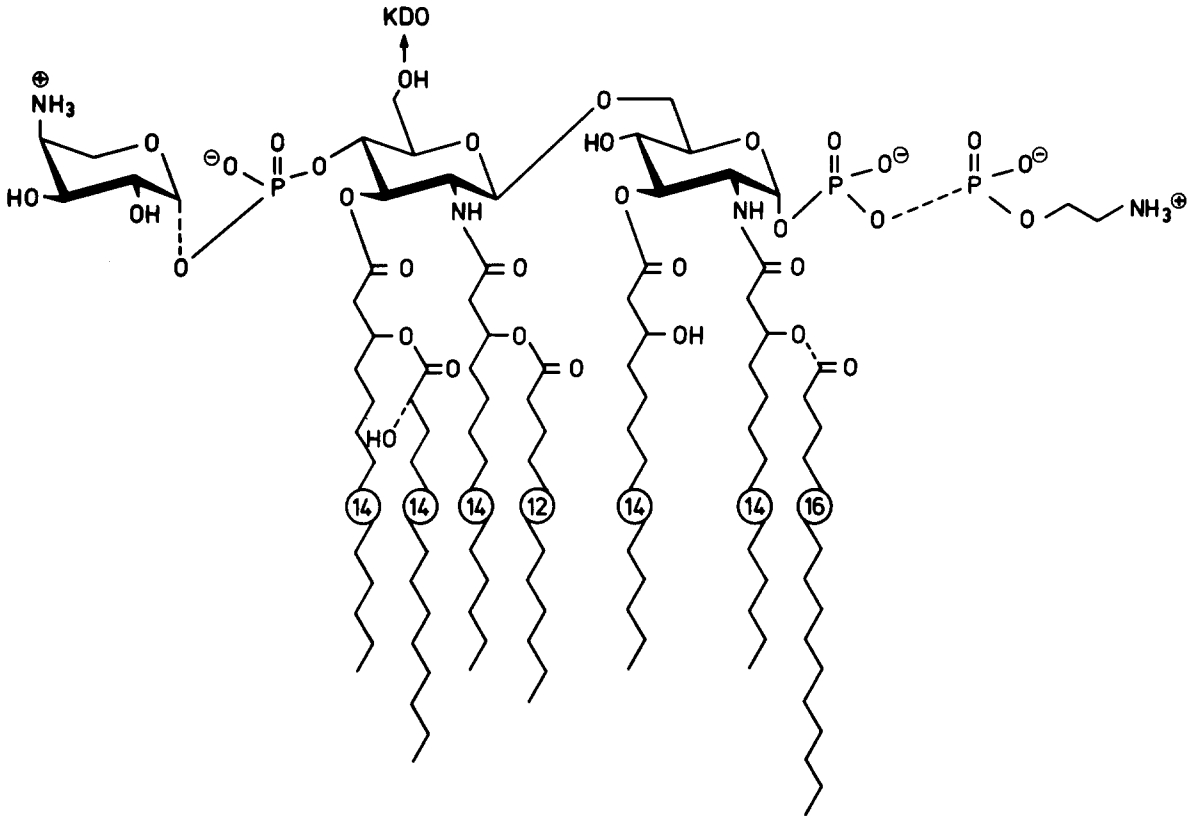

Fig. 2. Structure of lipid A of *S. minnesota*. The dashed lines indicate that the respective fragments are only partially synthesized by the bacterial cell ('microheterogeneity') [21].

the Na-salt form [16,22]. The various hydrolysis techniques differ in the degree of cleavage of 2-keto-3-deoxyoctonate and organic phosphate, HF- and HCl-treatment being more effective than acetic acid. Here, mostly the HF-treatment was applied.

Beside free lipid A, the compound lipid A (OH^-) was investigated which was obtained from free lipid A by NaOH treatment leading to the cleavage of the ester bound fatty acid residues and leaving only the two amide-bound acyl residues.

Dipalmitoyl-L-α-phosphatidylcholine (DPPC) as 'standard' phospholipids was purchased from Sigma (Munich, F.R.G.) and used without further purification, and the preparation of the lipid vesicles was done as described previously [23].

From the optical data (with *N*-phenylnaphthylamine) and the calorimetric data from bilayers the temperature T_c and the enthalpies ΔH_{cal},

$$\Delta H_{vH} = 4 \cdot R \cdot T_c^2 \left(\frac{d\theta}{dT}\right)_{T=T_c}$$

(R gas constant, θ ratio of melted to non-melted lipid) of the gel-liquid crystalline phase transition can be calculated as well as the cooperativity of the phase transition $\xi = \Delta H_{vH}/\Delta H_{cal}$.

From fluorescence polarisation measurements the 'polarisation' $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$ and the 'anisotropy' $r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$ can be determined. In addition to the equations presented in Ref. 14, the relation between r_∞ (residual fluorescence anisotropy for $t \to \infty$) and r was approximated via a least square fit of a polynom of 3rd order using the 81 empirical values from artificial and biological membranes collected by Van

Blitterswijk et al. [24]. The fit yields

$$r_\infty = -0.03r + 5.94r^2 - 8.47r^3$$

From that, the order parameter

$$S = (r_\infty / r_0)^{1/2}$$

($r_0 = 0.362$ for diphenylhexatriene) was calculated.

The values for S of all measured lipid systems turned out to deviate by maximal 1% from those calculated with the aid of the empirical relations presented by Pottel et al. [25] (especially Equation 14) using experimental values of 125 membrane systems.

The relative error was calculated according to

$$\Delta S/S = 0.6(1 - r/r_o)^2$$

containing all empirical values at a 90% confidence level. An estimate of the microviscosity, derived from the Perrin equation, is given by

$$\bar{\eta} = \bar{\eta}_{app} - \frac{2.4 \text{ poise} \cdot r_\infty}{r_0 - r_\infty}$$

$$\bar{\eta}_{app} = \frac{2P}{0.46 - P}$$

is the approximation of Shinitzky and Barenholz [26] resulting from the Perrin equation by neglecting r_∞. The error can be estimated to $\Delta\bar{\eta} = 2.9\ (r/r_0)^2$ at a 90% confidence level.

The monolayer measurements were evaluated by means of the two-dimensional Clausius-Clapeyron equation [27]

$$\Delta Q_c = T_c(A_e - A_c) \cdot \frac{d\pi_c}{dT}$$

to determine the latent heat ΔQ_c of transition.

A_e and A_c are the areas of the expanded and condensed phase, respectively, π_c the pressure at which the condensation occurs. A_e and A_c are determined from the intersections of the tangent of the extrapolated isotherm in the condensed phase and from the tangent of the isotherm at the beginning of the phase transition, respectively, with the tangent at $\pi = \pi_c$.

Results

For a better understanding of the results obtained for free lipid A or the various mutant lipopolysaccharides they are compared with the respective results obtained for DPPC with each method. In Fig. 3 the slopes of the fluorescence intensities dI_{425}/dT versus temperature obtained from free lipid A, lipopolysaccharides of wild-type (S form) and mutants mR595, mR60 are given and compared with that of DPPC. All preparations (labeled with the fluorophore *N*-phenylnaphthylamine) show pronounced phase transitions which can be assigned to the gel-liquid crystalline transition of the hydrocarbon chains in accordance to the main transition of DPPC at approx. 41 °C. Furthermore, free lipid A exhibits two additional transitions at approx. 30 and 40 °C. The former could be explained by assuming a small fraction of a lipid A-2-keto-3-deoxyoctonate compound corresponding to the lipopolysaccharide mR595 left in the free lipid A preparation after acid hydrolysis for cleaving 2-keto-3-deoxyoctonate [22].

This possibility is confirmed by results obtained from mixtures of free lipid A and lipopolysaccharide mR595 leading to more or less pro-

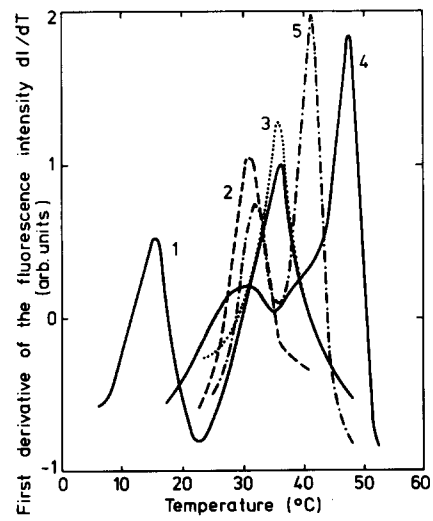

Fig. 3. First derivative of the fluorescence intensity after temperature dI_{425}/dT versus temperature T for free lipid A (4) and the lipopolysaccharides from wild-type (1) and mutants mR595 (2) and mR60 (3) as compared to DPPC (5). Fluorophore: *N*-phenylnaphthylamine.

nounced fluorescence increases at both transition temperatures (not shown) of as well lipid A as lipopolysaccharide mR595 depending on the mixing ratios. These findings,at the same time, strongly suggest that a mixture of these two lipids does not result in a homogeneous phase but leads to separate domains.

Whether the slightly pronounced transition in the free lipid A preparation at approx. 40 °C can be assigned to a $L_{\beta'} \rightarrow P_{\beta'}$ transition observed in DPPC at 32–36 °C [9,28] can at present not be decided definitely.

Interestingly, the lipopolysaccharide S form gives rise to two pronounced maxima which can in no way be explained by contaminations with other lipid components. While the second maximum can be assigned to the main phase transition of the hydrocarbon chains, the first maximum could originate from a conformational transition in the O-chain. This is backed by the observation that a respective peak cannot be observed for all the other lipopolysaccharide preparations, which differ from the S form in that they are missing the O-chain and, furthermore, by the results from fluorescence polarisation measurements with diphenylhexatriene (Fig. 4) giving no indication for the occurrence of the phase transition at approx. 16 °C. Due to its non-polar behaviour diphenylhexatriene, in contrast to *N*-phenylnaphthylamine, is a marker exclusively of the hydrocarbon moiety.

Also from Fig. 3 and from Table I, it can be taken, that only for the deep rough mutant lipopolysaccharides (especially mR595, mR4) the phase transition is completed at the physiological (growth) temperature of 37 °C, whereas the more smooth mutant lipopolysaccharides are partially in the gel state at this temperature. Interestingly, for the free lipid A a transition well above 37 °C is found.

Fig. 4 presents a plot of the fluorescence polarisation P versus temperature T for various mutant lipopolysaccharides and free lipid A in comparison with the respective measurement for DPPC. Obviously, the difference between the values for P in the gel and in the liquid-crystalline states, respectively, is highest for free lipid A and decreases with increasing length of the polysaccharide moiety. Also, from this figure the phase transition temperature can be taken. In Table I these values are summarized and compared with the respective data from the other technique applied. The order parameter S (calculated for 37 °C from the fluorescence polarisation data in dependence on the molecular weight (Fig. 5)) shows the highest value for free lipid A, and after traversing a minimum for the deep rough mutant lipopolysaccharides increases to the smooth mutant lipopolysaccharides again. The calculation of the approximation for the microviscosity $\bar{\eta}$ (see Methods) yielded that this quantity should be used only in the liquid-crystalline state of the hydrocarbon chains, i.e. for anisotropy values $r < 0.13$ (or $P < 0.18$) the relations $\Delta\bar{\eta}/\bar{\eta} < \Delta S/S$ and $\Delta\bar{\eta}/\bar{\eta} < 0.5$ hold true. In a more ordered state S seems to be more adequate especially for high r values (see error bars in Fig. 5) because (as has been stated by Pottel et al. [25]) the fluorescence polarisation measurements with diphenylhexatriene give rather information on the packing than on the membrane viscosity. A pretransition at $T_c' = 40$ °C for the free lipid A is also indicated by depicting the slope of the order parameter $-(dS/dT)$ versus temperature in the transition region (not shown).

Examples for measurements with differential thermal analysis are presented in Fig. 6, where scans of free lipid A and of the mutant lipopolysaccharide mR4 are compared with the scan of a DPPC sample. The scan of the free lipid A sample shows two endothermic peaks. The one at the

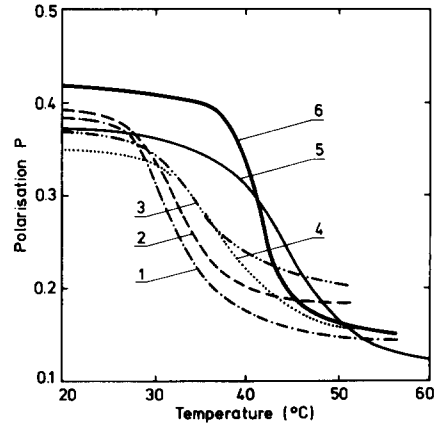

Fig. 4. Fluorescence polarisation P versus temperature T for free lipid A (5) and various lipopolysaccharides from mutants of *S. minnesota* mR595 (1), mR4 (2), mRz (3), mR60 (4) as compared to DPPC (6). Fluorophore: diphenylhexatriene.

TABLE I

PHASE TRANSITION TEMPERATURES T_c OF VARIOUS MUTANT-LIPOPOLYSACCHARIDES FROM *S. MINNESOTA* MEASURED BY DIFFERENT OPTICAL TECHNIQUES AND DIFFERENTIAL THERMAL ANALYSIS

n.v., no (closed) vesicles; –, not measured. The standard deviation for the measurement of T_c amounts to 0.5 deg. C due to variations between different samples.

Mutant lipopolysaccharides *S. minnesota*	T_c (°C)			
	from binding of *N*-phenyl-naphthylamine	from binding of diphenyl-hexatriene	from release of 6-carboxy-fluorescein	from differential thermal analysis
Lipid A TEN form	46.5	45.0	n.v.	47.5
Lipid A (OH^-)	34.0... 36.0	–	38.0	–
Lipopolysaccharides from mutants				
mR595	32.0	31.5	n.v.	30.0
mR4	29.5	33.0	(31.0) [a]	29.5
mR7	33.0	–	32.0	33.0
mRz	33.5	35.0	–	33.0
mR5	34.0	33.5	34.5	34.0
mR60	36.5	37.0	38.0	36.0
S form	37.0	38.0	38.5	38.5

[a] only slight increase in I_{520}.

higher temperature can be assigned to the gel-liquid crystalline transition and the one at the lower temperature again to a possible contribution of a 2-keto-3-deoxyoctonate-containing portion. Furthermore, beside for free lipid A and lipopolysaccharide mR595, the phase transition enthalpy (Fig. 7) is independent of the length of the polysaccharide side chain indicating that for these lipopolysaccharides the polysaccharide does not influence the conformation of the lipid A moiety within the membrane preparations.

As described in Methods, from a combination of ΔH_{vH} (fluorometric measurement) and ΔH_{cal}

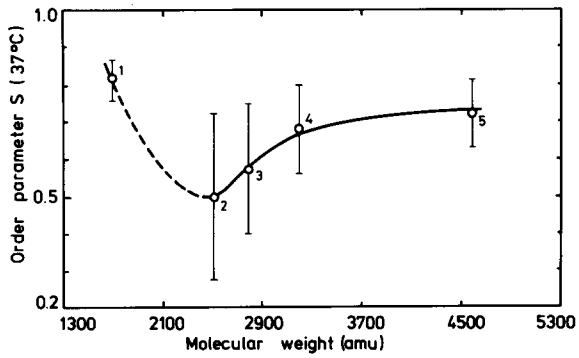

Fig. 5. Order parameter S (37 °C) versus molecular weight for free lipid A (1) and some lipopolysaccharides from mutants mR595 (2), mR4 (3), mRz (4) and mR60 (5). The error bars ($\pm \Delta S$) were calculated according to the formula presented in Methods.

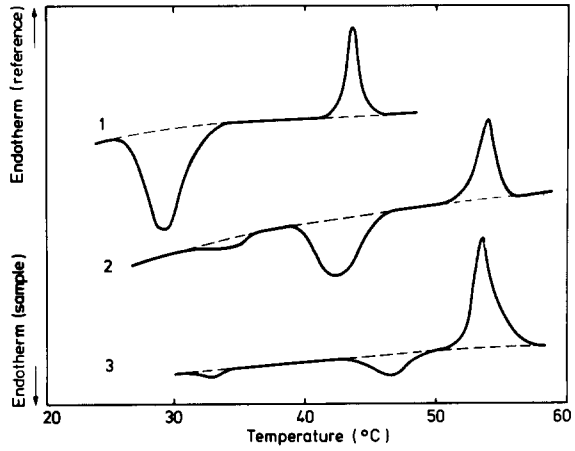

Fig. 6. DTA heating scans for free lipid A (3) and lipopolysaccharide of mutant mR4 (1) as compared to DPPC (2). The downward endotherms result from 47.3 mg lipopolysaccharide mR4 (1), 7.2 mg DPPC (2), and 23.6 mg free lipid A (3), respectively. The upward endotherms were obtained from 1.26 mg dodecanoic acid (1), 1.00 mg tetradecanoic acid (2), and 2.44 mg tetradecanoic acid (3), respectively.

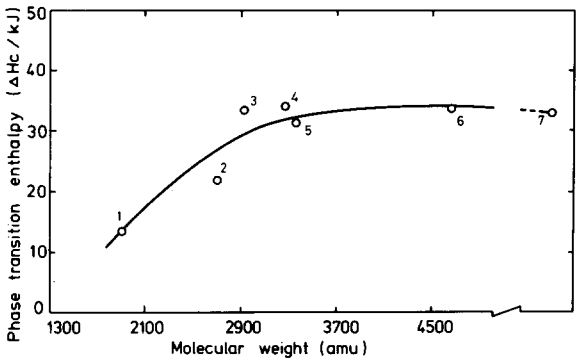

Fig. 7. Phase transition enthalpy ΔH_c versus molecular weight for free lipid A (1) and various lipopolysaccharides from mutants mR595 (2), mR4 (3), mR5 (4), mRz (5), mR60 (6), and wild-type (7). The value for the *S* form lipopolysaccharide was obtained assuming a molecular weight of 20000 amu. The standard deviation of the measurements amounts to approx. 10% due to variations between different samples.

(calorimetric scan) the cooperativity of the phase transition can be calculated and shows the highest value for free lipid A ($\xi = 26$) and decreasing values for the lipopolysaccharides with longer polysaccharide chains (lipopolysaccharide from mR595, $\xi = 15$, and from mR60, $\xi = 10$).

Interesting results were obtained from the fluorometric monitoring of the release of 6-carboxyfluorescein which usually starts at the beginning of fluidization after incorporation in closed lipid structures. Most of the preparations except those from the two lipid A forms (Na-salt- and TEN-form) and from the lipopolysaccharide mutant mR595 exhibit a steep increase in the fluorescence intensity I_{520} just before reaching T_c and should, therefore, form closed spherical structures. The mutant mR4 seems to be a border case showing, if any, only a slight increase in I_{520}.

In Table I the values for T_c obtained from the various techniques are listed showing satisfactory agreement. There seems to be no obvious deviation for the values of any single technique.

Fig. 8A gives pressure-area isotherms from film balance measurements with free lipid A (HF-treated) as compared to DPPC (Fig. 8B). Similar curves were obtained with some other mutant-lipopolysaccharide preparations, which showed, however, only slightly pronounced or even vanishing transitions.

As presented in Table II, monolayers were formed by all lipopolysaccharide preparations except those of the mutant mR60 and the wild-type (S form). This is in agreement with observations made earlier that native (smooth form) lipopoly-

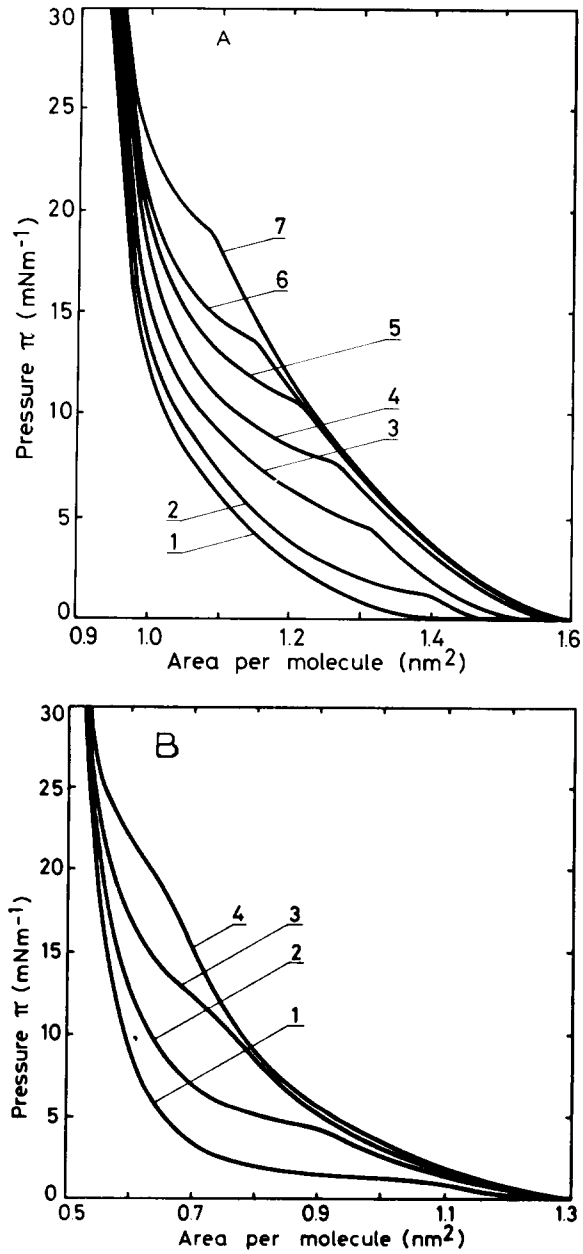

Fig. 8. (A) Monolayer isotherms for dephosphorylated lipid A of *S. minnesota*. (1) 9 °C, (2) 13 °C, (3) 20 °C, (4) 25 °C, (5) 30 °C, (6) 35 °C, (7) 45 °C. (B) Monolayer isotherms for DPPC. (1) 15 °C, (2) 22 °C, (3) 30 °C, (4) 37 °C.

TABLE II

THE LIMITING AREA PER MOLECULE AND THE TWO HIGHEST VALUES FOR THE HEAT OF TRANSITION ΔQ_c AND THE RESPECTIVE TEMPERATURES T CALCULATED FROM THE CLAUSIUS-CLAPEYRON EQUATION FOR DIFFERENT LIPID A's AND LIPOPOLYSACCHARIDES OF *S. MINNESOTA* AS COMPARED TO DPPC

Compound	Limiting area per molecule (ξ/nm^2)	ΔQ_c (kJ)	at T (°C)
DPPC	0.53	43.7	22
Lipid A [a]			
HAc treated	0.72	25.5	15
		18.1	20
HCl treated	0.92	33.7	15
		30.7	23
HF treated	0.90	29.7	20
		24.6	25
Lipid A (OH^-)	0.35	[b]	
Lipopolysaccharide			
from mutant mR595 [a]	1.05	43.9	10
		32.0	15
from mutant mR4 [a]	1.07	40.2	8
		23.0	11
from mutant mRz [a]	1.22	[b]	
from mutants mR5 [a]	1.18	[b]	
from mutant mR345 [a]	1.25	[c]	
from mutant mR60	no monolayer		
S form	no monolayer		

[a] In phenol/chloroform/petroleum ether (2:5:8, by vol.).
[b] Phase transition too weak for a reliable evaluation.
[c] No phase transition.

saccharide does not form monomolecular films [29]. It must be noted that the distinctness of the transition regions is significantly higher with a low phosphate content in the lipid A moiety.

The values calculated from these measurements for the limiting area per molecule ρ and the heat of transition ΔQ_c according to the two-dimensional Clausius-Clapeyron equation are summarized in Table II. It is worth mentioning that for free lipid A a decrease in the pH value from 7 to 2.9 led to a marked increase in ΔQ_c but left the ρ value nearly unchanged. A similar behaviour was observed for *Escherichia coli* lipid A. For this, in any case a significantly lower ΔQ_c-value was measured. This might be explained by the absence of a hexadecanoic fatty acid residue [30].

Furthermore, for the deep-rough mutant-lipopolysaccharide of mR595 and mR4, the temperature for which a maximum value of ΔQ_c was calculated according to the Clausius-Clapeyron equation are more than 10 °C lower than that for free lipid A in analogy to the values of T_c measured from the bilayer system. In a similar way, these compounds show a significantly higher ΔQ_c than free lipid A.

Discussion

From the presented results the following conclusions can be drawn.

1. Phase transition temperature T_c

All free lipid A- and lipopolysaccharide-preparations exhibit under physiological conditions, i.e. under high water content (> 99%) a phase transition, which can be assigned to the order-disorder transition $\beta \rightarrow \alpha$ of the hydrocarbon chains as measured with fluorometric techniques. The same transition can also be observed with a lower water content (80 to 95%) applying differential thermal analysis. The transition temperature T_c have in all cases (except for free lipid A) values around 37 °C or lower. For free lipid A, the T_c value is higher by several degrees, the absolute value depending on the content of organic phosphate and on 2-keto-3-deoxyoctonate not being cleaved by acid hydrolysis.

Also, in the monolayer system a phase transition corresponding to that of the bilayer system is observed. The T_c values calculated according to the Clausius-Clapeyron equation show a similar tendency: The free lipid A giving rise to highest and the mutant-lipopolysaccharides to lower T_c values.

The observation of a T_c well above 37 °C for free lipid A is consistent with the fact that this compound is no constituent of the membrane and that therefore no regulatory role can be assigned to this molecule. The amount of organic phosphate, mainly in position 1 of the reducing glucosamine part, and the amount of sugar strongly

influence some of the measured quantities (e.g. T_c, ΔQ_c). Even though at the moment no figures can be given for the absolute amount of phosphate and 2-keto-3-deoxyoctonate left after the various acid hydrolysis procedures it is known that the acetic acid treatment should produce free lipid A still containing considerable amounts of 2-keto-3-deoxyoctonate and phosphate. A comparison of the presented T_c values with data from literature could not be performed because up to now investigations in this respect have been published only for some wild-type lipopolysaccharides. Liu et al. [31] who investigated the effect of a special endotoxin (i.e. lipopolysaccharide mR595) on the phase transition of different phospholipids, did not comment the slight increase in fluorescence intensity of *N*-phenylnaphthylamine (presented in their paper in Fig. 1) at app. 33°C, which is possibly due to a transition of the lipopolysaccharide within a DPPC/lipopolysaccharide mR595 (1 : 8, molar ratio) mixture.

The few results for *E. coli* lipopolysaccharide (smooth forms) show variations of T_c between 22°C and 33.5°C [6–8]. Only very recently, however, Coughlin et al. [32] found two 'structural transitions' at 20 and 40°C for *E. coli* lipopolysaccharide measured with two ESR spin probes. The authors termed these transitions 'thermotropic' without specifying them more precisely, but possibly there is a connection to the two transitions of the S form lipopolysaccharide presented in Fig. 3.

Anyway, the different T_c values for lipopolysaccharides described in literature cannot be explained by different preparations or measuring techniques alone but indicate that still some confusion concerning an interpretation exists.

2. Fluidity and state of order

As can be taken from Fig. 4 differences in P and therefore in S (see Methods) nearly vanish at temperatures well below and above 37°C. Thus, a comparison of the 'state of order' has necessarily to be carried out at a fixed temperature. Contradictory statements in literature concerning the influence of the polysaccharide chain on the 'state of order', 'mobility', or 'fluidity' may be explained by the fact that this necessity has not been taken into consideration. Some authors found the state of order of the lipid A portion of the lipopolysaccharide to be independent of the length of the polysaccharide chain [2], others, however, found a higher state of order for a lipopolysaccharide bearing a longer polysaccharide portion [10,11,33]. Both statements could be brought into agreement if the former is assumed to be valid outside and the latter within the transition region.

The order parameter S (37°C) calculated from fluorescence polarisation measurements at a fixed temperature of 37°C (Fig. 5) increases with increasing length of the polysaccharide side chain. A comparison with free lipid A is inadequate because at this temperature it is still in the gel-state while the mutant-lipopolysaccharides are predominantly in the liquid-crystalline state. The higher fluidity of the deep-rough mutant lipopolysaccharides at $T = 37$°C as compared to the other lipopolysaccharide preparations is in agreement with results from Tamaki et al. [34] and Wilkinson et al. [35] who observed an increase of the sensitivity between Rc and Rd mutants. This behaviour was explained by Nikaido [36] with an increase in the phospholipid content in the outer membrane (first observed by Smit et al. [37]) due to a decrease in the lipopolysaccharide content. The present observations do not exclude this possibility but point out that the increasing fluidity of the lipopolysaccharides of the deep rough mutants might be responsible for their sensitivity.

3. Physical structure

For free lipid A, the fatty acid moiety is the determining portion for the lateral space requirement of the molecule within the membrane. This follows from a comparison of the limiting areas for free lipid A and lipid A (OH^-) which were measured by the monolayer technique to be 0.95 and 0.35 nm^2, respectively. From this and the fact, that with the addition of 2-keto-3-deoxyoctonate and increasing length of the polysaccharide side chain the limiting areas reach a maximum value of 1.25 nm^2 per molecule, it is concluded that, for the lipopolysaccharide of the more smooth mutants, the determining portion for the lateral space requirement is the polysaccharide moiety. Furthermore, from comparison of the quantities S (37°C) (Fig. 5), ΔH_c (Fig. 7), and ξ a clear variation between free lipid A and lipopolysaccharide mR4 and a relative invariability for all the other mutant

lipopolysaccharides can be deduced. It therefore seems most likely that between free lipid A and lipopolysaccharide mR595 on the one side, and between lipopolysaccharide mR595 and mR4 on the other side, there must occur structural modifications which can be explained by an increase in the cross-sectional area of the hydrophilic region and a more parallel orientation of the hydrocarbon chains by the apposition of additional sugar residues, especially with a negative charge (2-keto-3-deoxyoctonate).

As described in Results (Table I), except for lipopolysaccharide mR595 and free lipid A for all other lipopolysaccharide preparations closed structures were obtained as was shown by the release of 6-carboxyfluorescein.

For the formation of closed lamellar bilayer structures the effective cross-section of the hydrocarbon moiety must be lower than that of the hydrophilic head-group region. This assumption is confirmed by an estimation of the 'critical packing parameter' according to a formula given by Israelachvili and co-workers [38] $x = v/a \cdot l$ (v volume of the hydrophobic part, a area of the hydrophilic head group, l length of the acyl chain) yielding an x value for free lipid A well above 1. Therefore, it can be expected that free lipid A and lipopolysaccharide mR595 (at least partially) do not form lamellar but inverted structures such as 'lipidic particles' or spherical inverted micelles which transform to the hexagonal H_{II} phase in thermodynamic equilibrium [39]. Inverted structures, especially the H_{II} phase, have been observed for several phospholipids (e.g. phosphatidylethanolamines) and phospholipid mixtures [39–45].

The assumption of a transition to or within an inverted structure for free lipid A is supported by the results from differential thermal analysis and fluorescence polarisation measurements. The former yield markedly lower ΔH_c values for free lipid A and lipopolysaccharide mR595 as compared to the other lipopolysaccharide preparations. This can be explained by the lower number of interacting sites of the opposing methyl end groups. Similarly, the observed higher increase in the fluidity (Fig. 4) at the phase transition for the same substances may be explained. Furthermore, by assuming that, at least partially, the structures are of the H_{II} type, the measured higher cooperativity values for free lipid A and lipopolysaccharide mR595 can readily be explained by the cylindrical geometry leading to a more parallel alignment of the lipid molecules in one dimension.

Therefore, for an understanding of the observed phase transitions of free lipid A at $T_c = 46\,^{\circ}C$ and $T_c' = 40\,^{\circ}C$ (e.g. Fig. 3), it may be assumed for the former that it originates from a transition within an inverted structure, e.g. H_{II}; the latter in a transition between different structures, e.g. cubic-hexagonal (with cubic structure units similar to Schwarz's minimal surface [43]). This would be confirmed by the low values of the various measured quantities (see dI/dT in Fig. 3, dS/dT, and $H_c' < 1$ kJ).

It must be noted at this point that the presented results give strong evidence for the existence of inverted structures at least for free lipid A. This is confirmed also by the results of some electron micrographs. An absolute proof, however, cannot be given at present. Unfortunately, up to now there is no direct evidence for the existence of inverted structures such as 'lipidic particles' or 'inverted micellar intermediates' [39]. Even established methods such as the NMR technique may indicate isotropic or hexagonal phases, although other techniques definitely confirm the existence of only one lamellar phase [46].

The proposed structural models for free lipid A and lipopolysaccharides allow some conclusions on possible mechanisms for the translocation of the lipopolysaccharide molecule from its locus of biosynthesis in the inner half of the cytoplasmic membrane to its final destination in the outer leaflet of the outer membrane. Some existing models assume that from the cytoplasmic membrane a vesicle is released and subsequently fuses with the outer membrane at special sites [1,47]. A severe objective to this model seems to be the diameter of these vesicles of as large as 40 nm, which have to pass the periplasmic space inclusive the peptidoglycan layer.

Other models propose a direct fusion between the two membranes [1,47,48]. Here, a lipopolysaccharide molecule is assumed to be synthesized in or to diffuse to a special domain in the inner leaflet of the cytoplasmic membrane. Due to the structure in the site of fusion, in which a lipidic particle may be absent or present, the lipopolysac-

charide molecule then reaches, by two flip-flop mechanisms, first the area where the outer monolayer of the cytoplasmic membrane is connected with the inner monolayer of the outer membrane and, subsequently, the outer monolayer of the outer membrane [1]. In favour of this model is the fact, that these particular adhesion zones (Bayer's junctions) between the two membranes have been observed in several rod-shaped microorganisms [49,50]. A rather strong argument against this model is the fact that the time required for a flip-flop of lipid molecules for example for dimyristoyl PC is 80 min or under the influence of an exchange protein still 31 min [51]. The assumption of two flip-flop processes seems therefore to be rather unimaginable for example for bacteria like *E. coli* with generation times of about 20 min. As has been reported by Mühlradt and co-workers [48] the time after which newly synthesized lipopolysaccharide can be detected on the bacterial surface is as low as 30 s.

Considering these contradictions, the following modifications of the model are suggested:

Not the complete lipopolysaccharide but lipid A + 2-keto-3-deoxyoctonate molecules are synthesized in particular domains of the inner monolayer of the cytoplasmic membrane. These domains bulge towards the outer membrane (Fig. 9A), are finally strangulated, and form inverted structures within the region of existing Bayer's junctions (Fig. 9B). These structures are considerably smaller in diameter than the corresponding geometries built up from complete lipopolysaccharide molecules. Finally, the incorporation into the outer leaflet of the outer membrane and the attachment of the polysaccharide chains take place under the influence of a specific protein, which has been observed to interact in the outer membrane with the lipid A portion of lipopolysaccharide but only to a low amount with the complete lipopolysaccharide [52]. The polysaccharide moiety, which (according to this model) is added in a last step in the outlined process, might be translocated to the outer membrane by interaction with proteins which, too, are known to be transferred within the sites of membrane adhesion [53].

The proposed model is related to the 'direct fusion model' [47] but does not presume flip-flop processes. Furthermore, the model is confirmed by

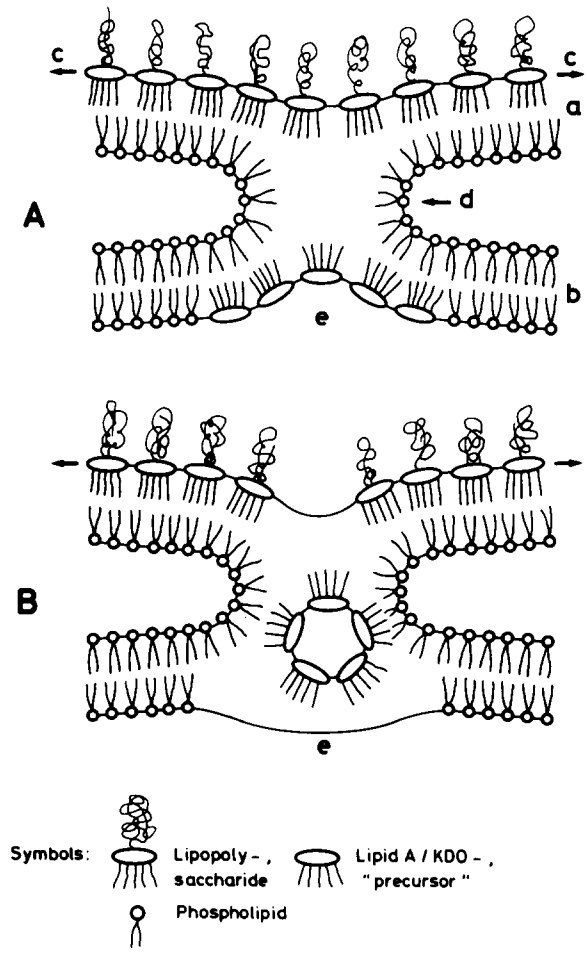

Fig. 9. Model for the translocation of a lipopolysaccharide 'precursor' into the outer membrane with subsequent attachment of the polysaccharide component (A) The first step is illustrated with lateral diffusing lipopolysaccharide molecules (c) in the outer membrane (a) at sites of adhesion (d) of outer and cytoplasmic (b) membranes. The lipopolysaccharide 'precursor' is biosynthesized in the inner leaflet of the cytoplasmic membrane (e). (B) The lipopolysaccharide 'precursor' in an inverted structure moves from the cytoplasmic towards the outer membrane while the gap in the inner leaflet of the cytoplasmic membrane is refilled with newly synthesized molecules (e). Finally, the cycle is completed by the incorporation of the 'precursor' into the outer leaflet of the outer membrane and the attachment of the polysaccharide component under the influence of a specific protein.

three observations.

(i) The rates of lipopolysaccharide production are identical for smooth and deep-rough mutants of Gram-negatives (e.g. mR595 and S form [37]).

This would mean identical rates of the insertion of the respective lipopolysaccharide into the outer monolayer of the outer membrane which can hardly be expected from the assumption of a translocation of the complete lipopolysaccharide molecules.

(ii) Osborn and co-workers [54] found the translocation to be unidirectional, i.e. an incompletely lipopolysaccharide, incorporated into the outer membrane under non-permissive conditions, was not completed following subsequent shift to permissive growth conditions. However, this irreversibility is (in contrast to the former models) just a particular demand of the model proposed in the present paper.

(iii) Osborn et al. [55] observed a very low translocation rate for a precursor of lipid A resembling closely lipid A (OH^-) structure described above. These authors assumed that for the effective translocation more complete lipid A-like structures should be necessary. This assumption again can readily be explained by the tendency of these lipid A-like molecules to form inverted structures which can be excluded for the lipid A (OH^-) molecules as described before.

In a recent paper Mulford and Osborn [56] suggest a mechanism in which the core-lipopolysaccharide and the O-chain are translocated separately to the periplasmic space where the attachment of the O-chain should take place. This assumption is deduced by the authors from ferritin labeling of the O-antigen chain at the exposed periplasmic face of the inner membrane. However, it has to be taken into account that the presence or absence of the lipid moiety of the lipopolysaccharide should not influence the antigenic properties of the O-chain.

Acknowledgement

The authors are indebted to Professor E.T. Rietschel and Dr. H. Brade for many helpful discussions especially regarding the chemistry of lipopolysaccharides and, not least, for supplying the various lipid A and lipopolysaccharide samples. They also thank Dr. B. Lindner for reading and Mrs. E. Hearn-Biernat for typing the manuscript and Mrs. M. Lohs for preparing the drawings.

References

1 Lugtenberg, B. and Van Alphen, L. (1983) Biochim. Biophys. Acta 737, 51–115
2 Rietschel, E.T., Wollenweber, H.W., Brade, H., Zähringer, U., Lindner, B., Seydel, U., Bradaczek, H., Barnickel, G., Labischinski, H. and Giesbrecht, P. (1984) in Handbook of Endotoxins (Proctor, R., ed.), Vol. 1, Chemistry of Endotoxin (Rietschel, E.T., ed.), Chapter 5, Elsevier, Amsterdam, in the press
3 Lüderitz, O. (1977) in Microbiology (Schlessinger, D. ed.), pp. 239–246, Am. Soc. Microbiol., Washington, DC
4 Rietschel, E.T., Hase, S., King, M.-I., Redmond, J. and Lehmann, V. (1977) in Microbiology (Schlessinger, D., ed.), pp. 262–268, Am. Soc. Microbiol., Washington, DC
5 Rottem, S., Markowitz, O. and Razin, S. (1978) Eur. J. Biochem. 85, 445–450
6 Nakayama, H., Mitsui, T., Nishikara, M. and Kito, M. (1980) Biochim. Biophys. Acta 601, 1–10
7 Emmerling, G., Henning, U. and Gulik-Krzywicki, T. (1977) Eur. J. Biochem. 78, 503–509
8 Van Alphen, L., Lugtenberg, B., Rietschel, E.T. and Mombers, C. (1979) Eur. J. Biochem. 101, 571–579
9 Tardieu, A., Luzzati, V. and Reman, F.C. (1973) J. Mol. Biol. 75, 711–733
10 Rottem, S. (1978) FEBS Lett. 95, 121–124
11 Rottem, S. and Leive, L. (1977) J. Biol. Chem. 252, 2077–2081
12 Van Alphen, L., Verkleij, A., Burnell, E. and Lugtenberg, B. (1980) Biochim. Biophys. Acta 597, 502–517
13 Brandenburg, K. and Seydel, U. (1983) Ann. Meeting of the Deutsche Gesellschaft für Biophysik, 10–12 Oct. 1983, Neuherberg, GSF-Bericht 5/83, ISSN 0721-1694
14 Brandenburg, K. and Seydel, U. (1983) Thermochim. Acta 69, 71–102
15 Brandenburg, K. and Meissner, J. (1980) Thermochim. Acta 40, 109–119
16 Galanos, C. and Lüderitz, O. (1975) Eur. J. Biochem. 54, 603–610
17 Lüderitz, O., Galanos, C., Risse, H.J., Ruschmann, E., Schlecht, S., Schmidt, G., Schulte-Holthausen, H., Wheat, R., Westphal, O. and Schlosshardt, J. (1966) Ann. N.Y. Acad. Sci. 133, 349–374
18 Lüderitz,O., Jann, K. and Wheat, R. (1968) Compr. Biochem. 26A, 105–228
19 Westphal, O., Lüderitz, O. and Bister, F. (1952) Z. Naturforsch. 7b, 148–155
20 Galanos, C., Lüderitz, O. and Westphal, O. (1969) Eur. J. Biochem. 9, 245–249
21 Lüderitz, O., Freudenberg, M.A., Galanos, C., Lehmann, V., Rietschel, E.T. and Shaw, D.H. (1982) Curr. Top. Membrane Transp. 17, 79–151
22 Brade, H., Galanos, C. and Lüderitz, O. (1983) Eur. J. Biochem. 131, 195–200
23 Seydel, U., Brandenburg, K., Lindner, B. and Moll, H. (1981) Thermochim. Acta 49, 35–48
24 Van Blitterswijk, W.J., Van Hoeven, R.P. and Van der Meer, B.W. (1981) Biochim. Biophys. Acta 644, 323–332

25 Pottel, H., Van Der Meer, W. and Herreman, W. (1983) Biochim. Biophys. Acta 730, 181–186
26 Shinitzky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367–394
27 Phillips, M.C. and Chapman, D. (1968) Biochim. Biophys. Acta 163, 301–313
28 Jacobsen, K. and Papahadjopoulos, D. (1975) Biochemistry 14, 152–161
29 Benedetto, D.A., Shands, J.W. and Shah, D.O. (1973) Biochim. Biophys. Acta 298, 145–157
30 Rietschel, E.T., Wollenweber, H., Russa, R., Brade, H. and Zähringer, U. (1984) Rev. Infect. Dis., in the press
31 Liu, M.S., Onji, T. and Snelgrove, N.E. (1982) Biochim. Biophys. Acta 710, 248–251
32 Coughlin, R.T., Haug, A. and Mc Groarty, E.J. (1983) Biochim. Biophys. Acta 729, 161–166
33 Burnell, E., Van Alphen, L., Verkleij, A., De Kruijff, B. and Lugtenberg, B. (1980) Biochim. Biophys. Acta 597, 518–532
34 Tamaki, S., Sato, T. and Matsuhashi, M. (1971) J. Bacteriol. 105, 968–975
35 Wilkinson, R.G., Gemski, P. and Stocker, B.A.D. (1972) J. Gen. Microbiol. 70, 527–554
36 Nikaido, H. (1976) Biochim. Biophys. Acta 433, 118–132
37 Smit, J., Kamio, Y. and Nikaido, H. (1975) J. Bacteriol. 124, 942–958
38 Israelachvili, J.N., Marcelja, S. and Horm, R.G. (1980) Q. Rev. Biophys. 13, 121–200
39 Siegel, D.P. (1984) Biophys. J. 45, 399–420
40 Singer, M. (1981) Chem. Phys. Lipids 28, 253–267
41 Seddon, J.M., Cevc, G. and Marsh, D. (1973) Biochemistry 22, 1280–1289
42 Hardman, P.D., (1982) Eur. J. Biochem. 124, 95–101
43 Larsson, K., Fontell, K. and Krog, N. (1980) Chem. Phys. Lipids 27, 321–328
44 Hui, S.W., Stewart, T.P., Yeagle, P.L. and Albert, A.D. (1981) Arch. Biochem. Biophys. 207, 227–240
45 Boni, L.T. and Hui, S.W. (1983) Biochim. Biophys. Acta 731, 177–185
46 Noggle, J.H., Marecek, J.F., Mandal, S.B., Van Venetie, R., Rogers, J., Jain, M.K. and Ramirez, F. (1982) Biochim. Biophys. Acta 691, 240–248
47 Osborn, M.J. (1979) in Bacterial Outer Membranes, Biogenesis and Functions (Inouye, M., ed.), pp. 15–34, Wiley-Interscience, New York
48 Mühlradt, P.F., Menzel, J., Colecki, J.R. and Speth, V. (1973) Eur. J. Biochem. 35, 471–481
49 Bayer, M.E. (1968) J. Virol. 2, 346–356
50 Bayer, M.E. (1979) in Bacterial Outer Membranes, Biogenesis and Functions (Inouye, M., ed.), pp. 167–202, Wiley-Interscience, New York
51 De Kruijff, B. and Van Zoelen, E.J.J. (1978) Biochim. Biophys. Acta 511, 105–115
52 Schweizer, M., Hindennach, J., Garten, W. and Henning, U. (1978) Eur. J. Biochem. 82, 211–217
53 Smit, J. and Nikaido, H. (1978) J. Bacteriol. 135, 687–702
54 Osborn, M.J., Gander, J.E. and Parisi, E. (1972) J. Biol. Chem. 247, 3973–3986
55 Osborn, M.J., Rick, P.D. and Rasmussen, N.S. (1980) J. Biol. Chem. 255, 4246–4251
56 Mulford, C.A. and Osborn, M.J. (1983) Proc. Natl. Acad. Sci. USA 80, 1159–1163