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A comment on the preparation of liposomes from and on the $\beta \leftrightarrow \alpha$ acyl chain melting behaviour of rough mutant lipopolysaccharide

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We would like to comment on the investigations of Vaara, M., Plachy, W.Z. and Nikaido, H. (Vaara, M. et al. (1990) Biochim. Biophys. Acta 1024, 152-158) on the partitioning of hydrophobic probes in lipopolysaccharide bilayers. These authors reported that they did not succeed in preparing closed vesicles (liposomes) from rough mutant lipopolysaccharide. We describe the conditions under which lipopolysaccharide liposomes are formed most readily. We, furthermore, summarize data which strongly support the existence of thermotropic phase transitions of lipopolysaccharides (with transition temperatures lying in the range of 30-36°C) contradictory to Vaara et al. who argue that such transitions are artefacts. Exemplary measurements of the $\beta \leftrightarrow \alpha$ acyl chain melting for lipopolysaccharide from *Escherichia coli* deep rough mutant (strain F515) as compared to synthetic and natural phospholipids are presented using fluorescence spectroscopy, Fourier-transform infrared spectroscopy and differential scanning calorimetry. These results unequivocally prove the necessity to perform experiments at 37°C for a determination of the outer membrane permeability under physiological conditions.

Vaara et al. [1] reported on experiments for the preparation of closed vesicles (liposomes) from *Salmonella* rough mutant lipopolysaccharides (LPS). The authors proposed that bilayers from these lipopolysaccharides could be stabilized at high temperatures, by the addition of divalent cations, and the use of very defective Re-type LPS. Considering these parameters, Vaara et al. stated that they still were not able to entrap hydrophilic substances, e.g., radioactive sucrose, and therefore concluded that LPS does not easily form closed vesicles.

Our experiments with various rough mutant lipopolysaccharides from *S. minnesota* and *Escherichia coli* show that spherical vesicles are most readily formed – usually also without, but particularly at low concentrations of Mg^{2+} (molar ratio $[LPS]:[Mg^{2+}] = 50:1$ to $5:1$) simply by applying the so-called swelling assay, i.e. essentially by swelling the dry lipid at least for several hours (better: for some days) in 50 to 60°C hot water with intermediate gentle shaking of the sample for a few seconds [2]. As an example of the large LPS vesicles obtained in this way, in Fig. 1 a micrograph under dark-field illumination of a $2 \cdot 10^{-3}$ M lipopolysaccharide preparation of Rd-lipopolysaccharide from *S. minnesota* strain Rz is shown. However, these large spherical structures are not necessarily closed. As shown previously [3], the entrapment of hydrophilic solutes like 6-carboxyfluorescein (CF) was successful – i.e. closed vesicles (liposomes) are formed – for all rough mutant LPS from *S. minnesota* except for deep rough mutant R595. In further experiments we found that CF may also be entrapped by Re-LPS, but only to a small extent. This was proven by a slight increase of the CF fluorescence emission intensity at 516 nm during long-term incubation at 37°C. The reduced ability of Re-LPS to form closed vesicles may be explained by its tendency – particularly under physiological conditions – to adopt non-lamellar cubic structures (unpublished data).

Our findings that rough mutant LPS may form closed vesicles are in accordance with those of Nixdorff et al. [4] who succeeded in entrapping ^{14}C-labelled saccha-

Abbreviations: LPS, lipopolysaccharide; NPN, *N*-phenylnaphthylamine; CF, 6-carboxyfluorescein; DPPC, dipalmitoyl phosphatidylcholine.

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rides like sucrose in liposomes from Rc- and Re-mutants also from *S. minnesota*.

The addition of higher amounts of Mg^{2+} (molar ratio [LPS]:[Mg^{2+}] < 1) to liposome preparations leads to their agglomeration into very large aggregates which tend to precipitate rapidly. This process is a result of the formation of large multilamellar stacks, which was proven by X-ray small-angle diffraction [5]. These aggregates, however, have a much lower surface/volume ratio than well-separated liposomes and thus the entrapment and, with that, the release of entrapped solutes into the outer medium is rather ineffective. These conditions are thus not favourable for proving the formation of closed vesicles.

Furthermore, although higher temperatures favour the formation of LPS-liposomes, once they are formed they are stable at room temperature for at least some days. Storage at room temperature or below is highly recommended for the following reason: rough mutant LPS from enterobacterial strains exhibit a gel (β) to liquid crystalline (α) phase transition of the hydrocarbon chains at 30 to 36 °C [6]; and it is known that the permeability of liposomes increases drastically with this phase transition [7].

The existence of a $\beta \leftrightarrow \alpha$ phase transition for lipopolysaccharides is a further point of controversy with the statements of Vaara et al. The authors assume "that the reported transitions are artefacts perhaps caused by the imperfect packing of LPS molecules". However, various groups including our own have provided numerous experimental data which unequivocally prove the existence of a $\beta \leftrightarrow \alpha$ phase transition in lipopolysaccharide and in free lipid A preparations [3,5,6,8–18]. In Fig. 2 experimental data are presented for a lipopolysaccharide preparation of *E. coli*-F515 (deep rough mutant Re) and, in some cases, compared with respective data of the synthetic phospholipid dipalmitoyl phosphatidylcholine (DPPC) and the natural *Salmonella*-type phospholipid mixture (consisting of 82% phosphatidyl ethanolamine, 17% phosphatidyl glycerol, and 2% cardiolipin) showing the acyl chain melting with fluorescence polarization utilizing the fluorophore diphenylhexatriene (a), with Fourier-transform infrared spectroscopy evaluating the shift of the peak position of the symmetric stretching vibration of the methylen groups (b), and with differential scanning calorimetry (c). In Fig. 2a, the reversibility of the phase transition of LPS is demonstrated expressing itself in a slightly lower temperature in the cooling scan than in the heating scan (scan rates in the range 0.5 to 2.0 °C), which is indicative for a cooperative process.

At approx. 42 °C for DPPC and 32 °C for LPS, a sudden decrease of fluorescence polarization (Fig. 2a) and an abrupt increase in the wavenumber of the peak position of the symmetric stretching vibration of the methylen groups (Fig. 2b) are observed. For DPPC it is generally accepted that the changes in these parameters around the phase transition temperature T_c can be attributed to a drastic increase in the conformational disorder of the acyl chains by the introduction of an increasing number of *gauche-conformers* substituting the ordered *all-trans* conformation of the hydrocarbon chains present at temperatures $T < T_c$. There seems to be no readily intelligible reason why this interpretation should not be valid to describe also the very similar changes in LPS.

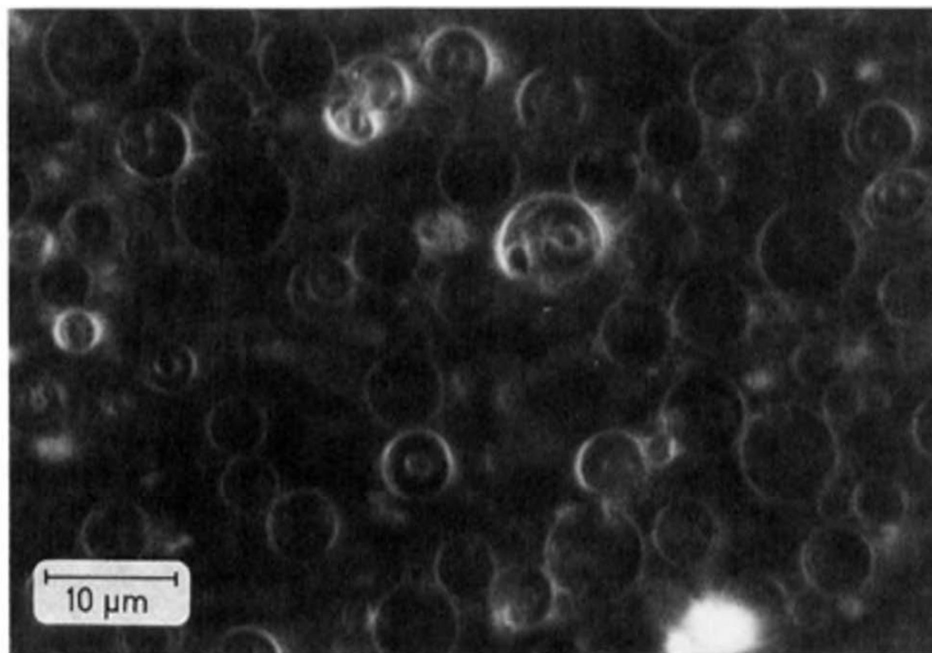

Fig. 1. Light micrograph of a liposome preparation from lipopolysaccharide of a Rd-mutant of *Salmonella minnesota* (strain Rz) observed under dark-field illumination.

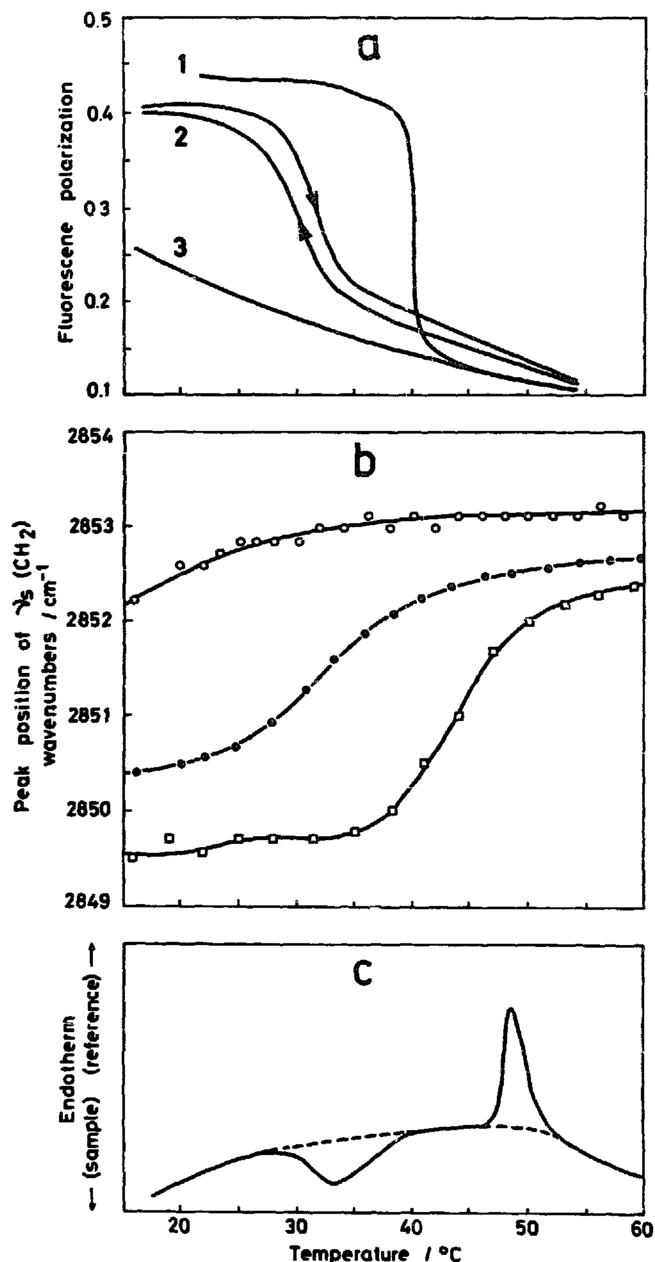

Fig. 2. Phase behaviour of a lipoplysaccharide preparation from *Escherichia coli* deep rough mutant F515 and, in some cases, also of synthetic and natural phospholipids in dependence on temperature monitored with three different techniques. The water contents of the LPS:water dispersions are >90% in each case. (a) Fluorescence polarization $P=(I_{\parallel}-I_{\perp})/(I_{\parallel}+I_{\perp})$, $I_{\parallel}$, $I_{\perp}$ intensities of the parallel and perpendicularly polarized light, respectively, for Re-LPS (2) as compared to dipalimtoyl phosphatidylcholine (1) and the natural phospholipid mixture from *Salmonella* (3). Fluorophore: diphenylhexatriene. (b) Peak position of the symmetric stretching vibration of the methylene groups $\nu_s(CH_2)$ for LPS (●) as compared to DPPC (□) and the natural phospholipid mixture (○). (c) Calorimetric endotherms of 8.0 mg lipopolysaccharide and of 2.5 mg dimyristoyl phosphatidic acid. From the known molar enthalpy change of the latter (26 kJ), the comparison of the peak areas yields a value of approx. 28 kJ/mol for LPS (see also Refs. 3 and 15).

The transition into a disordered state is connected with an enthalpy change typical for a first-order melting process. In Fig. 2c, this enthalpy change is compared for LPS with the respective behaviour of dimyristoyl phosphatidic acid, exhibiting the chain melting transition around 50 °C, which was chosen because of the close proximity of the transition ranges of LPS and DPPC.

Striking in this comparison is that infrared spectroscopy yields a significantly wider transition region than the other techniques. This is true also for other LPS and free lipid A preparations [6,14,16] and indicates that the vibrations of the methylene groups – beside the symmetric stretch also those of the antisymmetric stretch at 2920 cm^{-1}, the bending vibration at around 1467 cm^{-1}, the wagging progression bands in the range 1320 to 1180 cm^{-1}, and the strongest rocking progression band at 720 cm^{-1} – are the most sensitive markers of lipid order or disorder.

Vaara et al. claim "that the presence of transitions does not immediately indicate that the fluidity above the transition point is very high, and thus our data are not necessarily at conflict with these published results". However, when comparing the temperature dependence of the fluorescence polarization P or the peak position of $\nu_s(CH_2)$ and, with that, the state of order of the respective lipid assemblies in the temperature range 20 to 40 °C, for the natural phospholipid mixture a more or less continous decrease of the state of order over the entire temperature range takes place in contrast to the abrupt changes observed for LPS and DPPC at their phase transition temperatures. Particularly, at room temperature, the states of order show strong differences between the phospholipid mixture and LPS, but are only marginal at 37 °C. From these observations it follows that a determination of the membrane permeability under physiological conditions should only be performed at 37 °C.

These and other techniques – like fluorescence spectroscopy with anilinonaphthalensulfonate (ANS) and *N*-phenylnaphthylamine (NPN) [3,12], 90°-light scattering [13,15], small- and wide-angle X-ray diffraction [5,14] and monolayer techniques [3,6] – unequivocally reveal the existence of a phase transition of LPS lying in the range 30 to 36 °C for enterobacterial rough mutant, and at 36 to 42 °C for wild-type lipopolysaccharides, respectively [3,6]. Moreover, from the amphiphilic nature of LPS and lipid A, and from the fact that they carry nearly exclusively saturated fatty acid residues the existence of a relatively sharp $\beta \leftrightarrow \alpha$ transition should be expected. In particular, for the film balance measurements with monolayers at the air/water interface artefacts from 'an improper packing of the LPS molecules' can totally be excluded as an explanation for the observed melting transition.

It was shown that this phase transition cannot only be observed in samples with isolated LPS, but also in preparations of the outer membrane and of the cell envelope [8,15,18,19]. The temperature of this transi-

tion may have a considerable physiological relevance for the bacterial organism and, most likely, also for its endotoxic acitivity, i.e. its ability to interact with host cell membranes. For the biological activity of lipopolysaccharides and lipid A with varying length of the sugar side chain – corresponding to different T_c's – the fluidity of the acyl chains at 37 °C was shown to be an important parameter [6,20].

The reasons why Vaara et al. [1] or Nikaido et al. [21], respectively, could not detect any phase transition, might relate to the applied technique (ESR) and to the fact that Nikaido et al. used in their LPS preparations significant amounts of divalent cations (10 mM). As we have shown previously [12] – and this is in accordance with the observations of Emmerling et al. [8] – the phase transition of R-form LPS from *S. minnesota* could not – or only weakly – be detected with the fluorophore NPN in the presence of higher amounts of Mg^{2+}, although with calorimetry and infrared spectroscopy the existence of a $\beta \leftrightarrow \alpha$ phase transition could be unequivocally proven. Possibly, the partitioning of ESR probes or of fluorophores like NPN into LPS bilayers may be inhibited or masked at higher concentrations of Mg^{2+} or Ca^{2+}.

Also Labischinski et al. [22], in an earlier investigation, did not find any phase transitions for dry and – possibly – not fully hydrated samples of rough mutant and smooth form lipopolysaccharide from *Salmonella*. Later findings of the same group [17], however, were then in full accordance with the above cited investigations. These contradictory results can be explained by the extremely strong lyotropism of the phase behaviour of lipopolysaccharides [6], leading to a reduction or even abolishment of the changes in the parameters serving as probes for the phase transitions in the lower water concentration range. This is apparently connected with the fact that in LPS preparations free water exists only at water concentrations above 50%. Below this value the ice-water endotherm cannot be observed [6].

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