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Effects of temperature variation and phenethyl alcohol addition on acyl chain order and lipid organization in *Escherichia coli* derived membrane systems. A ^{2}H- and ^{31}P-NMR study

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Using ^{2}H- and ^{31}P-NMR techniques the effects of temperature variation and phenethyl alcohol addition were investigated on lipid acyl chain order and on the macroscopic lipid organization of membrane systems derived from cells of the *Escherichia coli* fatty acid auxotrophic strain K1059, which was grown in the presence of [11,11-$^{2}H_2$]oleic acid. Membranes of intact cells showed a gel to liquid-crystalline phase transition in the range of 4–20°C, which was similar to that observed for the total lipid extract and for the dominant lipid species phosphatidylethanolamine (PE). Phosphatidylglycerol (PG) remained in a fluid bilayer throughout the whole temperature range (4–70°C). At 30°C acyl chain order was highest in PE, followed by the total lipid extract, PG, intact cells, and isolated inner membrane vesicles. Acyl chain order in *E. coli* PE and PG was much higher than in the corresponding dioleoylphospholipids. *E. coli* PE was found to maintain a bilayer organization up to about 60°C, whereas in the total lipid extract as well as in intact *E. coli* cells bilayer destabilization occurred already at about 42°C. It is proposed that the regulation of temperature at which the bilayer-to-non-bilayer transition occurs may be important for membrane functioning in *E. coli*. Addition of phenethyl alcohol did not affect the macroscopic lipid organization in *E. coli* cells or in the total lipid extract, but caused a large reduction in chain order of about 70% at 1 mol% of the alcohol in both membrane systems. It is concluded that while both increasing temperature and addition of phenethyl alcohol can affect membrane integrity, in the former case this is due to the induction of non-bilayer lipid structures, whereas in the latter case this is caused by an increase in membrane fluidity.

Introduction

Membranes of *Escherichia coli* cells have a relatively simple phospholipid composition, which can easily be modified by making use of mutant strains (for review, see Ref. 1). Therefore, they are particularly suitable for investigation of biophysical properties of membranes in relation to membrane functioning. Studies using fatty acid auxotrophic mutant strains to alter the lipid acyl chain composition have shown that a number of functional membrane processes is sensitive to the physical state of the lipids [1–5]. One example is translocation of proteins across membranes, which is inhibited when the lipids are in a more ordered state [4,5].

In intact *E. coli* cells membane fluidity can be varied among other factors by changes in temperature [4,5] or by addition of drugs [6], such as phenethyl alcohol (PEA), which increases lipid fluidity and decreases lipid chain order [7,8]. In both cases membrane functioning is severely affected, as illustrated by the observation that exposure of cells to elevated temperatures as well as PEA addition results in translocation across the membrane of proteins that normally are not translocated [6,9]. This suggests that membrane in-

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Abbreviations: CSA, chemical shift anisotropy; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; EDTA, ethylenediaminetetraacetic acid; HPTLC, high performance thin-layer chromatography; PE, phosphatidylethanolamine; PEA, phenethyl alcohol; PG, phosphatidylglycerol; PIPES, 1,4-piperazinediethanesulfonic acid; POPE, 1-palmitoyl,2-oleoyl-*sn*-glycero-3-phosphoethanolamine.

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tegrity is affected by changes in lipid packing as a result of an increased fluidity. However, an alternative possibility is that these effects are caused by an increased tendency to form non-bilayer lipid structures. *E. coil* membranes are abundant in phosphatidylethanolamine (PE), which is a lipid that prefers organization in inverted (type II) non-bilayer structures [10,11]. These structures are believed to be involved as intermediates in functional membrane processes [11,12] and have been implied to play a role in protein-translocation [13]. Like membrane fluidity also the tendency to form non-bilayer lipid structures is generally enhanced by increasing temperature [10,11] and is promoted by PEA addition [14].

While previously some attention has been focussed upon lipid phase behaviour in *E. coli* systems [15–19], no consistent picture has emerged from these studies. The aim of the present study is to characterize lipid acyl chain order as well as polymorphic phase behaviour in *E. coli* cells and derived membrane systems. On one hand, this may serve as a basis for future experiments of which the objectives are to relate acyl chain order directly to functional membrane processes such as protein-translocation and on the other hand, it may lead to a further insight into the possible involvement of non-bilayer lipid structures in these processes. As a first step we investigated here the effects of temperature variation and phenethyl alcohol addition on lipid packing and macroscopic lipid organization in several *E. coli* derived membrane systems, including intact cells, inner membrane vesicles, total lipid extracts and isolated lipid classes, by using ^{2}H- and ^{31}P-NMR techniques. Membranes containing ^{2}H-labelled lipids were obtained by making use of an unsaturated fatty acid auxotrophic mutant of *E. coli*, grown in the presence of deuterium-labelled oleic acid. The localization of the ^{2}H label in oleic acid was chosen at the 11-position next to the carbon double bond, because at this position the deuterons in the lipid are motionally equivalent and give rise to a relatively small quadrupolar splitting ($\Delta\nu_q$) [20], thereby optimizing the signal-to-noise ratio in the ^{2}H-NMR measurements.

The results will be discussed in the light of the importance of lipid fluidity and polymorphic phase behaviour for membrane functioning in *E. coli*.

Materials and Methods

Materials

Deuterated oleic acid ([11,11-$^2H_2$]oleic acid) was synthesized as described by Farren et al. [21] with modifications according to Chupin et al. [20]. 1,2-[11,11-$^2H_2$]Dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) and 1,2-[11,11-$^2H_2$]dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) were prepared by phospholipase D-mediated headgroup exchange [22] from the corresponding phosphatidylcholine, which was obtained by coupling of [11,11-$^2H_2$]oleic acid to glycerophosphocholine according to standard methods [23]. Unlabelled phospholipids were obtained according to the same standard procedures [22,23]. Deuterium-depleted water was obtained from Sigma (St. Louis, MO). Phenethyl alcohol (2-phenylethanol) was from Merck (Darmstadt, FRG). All other chemicals were of analytical grade.

Preparation of E. coli cells

The double mutant *E. coli* strain K1059, which is unable to synthesize or degrade unsaturated fatty acids [24] was a kind gift of Dr. P. Overath. Cells were grown at 37°C in Cohen-Rickenberg (CR) mineral salts medium [25], supplemented with 0.5% (v/v) glycerol, 0.3% (w/v) casamino acids, 0.2% (v/v) Brij-35 and 0.01% (v/v) [11,11-$^2H_2$]oleic acid. Cells were grown till the late logarithmic phase after which they were chilled on ice and harvested by low-speed centrifugation. The pellets (approx. 2.3 g wet wt./l culture) were washed twice with an ice-cold 0.9% (w/v) NaCl solution and stored at −80°C after rapid freezing in liquid nitrogen.

For NMR measurements the pellets were suspended in ice-cold deuterium-depleted buffer (100 mM NaCl, 10 mM Pipes at pH 7.4) in amounts of 2 ml buffer per gram of pellet. Samples containing 2 g of wet cells were incubated for 10 min on ice to allow D–H exchange and centrifuged for 10 min at 12000 × *g* at 4°C. The pellets were washed once with the same buffer and transferred to a 10 mm NMR tube. The samples were equilibrated for 15 minutes at the desired temperature before spectra were accumulated.

Preparation of inverted inner membrane vesicles

Inverted inner membrane vesicles were prepared as described by De Vrije et al. [26], by passing the *E. coli* suspensions through a French press at 8000 psi and isolating the inner and outer membrane fractions on a sucrose gradient. Pelleted inner membrane fractions of a total of 4 litres culture were washed once in 2 ml deuterium-depleted buffer (100 mM NaCl, 10 mM Pipes at pH 7.4), after which they were taken up in 1 ml of this same buffer and transferred to NMR tubes.

Isolation of total lipid extracts and purification of lipids

Total phospholipid extracts of *E. coli* cells and inner membrane vesicles were isolated by an extraction according to Bligh and Dyer [27] and purified by chromatography on a silica (Polygosil 63–100 mm) column. The column was first eluted with chloroform to remove neutral lipids and next phospholipids were eluted with chloroform/methanol (1 : 1, v/v). The phosphate content of the total lipid extracts was determined accord-

ing to Rouser et al. [28]. Typically, about 15–20 mg of phospholipid was recovered per litre of culture.

E. coli phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) were purified from the total lipid extract on a silica column (Polygosil 63–100 μm) with chloroform/methanol/water/ammonia (68:28:2:2, v/v) as eluens, under which conditions cardiolipin eluted first, followed by PG and then PE. The purity of the lipids was checked by HPTLC using the same eluens.

For preparation of NMR samples 10 μmol of phospholid were hydrated with 1 ml deuterium-depleted 100 mM NaCl, 10 mM Pipes, 2 mM EDTA buffer at pH 7.4. The samples were incubated for 1 h at 30°C, after which they were gently vortexed and subjected to several cycles of freeze-thawing. Samples were either measured directly or stored at −20°C.

Determination of phospholipid and fatty acid composition

The phospholipid composition of the total lipid extract of *E. coli* cells and inverted inner membrane vesicles was determined by 2-dimensional HPTLC on silica-plates (Kieselgel 60, Merck) with chloroform/methanol/water (65:25:4, v/v) as the first and chloroform/methanol/acetic acid (65:25:10, v/v) as the second eluens. The phosphate content in each spot was determined according to Rouser et al. [28]. The phospholipid composition (mol%) of the cells was found to be: 79% PE, 15% PG and 6% cardiolipin. Inner membrane vesicles contained 74% PE, 19% PG and 7% cardiolipin.

The fatty acid composition was determined by gas chromatography using methyl esters of the fatty acids. The fatty acyl composition (mol%) of the phospholipids extracted from the whole cells was: 14:0 (7.0%), 16:0 (34.2%), 16:1 (1.7%), 18:0 (2.9%), 18:1 (51.0%) and 18:2 (3.1%), and from the inner membrane vesicles: 14:0 (5.4%), 16:0 (29.5%), 16:1 (1.5%), 18:0 (1.2%), 18:1 (58.9%) and 18:2 (3.5%). The presence of small amounts of unsaturated fatty acids other than oleic acid is ascribed to uptake of these fatty acids from the medium, in which they were found to be present in trace amounts. About 10% of the total amount of oleic acid present in the medium became incorporated into the *E. coli* membranes.

Titration with phenethyl alcohol

Phenethyl alcohol (PEA) was added from a suspension of 2 vol% in the same buffer as used for preparation of the NMR samples. When during titrations the PEA concentration in the NMR sample reached 1 vol%, further aliquots of the alcohol were added from a 10 vol% dispersion of PEA in buffer. After each addition the sample was mixed by gentle rotation of the NMR tube in a near vertical position and allowed to equilibrate for 10 min at 30°C. No further spectral changes were observed upon prolonging the incubation time. Control experiments showed that addition of buffer in the absence of PEA did not affect the ^{2}H-NMR spectra.

NMR measurements

^{2}H-NMR spectra were recorded at 46.1 MHz on a Bruker MSL 300 spectrometer using a quadrupolar echo pulse sequence [29] with a 11 μs 90° pulse, a 2K time domain, an echo delay of 35 μs and a 100 ms delay between pulses. The spectral width was 71.5 kHz and typically 36 K scans were accumulated. The signal-to-noise ratio was reduced by applying an exponential multiplication prior to Fourier transformation, resulting in a 200 Hz linebroadening.

^{31}P-NMR spectra were recorded at 121.4 MHz on the same spectrometer, using a 12 μs 90° pulse, a 38.5 kHz spectral width, 4K data points and a 1-s interpulse time. A gated decoupling program was used with an input power of 3 W during 2.6% of the interpulse time. 5000 free induction decays were accumulated and an exponential multiplication was applied resulting in 100 Hz line broadening. All spectra shown are normalized to the same height.

Results

The properties of the *E. coli* cell membranes and derived membrane systems were first characterized as a function of temperature. ^{2}H-NMR spectra of intact *E. coli* cells are shown in Fig. 1. At 30°C a rather broad powder pattern is observed for the [^{2}H]oleic acid containing membranes, with two peaks separated by a $\Delta\nu_q$ of 6.7 kHz (Fig. 1B) and a sharp isotropic signal which is ascribed to a small amount of residual HOD that was not removed by the exchange procedure. $\Delta\nu_q$ is related to the segmental order of the C–D bond, as described [30]. The large intrinsic line width is typical for protein-containing bilayers [31].

The spectra exhibited a pronounced temperature dependence. Upon lowering the temperature below 20°C a gradual decrease in signal intensity was observed, accompanied by the appearance of a broad underlying component, as shown in Fig. 1A for the spectrum obtained at 4°C. This behaviour is typical for lipids undergoing a phase transition from the liquid-crystalline phase to the gel state and in agreement with previous observations in other *E. coli* fatty acid auxotrophs [17,32–34]. Increasing the temperature to 42°C also led to changes in the ^{2}H-NMR spectra. Superimposed on the powder pattern in time gradually a rather broad isotropic signal emerged (Fig. 1C), that did not disappear upon subsequent cooling of the samples (not shown). This component was not observed at the growth temperature of 37°C. $\Delta\nu_q$ in the intact cells decreases

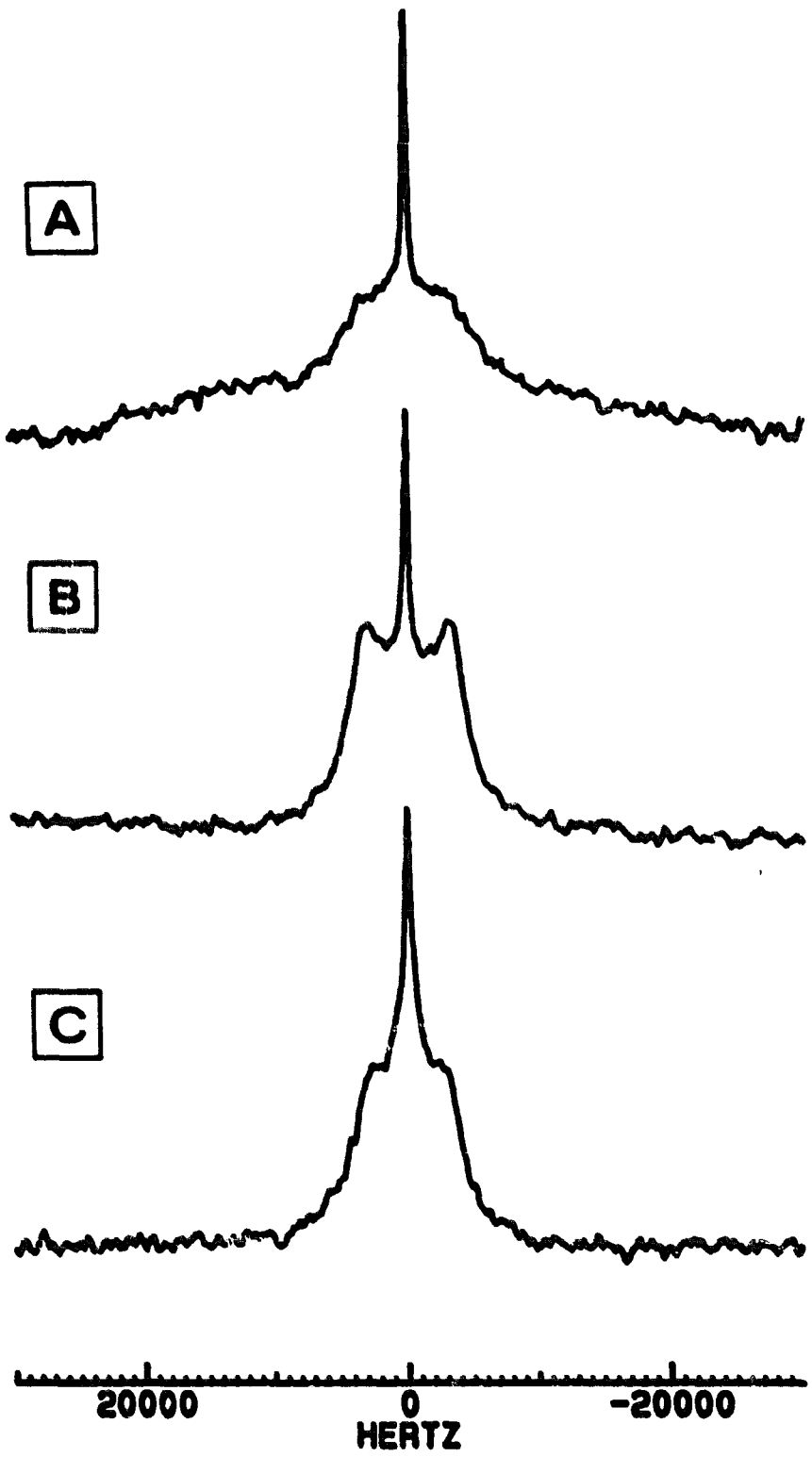

Fig. 1. ^{2}H-NMR spectra of *E. coli* K1059 cells at 4°C (A), 30°C (B) and 42°C (C). Cells were grown in the presence of [11,11-$^2H_2$]oleic acid. All spectra are normalized to the same height. For details see text.

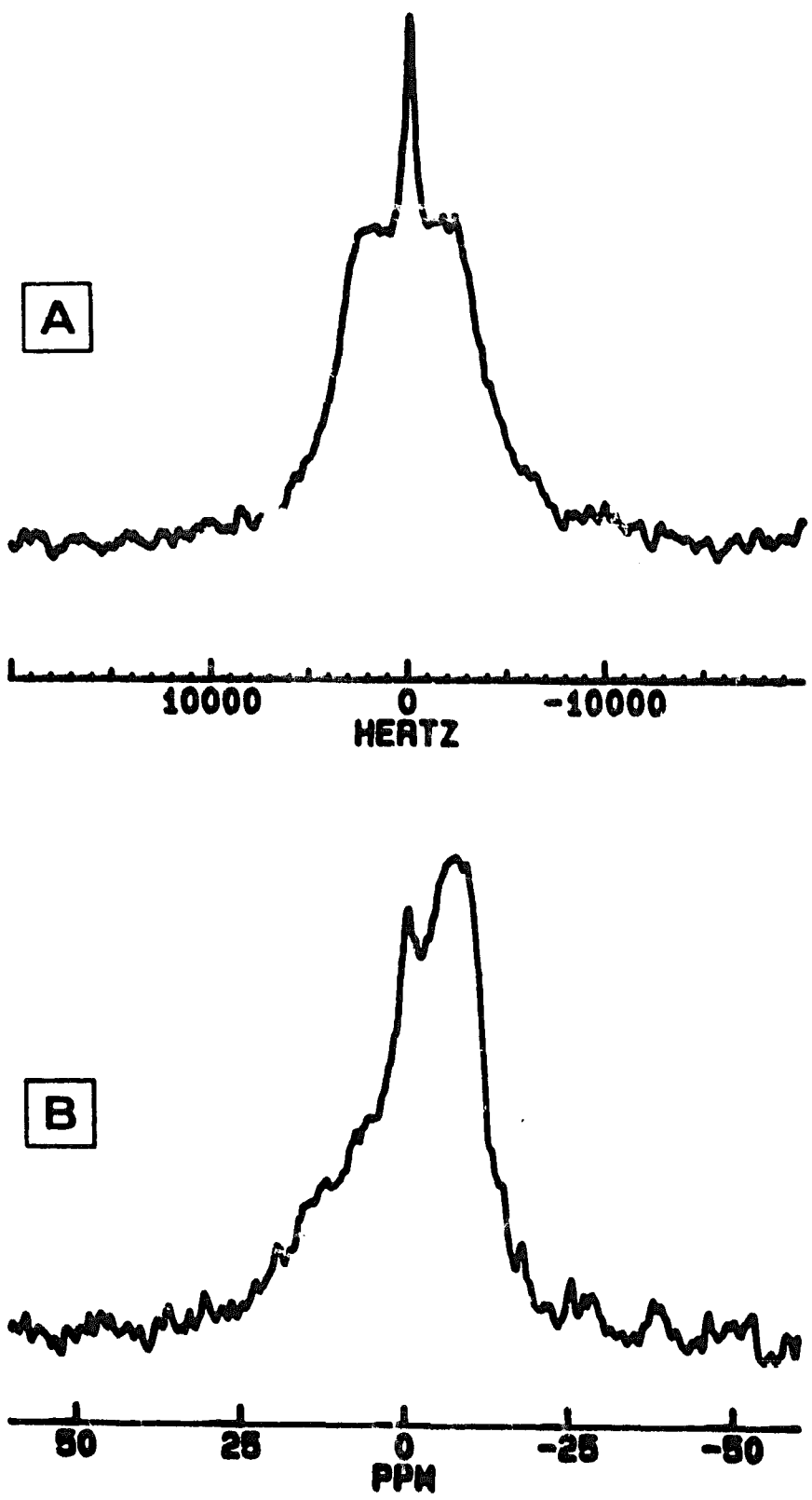

Fig. 2. ^{2}H-NMR (A) and ^{31}P-NMR (B) spectra at 30°C of isolated inner membrane vesicles from *E. coli* K1059 cells, grown on [11,11-$^2H_2$]oleic acid.

with increasing temperature and is about 5.7 kHz at 42°C.

As compared to the intact cells the ^{2}H-NMR spectrum of isolated inner membrane vesicles at 30°C (Fig. 2A) has a smaller $\Delta\nu_q$ of about 4.8 kHz and appears to have a larger intrinsic linewidth. The ^{31}P-NMR spectrum of these inner membrane vesicles (Fig. 2B) consists of one major component, characterized by a low-field shoulder and a high-field peak, and a small isotropic component. The lineshape of the major component is indicative of lipids in a liquid-crystalline bilayer in which the chemical shift interaction is averaged by fast long axis rotation of the lipids [30,35]. A residual CSA of 29 ppm is observed, which is small as compared to that of the total lipid extract (36 ppm) (Fig. 3D). The isotropic component is most likely due to the presence of a minor amount of small vesicles, in which lateral diffusion of the lipids and vesicle tumbling result in a complete motional averaging of the CSA [37].

The total lipid extract at 30°C (Fig. 3B) gives rise to a ^{2}H-NMR spectrum with two sharp peaks, separated by 9.9 kHz, which is much larger than $\Delta\nu_q$ of the lipids in the membrane of the intact cell at this temperature. The lineshape is not typical for that of randomly oriented dispersions, but indicates a magnetic orientation of the lipids, as described previously for lipid extracts from *E. coli* [36]. This magnetic orientation is also evident in the corresponding ^{31}P-NMR spectrum at 30°C (Fig. 3D) from the lack of a low-field shoulder.

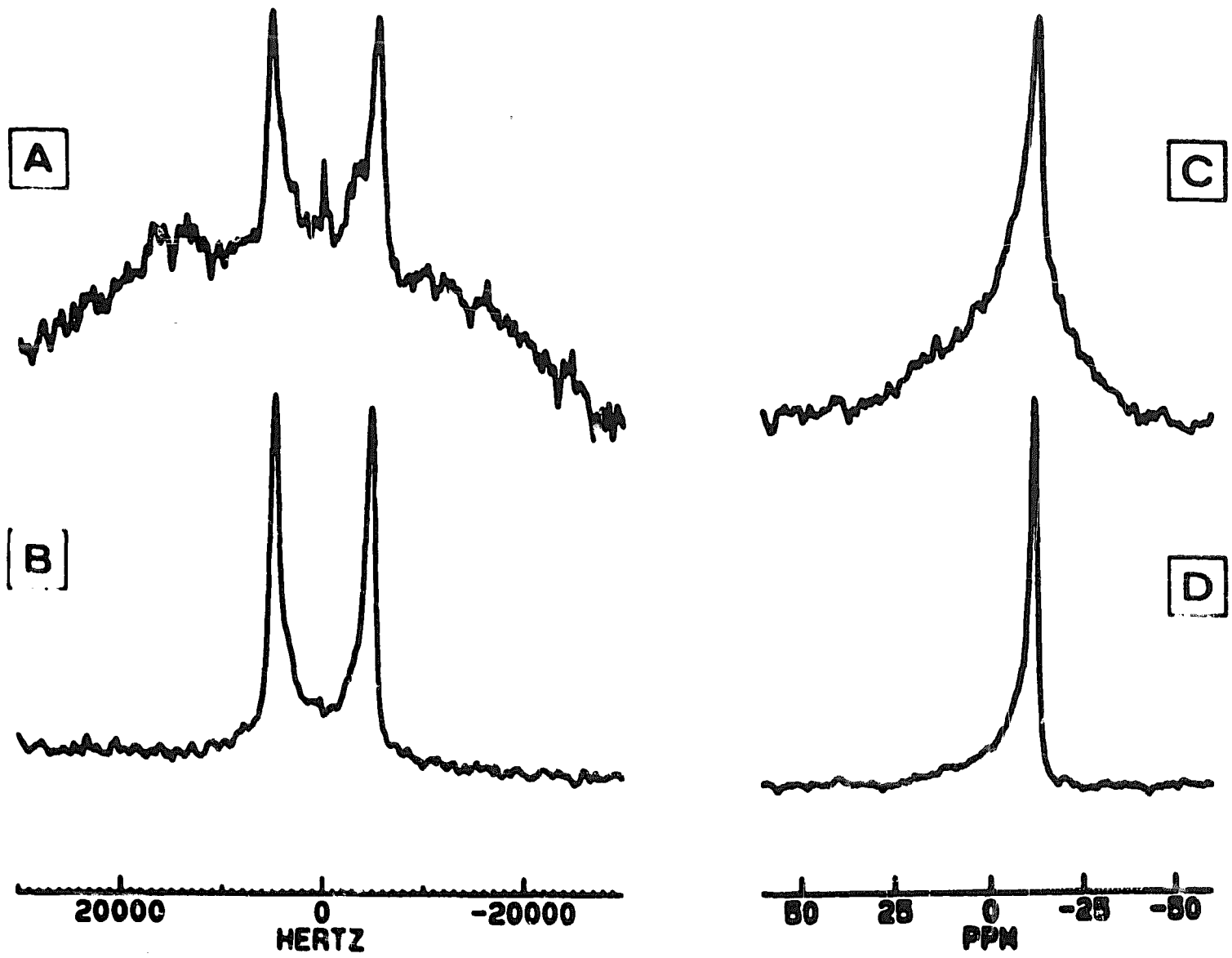

Fig. 3. ^{2}H-NMR (A, B) and ^{31}P-NMR (C, D) spectra of dispersions of the total lipid extract of *E. coli* K1059 cells, grown on [11,11-$^{2}H_2$]oleic acid, at 4°C (A, C) and 30°C (B, D).

Upon lowering the temperature gradually an underlying broad component appears in the ^{2}H-NMR spectra. This is similar to the behaviour in intact cells and indicative of a phase transition of the lipids from a liquid-crystalline to a gel state, which has been reported to occur in this temperature region [17,33,34]. Fig. 3A shows the ^{2}H-NMR spectrum at 4°C, in which superimposed on this broad component a doublet with $\Delta\nu_q$ of 10.8 kHz is observed, originating from a small part of the lipids that is still present in the liquid-crystalline phase. The ^{31}P-NMR spectrum of the total lipid extract at 4°C (Fig. 3C) shows a broadening, consistent with a large part of the lipids being present in the gel state.

Upon increasing the temperature both ^{2}H- (not shown) and ^{31}P-NMR measurements (Fig. 4) showed a bilayer to isotropic phase transition in the total lipid extract. While at 37°C the ^{31}P-NMR spectrum of the total lipid extract is still mostly representative of a bilayer organization, a slight increase in intensity occurs in the region of about 0 to −10 ppm (Fig. 4A). Upon raising the temperature to 42°C a rather broad isotropic component emerges, superimposed on the bilayer component (Fig. 4B). In time the bilayer signal slowly disappears and the isotropic signal becomes sharper (Fig. 4C), indicating that the lipids now undergo isotropic motion with a correlation time of less than 10^{-5} s [37]. These same spectral changes occurred also at 37°C, but much slower. A lineshape typical for a bilayer organization of lipids could be regained only by freeze-thawing of the samples (not shown). This behavior is very similar to that of mixtures of synthetic DOPE and DOPG (3 : 1, molar) for which also at about 40°C a slow transition from a bilayer organization to an isotropic phase was observed [13]. This isotropic phase was assigned to an inverted (type II) organization with a net concave surface curvature, which most likely represents a cubic phase [43]. That a similar isotropic phase is formed in the *E. coli* total lipid extract is supported by the observation (not shown) that, like in the synthetic lipid mixture [13] the bilayer configuration is stabilized by addition of palmitoyllyso-PC, which is a typical type I lipid that mitigates against the formation of type II structures [50].

Next, the acyl chain order and lipid phase behaviour of the total lipid extract was compared to that of the major lipids, PE and PG. Fig. 5 shows that for the total lipid extract $\Delta\nu_q$ initially increases with temperature, and, after reaching a maximum at about 20°C decreases again. At first sight this behaviour at low temperatures is highly unusual. However, it may be explained by a contribution to $\Delta\nu_q$ only from those lipids

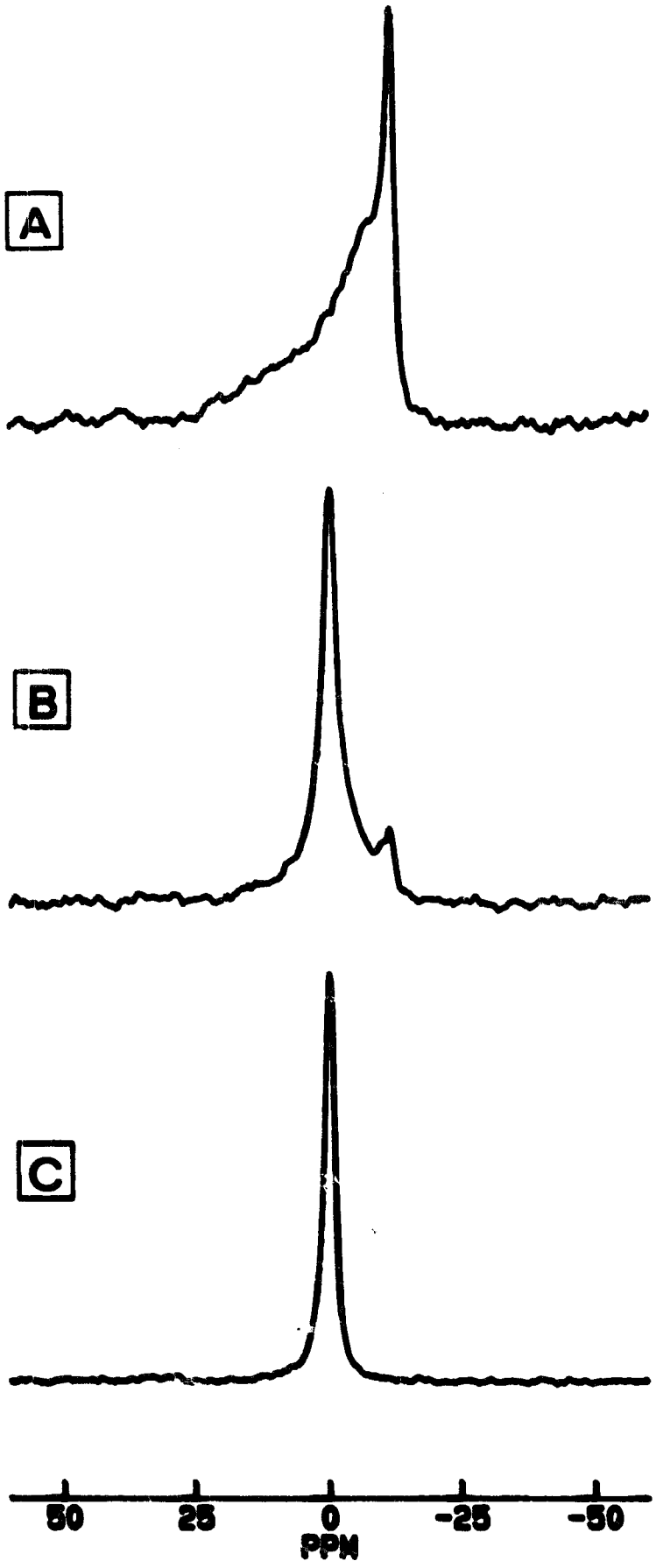

Fig. 4. ^{31}P-NMR spectra of a dispersion of the total lipid extract of *E. coli* K1059 cells, grown on [11,11-$^2H_2$]oleic acid, at 37°C (A), at 42°C after 15 min equilibration time (B) and at 42°C after a total incubation time of approx. 90 min. (C).

that are in the liquid-crystalline state. Since lipids with higher transition temperatures can be expected to have more ordered acyl chains, as will be illustrated below by comparison of *E. coli* PE and synthetic DOPE, their subsequent melting upon raising the temperature may increase the value of $\Delta\nu_q$. At 20°C nearly all the lipids are in the liquid crystalline state and, as expected, further increasing the temperature then decreases chain order in all lipids. At temperatures of 42°C and higher an isotropic phase was induced.

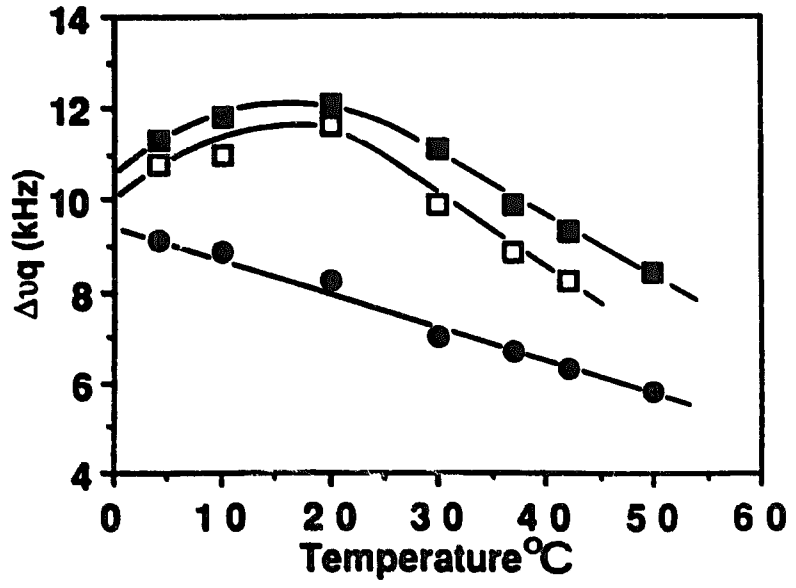

Fig. 5. Temperature dependence of the quadrupolar splitting ($\Delta\nu_q$) in dispersions of the total lipid extract of *E. coli* K1059 cells, grown on [11,11-$^2H_2$]oleic acid (□) of isolated *E. coli* PE (■) and of *E. coli* PG (●).

A qualitatively similar melting behaviour is shown in Fig. 5 for *E. coli* PE, which at all temperatures has a slightly higher value of $\Delta\nu_q$. ^{31}P- and ^{2}H-NMR lineshapes (not shown) were indicative of a broad gel to liquid-crystalline phase transition in the range of 4–20°C and showed a bilayer to H_{II} phase transition at about 65°C, similar to the behaviour of PE of other *E. coli* strains [15,16]. In contrast, $\Delta\nu_q$ of *E. coli* PG is much smaller than that of PE, and gradually decreases with increasing temperature. In line with this, the ^{2}H- and ^{31}P-NMR lineshapes (not shown) indicate that PG is in a liquid-crystalline bilayer throughout the whole temperature range of 4 to 70°C.

Since in spite of the presence of these different types of lipids only one value of $\Delta\nu_q$ is observed for the total lipid extract of *E. coli*, the acyl chains must be homogeneously packed. The value of $\Delta\nu_q$ then can be expected to be an average value that is determined by the lipid composition. For mixtures of synthetic deuterium-labelled DOPE and DOPG it could be demon-

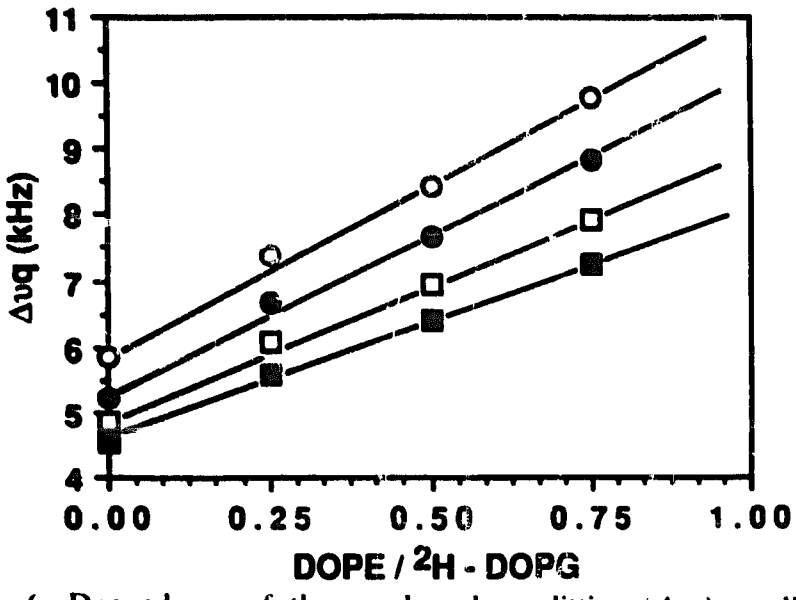

Fig. 6. Dependence of the quadrupolar splitting ($\Delta\nu_q$) on lipid concentration in mixtures of DOPE and ^{2}H-labelled DOPG at 10°C (○), 20°C (●), 30°C (□), and 40°C (■).

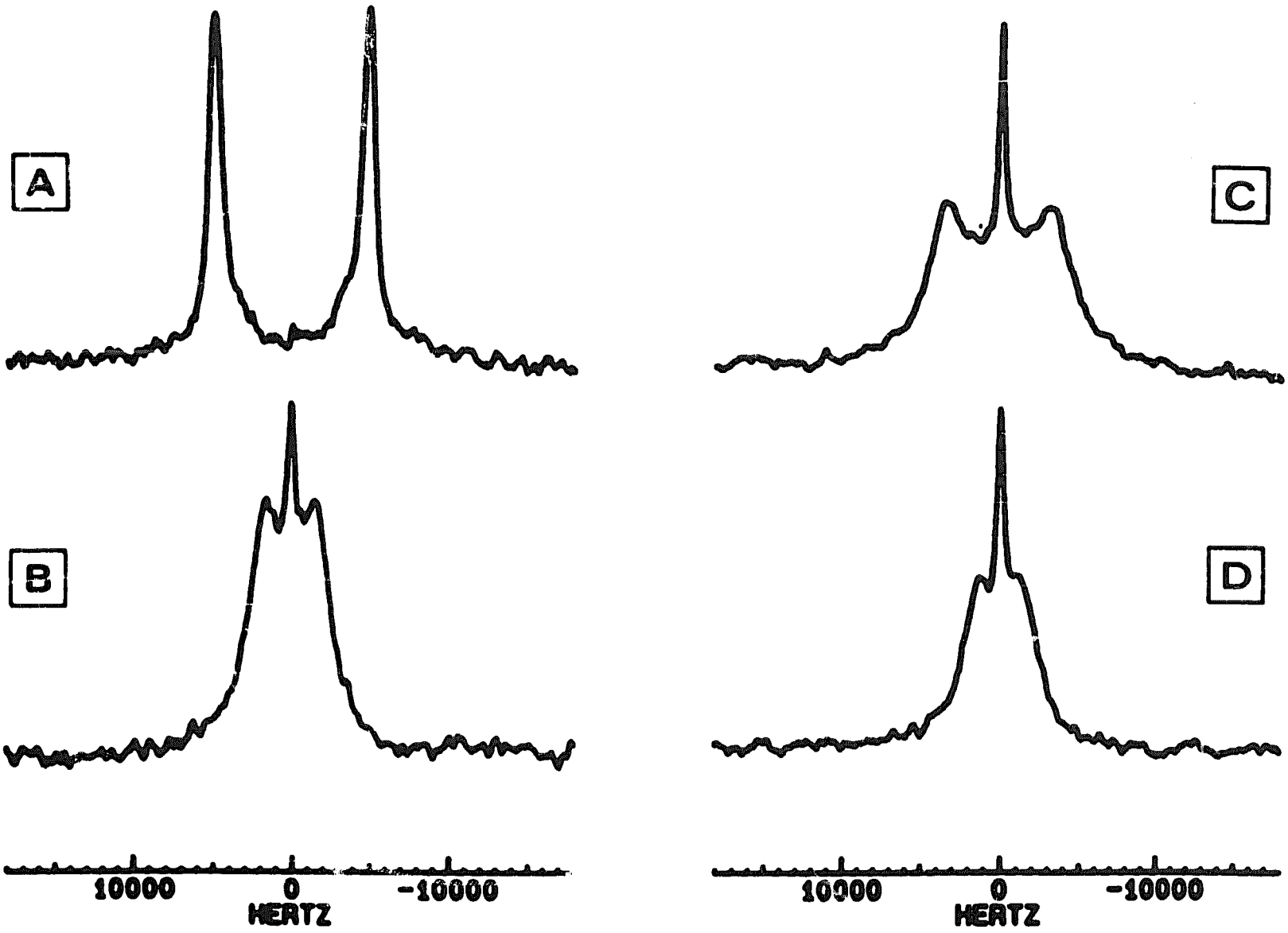

Fig. 7. ^{2}H-NMR spectra at 30°C of the total lipid extract of *E. coli* K1059 cells, grown on [11,11-$^2H_2$]oleic acid (A, B) and of intact cells (C, D) in the absence (A, C) and presence (B, D) of 1 volume % phenethyl alcohol.

strated that $\Delta\nu_q$ indeed directly reflects the lipid composition, since it is linearly related to the DOPE concentration. This is shown in Fig. 6 for mixtures of DOPE and ^{2}H-labelled DOPG at various temperatures.

Like for the *E. coli* lipids also for the dioleoylphospholipids $\Delta\nu_q$ in PE is much larger than in PG. At 0°C, with both lipids being in a liquid-crystalline bilayer, values of $\Delta\nu_q$ are found of 12.0 and 6.6 kHz, respectively. Furthermore, DOPG has a smaller value of $\Delta\nu_q$ than the *E. coli* lipid with its more saturated character, indicating that the acyl chains are more disordered. At 30°C the difference in $\Delta\nu_q$ between both lipids is about 2 kHz. Similarly, extrapolation in Fig. 6 yields a theoretical value of $\Delta\nu_q$ of 8.6 kHz for DOPE in the liquid-crystalline bilayer at 30°C, which is much smaller than that of the *E. coli* PE (11.2 kHz).

Next investigated was how temperature-induced changes in lipid packing compare with those induced by addition of PEA. Fig. 7 shows ^{2}H-NMR spectra of the total lipid extract and of intact *E. coli* cells before and after addition of 1 mol% PEA. In the *E. coli* total lipid extract (Fig. 7A and B) PEA addition results in a dramatic reduction in $\Delta\nu_q$ from 9.9 to 2.9 kHz, corresponding to a 70% decrease in lipid chain order. The origin of the small isotropic signal that is observed after addition of PEA is not known, but most likely it arises from residual naturally abundant deuterons present in the alcohol. Interestingly, also in intact *E. coli* cells (Fig. 7C and D) addition of PEA results in a strong decrease of $\Delta\nu_q$ from 6.7 to 2.5 kHz, corresponding to a decrease in lipid chain order of 63%.

The influence of PEA addition on $\Delta\nu_q$ was further investigated in the *E. coli* total lipid extract as a

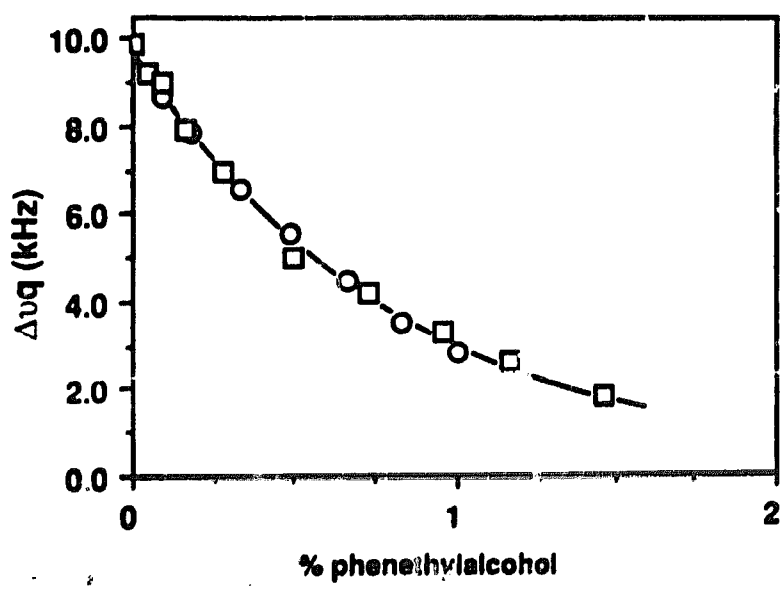

Fig. 8. Effect of phenethyl alcohol addition on the quadrupolar splitting in dispersions of the total lipid extract of *E. coli* K1059 cells, grown on [11,11-$^2H_2$]oleic acid, at 30°C. Symbols represent separate titrations of two different lipid extracts.

function of the concentration of the alcohol. Fig. 8 shows that at low concentrations $\Delta\nu_q$ decreases approximately linearly with the amount of PEA present. At higher concentrations of PEA the decrease in $\Delta\nu_q$ appears to level off. ^{31}P-NMR spectra of the total lipid extract were not affected by PEA addition (not shown), suggesting that the alcohol does not affect lipid organization in these systems. However, upon freeze-thawing of samples of the total lipid extract to which PEA was added no longer a spectrum typical for a bilayer organization was obtained, but both ^{31}P- and ^{2}H-NMR measurements yielded a sharp isotropic signal (not shown).

Discussion

In this study we used an unsaturated fatty acid auxotrophic mutant to incorporate ^{2}H-labelled oleic acid in *E. coli* membranes and we characterized lipid organization and lipid acyl chain order in intact cells and derived membrane systems by NMR techniques.

The membranes of intact cells showed a broad gel to liquid-crystalline phase transition and were completely fluid at 30°C. At this temperature a value of $\Delta\nu_q$ was observed that was 30% lower than in the total lipid extract, indicating that the presence of proteins in the intact membranes significantly reduces lipid chain order. Such a 30% difference in acyl chain order is rather large as compared to previous reports [17,33,34]. Since the sensitivity of $\Delta\nu_q$ towards particular changes in lipid packing depends upon the position of the label in the acyl chain [38,39], a possible explanation is that this sensitivity is relatively high for the 11,11-position in oleic acid under the present conditions.

In the isolated inner membrane vesicles an even smaller value of $\Delta\nu_q$ was observed, which was about 25% reduced as compared to $\Delta\nu_q$ of the intact cells. This is similar to the difference in acyl chain order between outer and inner membrane vesicles as reported by Nichol et al. [32]. The small value of $\Delta\nu_q$ could be a result of the different composition of the inner membrane, which contains less PE, has a higher content of unsaturated acyl chains and is more fluid than the outer membrane [17,32,34,40]. However, it is more likely that $\Delta\nu_q$ is reduced because vesicle tumbling and lateral diffusion of the lipids results in additional motional averaging of the quadrupolar interaction due to the relatively small size of the inner membrane vesicles. Their average diameter is about 100 nm, with a rather inhomogeneous size distribution, varying from 40 nm to about 300 nm as estimated from freeze-fracture electron-microscopy experiments (not shown). Similarly the relatively small residual CSA and the apparent linebroadening in the ^{31}P-NMR spectra of these inner membrane vesicles (Fig. 2B) could result from motional averaging due to a small and heterogeneous vesicle size [37]. The isotropic signal most likely originates from a minor percentage of even smaller vesicles.

The broad gel to fluid transition that was observed in the intact *E. coli* cells as well as in the total lipid extract appeared to be dictated by the phase behaviour of the dominant lipid PE. PE is a typical non-bilayer structure preferring lipid in which, due to the low headgroup hydration and strong intermolecular headgroup interactions, the average cross-sectional area per headgroup is small compared to that of PG [10,11]. As a result the lipids tend to organize in highly curved structures, i.e. they have a high spontaneous surface curvature [10,41]. When PE is present in a bilayer this will lead to an increased lateral pressure in the hydrophobic part of the bilayer as a result of which the acyl chains will stretch. The large value of $\Delta\nu_q$ in PE as compared to PG with a similar acyl chain composition is indicative of this larger effective length of the acyl chain [38]. The lateral stress can be relieved by organization of the lipid in structures with a concave surface curvature at the lipid/water interface, such as the hexagonal H_{II} phase. However, this phase can only be formed at the cost of free energy due to packing constraints at the intersections of the tubes in the H_{II} phase [10,41]. For *E. coli* PE used in this study apparently the H_{II} phase becomes energetically more favourable at high temperatures of about 65°C. This phase behaviour of *E. coli* PE is rather similar to that of POPE, which is expected to be the prevailing lipid species [1] and which undergoes a gel to liquid-crystalline phase transition at 27°C and a bilayer to H_{II} phase transition at 71°C [42].

Replacement of both acyl chains by oleic acid, as in DOPE, increases the lipid fluidity and results in a large decrease in $\Delta\nu_q$. The bilayer to H_{II} phase transition is decreased to about 8°C [20] and addition of DOPG stabilizes the bilayer [13]. A bilayer stabilizing activity of PG in mixtures with PE has been reported previously [44,45] and can be easily understood on the basis that PG is a negatively charged, bilayer preferring lipid, which decreases the spontaneous curvature when mixed with PE.

In view of this expected bilayer stabilizing activity of PG, the difference between the phase behaviour of *E. coli* PE, which forms a bilayer up till 60°C, and the total lipid extract, which besides PE contains PG and CL, and which undergoes a bilayer to isotropic transition at about 40°C, is puzzling. Apparently the presence of PG and small amounts of CL, which in the absence of divalent cations is a bilayer preferring lipid [11], now destabilizes the bilayer. The only logical conclusion is that apparently in the isotropic phase the need is satisfied for a curved interface, with a different curvature than is required for pure PE, and that the free energy cost due to packing constraints in this

isotropic structure is considerably lower than for *E. coli* PE in the H_{II} phase.

Also surprising is the observation that total lipid extracts of *E. coli* cells show a similar bilayer to isotropic transition at about the same temperature as DOPE/DOPG (3:1) mixtures [13], in spite of their very different acyl chain composition. Likewise, the phase behaviour of a total lipid extract from a wild-type strain SD12, which has a different fatty acid composition, is very similar close to growth temperature (not shown). A likely explanation is that it is mainly the PE content that determines the spontaneous curvature of the lipid mixture, and that this curvature dictates the transition temperature, rather than the energetics of lipid packing in the non-bilayer phase, which for a large part is determined by the acyl chain composition. Since the transition occurs close to the growth temperature, these data suggest that it may be important for functioning of *E. coli* to maintain a situation in which the membrane lipids are close to the bilayer to non-bilayer transition and that this may be easily accomplished by keeping the PE content approximately constant. Another, very different indication, that in *E. coli* the bilayer to non-bilayer lipid ratio is regulated, is the recent observation that *E. coli* mutants, unable to synthesize PE, contain high levels of PG and CL instead and are auxotrophic for divalent cations [46]. This suggests that PE fulfills a structural role that can be taken over by CL and PG only when divalent cations are present, which is precisely the condition under which CL is able to organize in an H_{II} phase [10,11].

Non-bilayer structures have been previously observed in *E. coli* derived membrane systems [13,16,18,19] and have been implied as intermediates in functional membrane processes [11,12], during which they are believed to be formed locally and transiently, while not affecting membrane integrity. In intact *E. coli* cells at higher temperatures a broad, non-reversible isotropic component is induced, that might be indicative of a more permanent formation of non-bilayer lipid structures. A similar component, also ascribed to non-bilayer lipid structures, had been reported previously [34]. Since the permanent formation of non-bilayer lipid structures is expected to affect membrane integrity, this could explain the observed phenomenon of translocation across the membrane of mature proteins upon exposure of *E. coli* cells to elevated temperatures [9].

Although also PEA is known to promote formation of type II structures [14], it does not appear to do so upon addition to either the total lipid extract or to intact *E. coli* cells. However, PEA induces a dramatic decrease in lipid chain order, which at 1 mol% of the alcohol is decreased by as much as 60–70%. This is much larger than the 15% change in acyl chain order found by Halegoua and Inoue [7], using 5-doxylstearate as a spin label, and slightly larger than the approximately 55% decrease observed upon addition of PEA to mixtures of [11,11-$^2H_2$]dioleoylphosphatidylcholine and phosphatidylserine, in which it could be directly related to an increased translocation efficiency of apocytochrome *c* [8]. The decrease in chain order most likely results from an interfacial localization of the alcohol, by which it can act as a spacer between the headgroups [8].

The only indication for a type II structure inducing activity of PEA in the present study is the observation that upon freeze-thawing of samples of the total lipid extract to which PEA was added an isotropic signal was induced. This suggests that freeze-thawing changes the localization of PEA in the lipid phase, which results in a different effect on lipid phase behaviour. The isotropic signal most likely reflects organization of the lipids in a cubic phase similar to that obtained upon heating the samples.

Thus, it is clear that both temperature variation and phenethyl alcohol addition have a large effect on lipid packing. However, the way in which both factors affect membrane properties is different. Phenethyl alcohol has a very large effect on membrane fluidity and this appears to be the primary mechanism by which it affects the properties of the membrane and for instance enhances translocation of proteins across the membrane [6,8]. In contrast, upon increasing temperature the lipid packing appears disturbed locally and (type II) non-bilayer structures are likely to be formed.

In *Acholeplasma laidlawii* it has been shown that both lipid fluidity and polymorphic phase behaviour are regulated [47], indicating their importance for membrane functioning. While previously it had been shown that lipid fluidity is regulated in *E. coli* [48,49], the results presented in this paper, together with data from other studies [46] suggest that a regulation mechanism to maintain a certain bilayer-to-non-bilayer lipid ratio exists also in *E. coli*.

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