Biochimica et Biophysica Acta, 1070 (1991) 409–418

BBAMEM 75455

The uptake of pristane (2,6,10,14-tetramethylpentadecane) into phospholipid bilayers as assessed by NMR, DSC, and tritium labeling methods

Klaus Gawrisch [1] and Siegfried Janz [2]

[1] *Physical Sciences Laboratory, Division of Computer Research and Technology, National Institutes of Health, Bethesda, MD (U.S.A.) and* [2] *Laboratory of Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD (U.S.A.)*

(Received 22 April 1991)
(Revised manuscript received 12 August 1991)

Key words: Pristane; Alkane; Dioleoylphosphatidylcholine; Liposome; Plasmacytomagenesis; NMR; DSC

Unilamellar dioleoylphosphatidylcholine (DOPC) liposomes (250 μM) incorporated 2 mol% of [^{3}H]pristane at 37 °C after addition of 50 μM pristane solubilized with β-cyclodextrin. Conventional solubilization in dimethyl sulphoxide resulted in much lower uptake. Premixing of perdeuterated pristane with DOPC and dipalmitoylphosphatidylcholine (DPPC) prior to the formation of multilamellar liposomes resulted in homogeneous incorporation of up to 5 mol% pristane at 22 °C and 50 °C, respectively, as observed by ^{2}H-NMR. Lipid order parameters measured by ^{31}P and ^{2}H-NMR remained unchanged after pristane uptake. Pristane induced the transformation of part of the dioleoylphosphatidylethanolamine (DOPE)/DOPC (3:1, mol/mol) liquid crystalline lamellar phase into an inverse hexagonal phase. 5 mol% pristane in DPPC bilayers decreased the midpoint of the main phase transition temperature of DPPC from 41.5 °C to 40.9 °C. Upon cooling in the temperature range from 41 °C to 36 °C, pristane was either displaced from the DPPC bilayer or the mode of incorporation changed. These results may aid in defining the mechanisms whereby pristane, an isoprenoid C_{19}-isoalkane, induces plasmacytomagenesis in mice.

Introduction

Pristane (2,6,10,14-tetramethylpentadecane) causes numerous detrimental effects in animal species [17,28,47,48]. It promotes skin carcinogenesis in C3H mice [20] and the development of 3-methylcholanthrene induced lymphoid malignancies in Copenhagen rats [4]. Furthermore, pristane induces histiocytomas in BALB/c and BALB/Mo mice [33] and plasmacytomas in BALB/cAnPt mice [1]. Murine plasmacytomagenesis is by far the most widely studied tumor system involving pristane (reviewed in Refs. 37 and 38). Hence, much of the current interest in this simple compound, an isoprenoid C_{19}-isoalkane, is derived from the desire to better understand plasmacytomagenesis in mice.

The tumor inducing activity of pristane, thought to be a non-genotoxic, 'complete' carcinogen, is still poorly understood. This is reflected by several diverse hypotheses on its putative effects *in vivo*. Changes in the conformation of DNA in lymphocytes [11] and hybridoma cells [12] after incorporation of pristane have been observed with propidium iodide staining. A subsequent fluorescence polarization study revealed pristane-induced changes in the membrane fluidity of rat lymphocytes [3]. The generation of oxidation products of pristane by reactive oxygen intermediates [22] may provide an additional component for the action of pristane under conditions of prolonged inflammation *in vivo* [41]. Immunosuppression [10,26,35] and formation of a specific histologic matrix for tumor origin and development [34] after the intraperitoneal administration of pristane may contribute to tumorigenesis through different pathways. Whether or not pristane metabolites that are generated *in vivo* [27] are involved in the tumor inducing activity of pristane is presently completely unclear.

Our interest is to elucidate the direct effects of pristane at the cellular level. It is likely that the uptake of pristane by cell membranes facilitates both the dis-

Abbreviations: β-CyD, β-cyclodextrin; D_2O, deuterium oxide; DMSO, dimethyl sulphoxide; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline.

Correspondence: K. Gawrisch, NIH, Building 10, Room 7N316, Bethesda, MD 20892, U.S.A.

tribution of the compound throughout the intracellular membrane network and alterations of membrane properties. Yet, quantitative data on the uptake of pristane by lipid membranes are not available. In this report, the incorporation of pristane into model membranes was studied with three physico-chemical methods. The experiments were aimed at quantifying the uptake of pristane into model membranes and determining the influence of the mode of solubilization on its incorporation into lipid bilayers. For the latter, we were particularly interested in comparing two methods; a novel solubilization procedure for pristane in β-cyclodextrin (β-CyD) [23] and the conventionally used method of dissolving pristane in the organic solvent dimethyl sulphoxide (DMSO). By employing NMR techniques we attempted to (i) localize pristane within the membrane, (ii) investigate changed membrane properties such as altered mobilities and conformations of lipid headgroups and fatty acids, (iii) study pristane induced lamellar-hexagonal phase transitions. DSC measurements were performed to measure the effect of the incorporation of pristane on the main phase transition temperature of DPPC. The results reported here should allow better comparison of the effects of pristane on lipid bilayers to the effects of other membrane-targeted compounds such as tumor promoting phorbol esters.

Materials and Methods

Chemicals

Synthetic L-α-dioleoylphosphatidylcholine (DOPC), L-α-dioleoylphosphatidylethanolamine (DOPE), and L-α-dipalmitoylphosphatidylcholine (DPPC) were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). The lipids were checked for impurities by thin layer chromatography and were judged to be at least 98% pure. Pristane (2,6,10,14-tetramethylpentadecane, 96% pure) was purchased from Aldrich Chemical Company (Milwaukee, WI, U.S.A.). The main contaminants in commercial pristane preparations were identified by GC/MS as *n*-alkanes and isoalkanes of similar chain length as pristane [21]. Perdeuterated pristane and deuterium oxide (minimal isotopic purity 99.9 atom% deuterium) were obtained from MSD Isotopes, Merck Division (Montreal, Canada). Deuterium depleted water was from Cambridge Isotope Laboratories (Woburn, MA, U.S.A.), Tris (enzyme grade) was from BRL (Gaithersburg, MD, U.S.A.), sodium chloride was from Mallinckrodt (Paris, KY, U.S.A.), and β-cyclodextrin was from Sigma (St. Louis, MO, U.S.A.).

NMR

Sample preparation

(A) The lipid mixture of DOPE/DOPC (molar ratio 3:1) was prepared by dissolving both lipids in chloroform/methanol (2:1, v/v). The solvent was first removed in a stream of nitrogen and then under vacuum. About 50 mg of dry lipid or lipid mixture were put into short Pyrex glass tubes with an outer diameter of 8 mm. Pristane was added to the dry lipid using a microsyringe. The exact amount of lipid and pristane was determined with a microbalance. Pristane concentrations given in mol% are calculated on the basis of the dry weight of the lipid. After the addition of pristane, the tubes were closed with rubber stoppers. On the next day, water was added to prepare a 50 wt.% lipid-in-water dispersion.

(B) Some samples (containing less than 10 mol% pristane) were prepared using stock solutions of pristane in chloroform. 50 mg of the matrix lipid were dissolved in an appropriate volume of the stock solution. The chloroform was removed first in a stream of nitrogen and then by vacuum (10 min). The samples were kept for a further 7 to 12 h in a desiccator under reduced pressure. The rate of evaporation of pristane was checked with a pure sample of pristane in the same desiccator and a loss of only 1.5% pristane was detected. For measurements on samples containing deuterated lipid or pristane, deuterium-depleted water was added. The glass tubes were sealed with a flame. Sample contents were mixed and pelleted by centrifugation. Prior to the NMR measurements, the samples were stored for several days at room temperature, except for DPPC samples which were kept at 50 °C.

Measurements

NMR measurements were carried out on a Bruker MSL 300 spectrometer using a high power probe with an 8 mm solenoidal sample coil which was doubly tuned for ^{31}P and ^{1}H at 121.513 MHz and 300.133 MHz, respectively. Gated broadband decoupled ^{31}P spectra were observed with a phase cycled Hahn echo sequence as described in [39]. A delay time between the 90° and 180° pulse of 100 μs was chosen. Accumulation was started at the top of the echo. Typically 1024 scans with a recycle delay time of 1 s were accumulated. ^{2}H-NMR spectra were observed using the same 8 mm solenoidal sample coil as for ^{31}P-NMR but the coil was tuned for a resonance at 46.073 MHz. ^{2}H-NMR spectra were obtained using a quadrupole echo sequence [8]. The delay time between the two 90° pulses was 100 μs and the recycle delay time 1 s. Sample temperatures were adjusted using a Bruker temperature control unit.

Calorimetry

A Perkin-Elmer-DSC-2 was used to perform differential scanning calorimetry experiments. Pristane was dissolved in chloroform prior to its addition to dry DPPC. The solvent was evaporated under a stream of nitrogen followed by application of vacuum. The dry

mixture of lipid and pristane was filled into aluminum pans. The weight of the lipid was determined with a Cahn electrobalance. Then, 5 μl of water were added. Samples were sealed and equilibrated for 5 h at 50 °C in the calorimeter. Measurements were performed in the cooling regime only. The calorimeter was calibrated against the known temperature and enthalpy of the main phase transition of DPPC [29]. Enthalpies of transitions were determined by integrating the DSC thermograms.

Solubilization of pristane

Pristane was solubilized by its molecular inclusion into β-cyclodextrin (β-CyD) in a novel coprecipitation procedure that was modified from a published method for complexing cinnarizine, a cerebral blood flow enhancer [43]. Briefly, a bolus of pristane (final concentration 2 mM) was pipetted into a 4 mM β-CyD solution in distilled, deionized water and vigorously stirred on a simple benchtop mixer at ambient temperature for 4 days. The crystalline inclusion complexes which precipitated out of solution were washed two times in PBS (pH 7.4, 2800 × g, 20 min) in order to remove remaining free β-CyD. Aliquots were stored at −20 °C. Working portions were kept at 4 °C. The time course of inclusion complex formation, data on the stability and dissolution properties of the inclusion complex, and results of its biological testing are given in Ref. 23. Alternatively, pristane was dissolved in plain DMSO. This can be accomplished without phase separation problems at an initial dilution factor of 1:200 (v/v) or greater. That corresponds to a stock concentration of pristane of 14.6 mM. Using tritiated pristane as a liquid scintillation probe, this dilution factor was determined in experiments aimed at estimating the solubility limit of pristane in plain DMSO and the requirements for its further dilution without phase separation in an aqueous phase (phosphate buffer) with progressively decreasing concentrations of DMSO (data not shown).

Preparation of unilamellar liposomes

250 mg of DOPC were dispersed in 25 ml of 10 mM Tris, 10 mM NaCl, 0.02% (w/v) sodium azide in D_2O. The pD was adjusted to 7.4 using 1 M HCl. After initial vortexing, the dispersion was sonicated with a Fisher Sonic Dismembrator, Model 300 (Artek Systems Corp., Farmingdale, NY, U.S.A.) with a tip sonicator at 60% power output for about 45 min. During sonication the dispersion was cooled using a ice-cold water bath. Prior to the experiments the dispersion was stored in a refrigerator at a temperature of 4 °C.

Uptake of pristane into liposomes

The incorporation of pristane into model lipid bilayers was studied using [^{3}H]pristane, DOPC liposomes, and Tris/NaCl/D_2O buffer. [^{3}H]Pristane (specific activity 30 mCi/ml) was prepared according to the catalytic hydrogen exchange method and was purified (final radiochemical purity > 95%) by silica gel chromatography using n-hexane as solvent (American Radiolabeled Co., St. Louis, MO, U.S.A.). The experiments were carried out in 13 × 51 mm polyallomer centrifuge tubes in a final volume of 3 ml. Liposome buffer (10 mM Tris, 10 mM NaCl, D_2O, pH 7.0/pD 7.4), liposomes and [^{3}H]pristane were pipetted in the given order into centrifuge tubes that were then vortexed and adapted (20 min) to a temperature of 37 °C. In different experiments, [^{3}H]pristane was added either solubilized in DMSO or as an inclusion complex with β-CyD. Samples were incubated between 1 and 12 h at 37 °C with intermittant vortexing every 15–20 min. Then the tubes were centrifuged (2 h, 150000 × g, 25 °C) in a Beckman L8-T70 ultracentrifuge using a SW-Ti55 rotor. After centrifugation a lower D_2O phase and an upper liposome phase were clearly separated. The lower D_2O phase was carefully aspirated in a plastic 3 ml syringe with a 20 gauge, 1.5 inch needle. The upper liposome phase was left in the tube. Triplicates of both phases were counted in a Packard Tri-Carb 1500 liquid scintillation analyzer. The activities in both phases were compared to the total activity, thus allowing estimation of the uptake of pristane into liposomes, the concentration of free pristane in the D_2O phase, and the amount of pristane adsorbed to the wall of the centrifuge tube.

Results

NMR

(a) DOPC + pristane-d_{40}

The ^{2}H-NMR spectrum of free pristane appears as a single resonance line with a linewidth of the order of 100 Hz indicative of isotropic motions. As a consequence of the incorporation of pristane into the lipid bilayers, the molecular motions aquire a certain degree of anisotropy. The ^{2}H-NMR lines split into doublets due to the angular dependent quadrupolar interaction between the deuterium nucleus and the internal electric field gradient of C-D (carbon-deuterium) bonds. The magnitude of splitting depends on the orientation of the particular C-D bond to the magnetic field and fast motions of that bond axis. The spectra in Fig. 1 represent so-called powder patterns of doublet splittings of pristane incorporated into non-oriented bilayers. It can be seen that the quadrupolar splittings of the methyl and methylene groups of pristane are poorly resolved and overlapping. While the largest of the overlapping splittings is in the order of 5 kHz, other quadrupolar interactions in the same molecule are only in the order of a few hundred Hz or less, thus con-

tributing to a broadened isotropic signal. Broadening of doublets may be caused by reorientation processes with a correlation time of the order of 10^{-4} s. Slow reorientations of pristane molecules during lateral diffusion along membranes with radii of curvature of 1 μm or less is the most appropriate explanation of the broadening [5,14]. In agreement with this explanation is the fact that the shape of the spectra depends to a certain extent on the sample preparation itself. We demonstrated previously that the preparation procedures decisively determine the fraction of liposomes in aqueous dispersions that have smaller radii of curvature [13,25].

The shape of ^{2}H-NMR spectra is independent of the amount of pristane at concentrations of up to 5 mol% in DOPC bilayers. Above this concentration, the quadrupolar splittings of pristane decrease slightly while the intensity of the broadened isotropic component increases (Fig. 1). Unfortunately, it is impossible to distinguish between the spectral contribution of pristane moving isotropically in a separate phase from the methyl group signals of incorporated pristane that also have very small quadrupolar splittings. For this reason we are not able to give the exact concentration of pristane at which a truly isotropic pristane signal can be seen for the first time. Nevertheless, all data indicate a change in the orientation and/or motion of pristane molecules at concentrations above 5 mol%.

It is noteworthy that we reproducibly observed differences between the spectra obtained after direct addition of pristane to the lipid matrix (see NMR, Sample preparation (A) in Materials and Methods) and spectra recorded after prior simultaneous dissolution of pristane and lipids in chloroform (see NMR, Sample preparation (B) in Materials and Methods). Direct addition results in a stronger isotropic component at concentrations as low as 3 mol%. On the other hand, the largest quadrupolar splittings, as reflected by the spectral width close to the baseline, at pristane concentrations as high as 16 mol% are still close to the values measured at 1 mol%. The ^{31}P-NMR spectra of DOPC in the presence of increasing concentrations of pristane do not show any significant changes in the effective anisotropy of chemical shift of the lipid phosphate groups upon pristane addition (Fig. 4). Indications of medium speed exchange processes that are connected to the decrease of the radii of curvature of liposomes after pristane addition can be found in both the ^{2}H NMR spectra of pristane-d_{40} in DPPC water dispersions and the ^{31}P-NMR spectra of lipids.

(b) DPPC + pristane-d_{40}

When DPPC is in the gel phase, the ^{2}H-NMR signal of pristane-d_{40} shows no indication of a quadrupolar splitting (5 mol% pristane in DPPC). Apparently, pristane is not incorporated in measureable quantities into DPPC bilayers in the gel phase. Alternatively, the incorporation does not cause anisotropic motions of pristane molecules. However, after heating the sample in the spectrometer to 50 °C (i.e., above the phase transition temperature at which DPPC enters the lamellar liquid crystalline phase), the isotropic resonance line of pristane slowly transformes into a quadrupolar split signal that reflects incorporation (Fig. 2). Typically, such changes in lineshape were observed during measurements over 2 h, a period of time that is characteristic for diffusion controlled processes. Upon cooling the sample at a rate of 4 C°/h, the isotropic pristane peak reappeared between 41 °C and 36 °C. Below 36 °C the pristane signal was always isotropic.

The quadrupolar splittings of deuterated pristane in DPPC (Fig. 2) are better resolved than in DOPC (Fig.

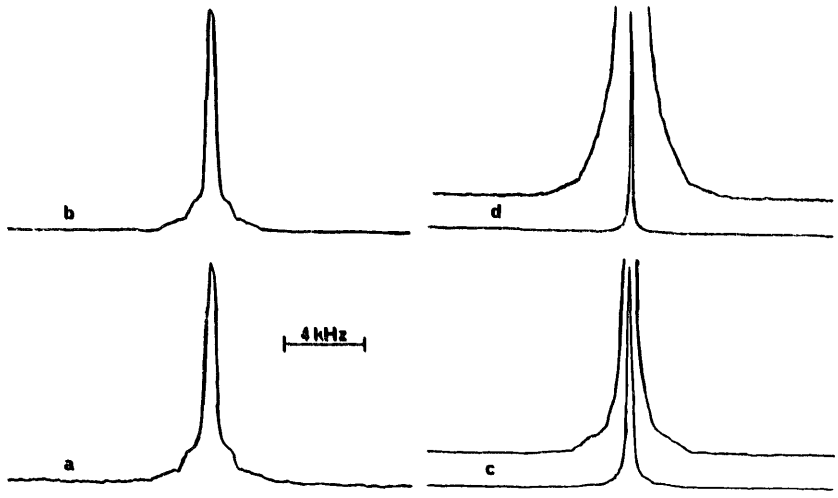

Fig. 1. ^{2}H-NMR spectra of perdeuterated pristane in DOPC-water dispersions (50 wt.% deuterium depleted water) at 22 °C. The concentration of pristane is given in mol% of the sum of the molar concentrations of pristane and phospholipid: a, 1.0 mol%; b, 9.7 mol%; c, 16.3 mol%; d, 34.6 mol%.

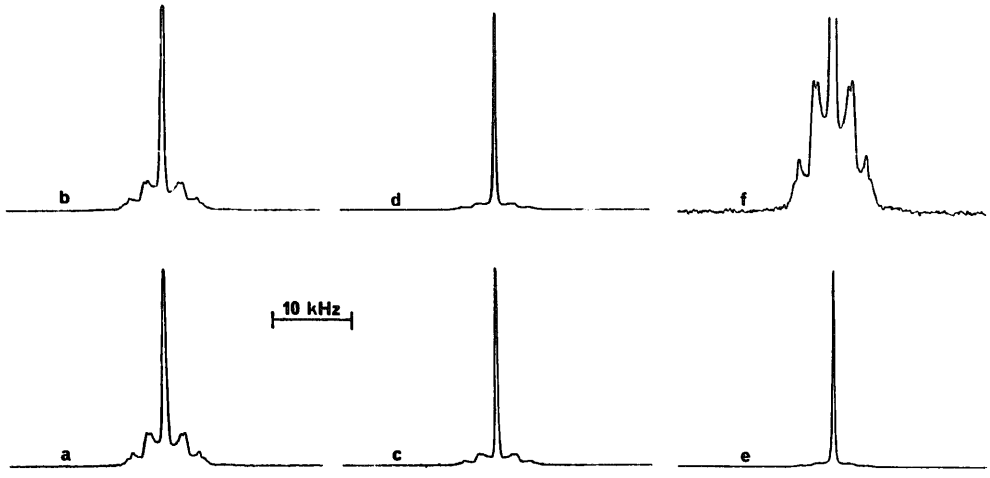

Fig. 2. ^{2}H-NMR spectra of pristane-d_{40} in DPPC-water dispersions (50 wt.% deuterium depleted water) at 50 °C (liquid crystalline lamellar phase). The pristane concentration is given in mol%: a, 2.5; b, 5.0; c, 7.5; d, 10.0; e, 12.5; f, 2.5 mol% as in (a) but shown on an extended y-scale. Spectrum (f) is a superposition of four different quadrupolar splittings with amplitudes of 0.6 kHz, 4.06 kHz, 4.94 kHz, and 8.76 kHz.

1). At 50 °C, four splittings with amplitudes of 0.6 kHz, 4.06 kHz, 4.94 kHz, and 8.76 kHz could be identified. The quadrupolar splittings increased slightly with decreasing temperature. In the range from 2.5 mol% to 12.5 mol% pristane, the splittings were independent of the concentration of pristane. However, at concentrations above 5 mol% an intense isotropic resonance signal of pristane was observed. This finding indicates that liquid crystalline DPPC bilayers can incorporate a maximum of 5 mol% pristane-d_{40}.

(c) DPPC-d_{62} + pristane

The influence of the incorporation of pristane on the fatty acids of the phospholipids was investigated at 50 °C using fatty acid deuterated DPPC-d_{62} in the presence of a large excess of pristane (74.3 mol%). In order to ensure incorporation of pristane into the lamellar liquid crystalline bilayers, samples were kept for five hours at 50 °C in the spectrometer prior to measurements. The quadrupolar splittings observed for the DPPC-d_{62} without added pristane were identical to the values reported in Ref. 7. Pristane addition resulted in a slight broadening of resonance lines but had no influence on the position of resonance peaks. As was the case in the ^{31}P-NMR mesurements, the broadening probably reflects the formation of liposomes with a radius of membrane curvature of less than 1 μm (Fig. 3).

(d) DOPE / DOPC (3:1, mol / mol) + pristane-d_{40}

Rand et al. [40] reported recently that addition of tetradecane to a DOPE/DOPC (3:1, mol/mol) mixture transformed the lipid mixture from a liquid crystalline lamellar to an inverse hexagonal phase. As shown in Fig. 5, pristane also caused the transformation of part of the lipid into a hexagonal phase in a dose-dependent fashion. Measurements of the anisotropy of chemical shift of the ^{31}P-NMR signal of DOPE in the hexagonal phase in the presence of pristane did not show any significant changes of lipid order parameters in comparison with samples not containing pristane. The ^{2}H-NMR spectrum of deuterated pristane in the hexagonal phase consists of poorly resolved overlapping quadrupolar splittings with a maximum splitting around 1–2 kHz (data not shown). Pristane-d_{40} was incorporated into both lamellar and hexagonal phases in the same sample as indicated by a quadrupolar splitting pattern in ^{2}H-NMR. Due to overlapping resonance signals it was not possible to determine the exact amount of pristane in both phases.

Calorimetry

Because of the longer time necessary to achieve equilibrium incorporation of pristane after heating the

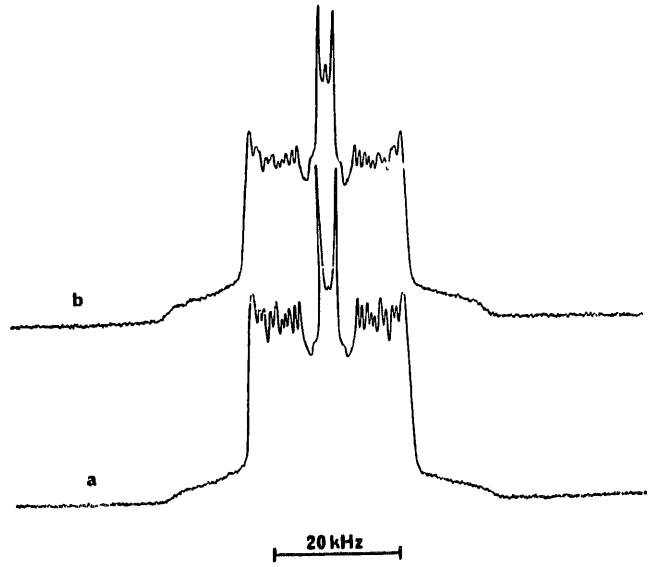

Fig. 3. ^{2}H-NMR spectra of (a) DPCC-d_{62} (50 wt.%) in water without pristane and (b) with pristane (74.3 mol%) at 50 °C.

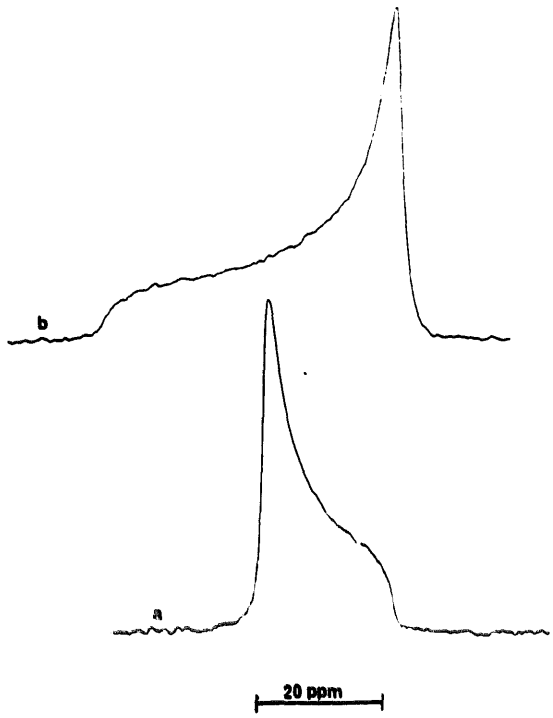

Fig. 4. ^{31}P-NMR spectra of DOPE (a, inverse hexagonal phase) and DOPC (b, lamellar phase) in the presence of pristane (a, pristane 4.2 mol%, b, pristane 3.7 mol%). The anisotropy of the chemical shift was +20.2 ppm (a) and −46.8 ppm (b).

samples into the liquid crystalline phase (see also NMR results on DPPC + pristane-d_{40}), samples were kept for several hours at 50 °C in the calorimeter prior to measurements that were performed in the cooling regime. Incorporation of 1–5 mol% pristane into liquid crystalline DPPC bilayers lowered the midpoint of the liquid crystalline-gel phase transition temperature from 41.5 °C to 40.9 °C. The DSC peaks were broadened slightly and were asymmetric, with a long tail towards lower temperatures. The tail ended always at 36 °C; i.e., at the same temperature at which the ^{2}H-NMR signal of pristane-d_{40} became completely isotropic (Fig. 6). Within an experimental error of 10%, the transition enthalpy was independent of the concentration of pristane. At pristane concentrations above 5 mol%, complex thermograms with intensities in the range from 41 °C to 36 °C were observed. The appearence of the thermograms at higher pristane concentrations depended strongly on the cooling rate and on sample handling (results not shown).

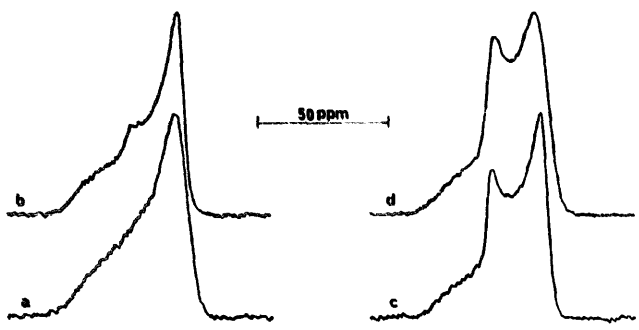

Fig. 5. Enhancement of the formation of an inverse hexagonal phase in DOPE/DOPC (3:1, mol/mol) mixtures by pristane. The concentration of pristane is given in mol% of the sum of molar concentrations of pristane and phospholipid: a, 5.5 mol%; b, 8.5 mol%; c, 12.7 mol%; d, 13.3 mol%.

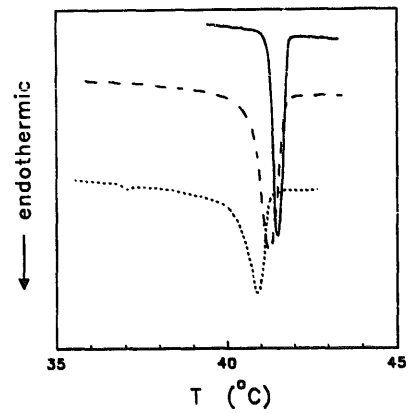

Fig. 6. DSC thermograms of dispersions of DPPC in water recorded in cooling regime: solid line, pure DPPC; dashed line, 2.5 mol% pristane in DPPC; dotted line, 5.0 mol% pristane in DPPC.

Uptake of pristane by liposomes

The uptake of pristane into model bilayer membranes was determined using tritiated pristane and small unilamellar DOPC liposomes with diameters of about 40 nm. The diameter of liposomes was determined by ^{31}P-NMR measurements after addition of the shift reagent $PrCl_3$. At ambient temperature, DOPC is in a liquid crystalline lamellar phase that is equivalent to the phase state of membrane phospholipids *in vivo*. Deuterium oxide was used instead of water since previous methodologic experiments showed a better separation between the liposome upper phase and the aqueous lower phase after ultracentrifugation (data not shown). Pristane was added as an aqueous suspension of β-CyD/pristane inclusion complexes. First, the reproducibility of the uptake of pristane by three different DOPC liposome preparations was measured. The uptake proved to be reproducible with variations of about 10% in the maximal amount of pristane taken up (data not shown). In order to estimate the kinetics of the uptake of pristane, the incubation time at 37 °C was varied from 1 to 12 h. Fig. 7 illustrates that the incorporation of pristane is complete after 1 h and that prolongation of the incubation time up to 12 h does not further increase the amount of pristane incorporated.

The difference between using two different modes of solubilizing pristane on its subsequent incorporation into DOPC liposomes was analyzed. Pristane was added either solubilized in DMSO or as a molecular inclusion complex with β-CyD. The latter method disperses pristane at the molecular level by individually encapsulat-

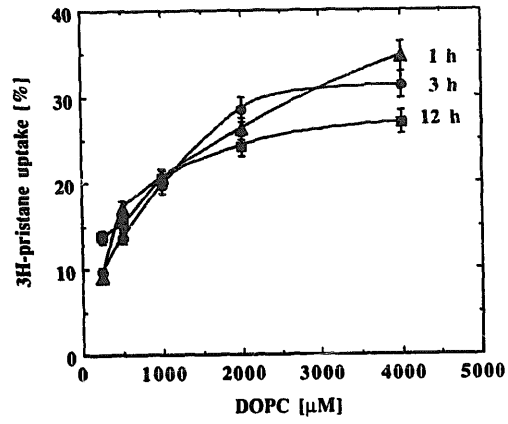

Fig. 7. Comparison of the uptake of pristane (final concentration 50 μM) solubilized in β-CyD into unilamellar DOPC liposomes during 1, 3, and 12 h at $T = 37\,^{\circ}$C. Measurements were made in duplicate.

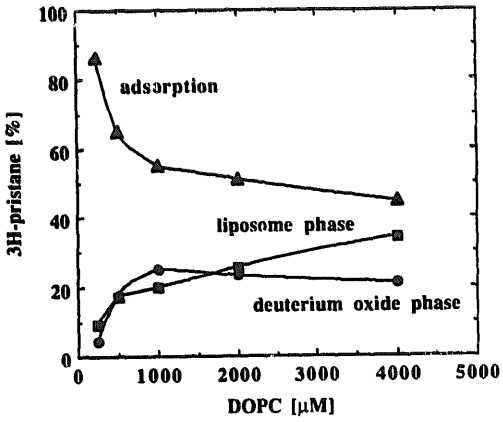

Fig. 8. Partitioning of β-CyD/pristane (final concentration 50 μM; $t = 12$ h; $T = 37\,^{\circ}$C) into the liposome phase (DOPC 250–4000 μM), the D_2O phase, and the surface phase of the plastic vial.

ing each hydrophobic pristane molecule with a hydrophilic β-CyD coat [23]. The amount of pristane in DOPC bilayers after incorporation *via* DMSO solubilization was less than 5% of the total pristane added (data not shown). Because of an experimental error of ±5% we were not able to determine the exact value. In contrast, solubilization of pristane in β-CyD led to substantially higher incorporation rates into liposomes. In Fig. 8 the partition of β-CyD/pristane into the three compartments of this simple system is given; i.e., the liposome phase, the D_2O phase, and the plastic surface of the centrifuge tube. When DMSO was used to solubilize pristane, no significant adsorption to the plastic surface was observed (data not shown).

Discussion

Incorporation of alkanes into lipid bilayers

Because of the wide use of alkanes in the formation of Black Lipid Membranes (BLM) numerous studies of *n*-alkane incorporation into lipid bilayers have been performed in the last 25 years. Pope et al. [36] found that *n*-alkanes with chain lengths of 12 carbon atoms or less could be dissolved in amounts above 10 mol% in all bilayers examined. The same authors showed that the solubility of the longer chain alkanes at concentrations around 10 mol% alkane increased with increasing acyl chain length of the matrix lipid. Cholesterol in the membranes reduced the solubility for the longer chain alkanes, whereas the solubility increased with increasing temperature. Using methylated alkanes, White [45] showed that the molecular volume of alkanes has only a second order effect on alkane incorporation.

Alkanes in general do not change the area per molecule in the liquid crystalline phase. Short chain alkanes, however, increase the thickness of the hydrophobic portion of the bilayer. Neutron diffraction measurements [46] show that *n*-hexane forms a layer in the middle between the two bilayers. The ^{2}H-NMR order parameters of fatty acid deuterated lipids in the liquid crystalline lamellar phase change little after addition of equimolar amounts of *n*-hexane [18,19]. The mode of incorporation and the amount of incorporated material seems to derive from a complicated interplay between the enthalpy and entropy difference of alkanes in a bulk phase and in ordered membranes. The enthalpy of interaction increases if the alkanes line up with the lipid molecule, while a less ordered state of alkane molecules in the middle of the bilayers is preferred entropically. With increasing alkane chain length the presence of alkanes in the center of bilayers is restricted to lower concentrations. The longer molecules are more likely to line up with the lipid fatty acid chains. Pope et al. [36] relate the solubility of alkanes to an increased motional freedom of lipid hydrocarbon chains in the middle of the bilayer. They conclude that alkanes dissolve in the disordered region in the middle of the membrane. The disordered region is larger for lipids with larger acyl chains and, therefore, these lipids incorporate more of the longer chain alkanes.

Incorporation of pristane into lipid bilayers

Addition of up to 5 mol% pristane to DOPC or DPPC liposomes *via* a chloroform solution results in the formation of a homogeneous lipid pristane mixture in which all pristane molecules occupy identical binding sites if the lipid is in the liquid crystalline lamellar phase. The apparent decrease of pristane order parameters in DOPC membranes at higher pristane concentrations is either connected with the occupation of binding sites of lower order in the same phase or the formation of other lipid structures containing increasing amounts of pristane. Possible candidates of other lipid structures containing pristane are pristane lenses covered by lipid monolayers and lipid micelles encapsulating pristane. How much of these structures is formed depends on the way pristane is added. Addition of plain pristane to the dry matrix lipid prior to the

addition of water results in formation of the above mentioned structures at pristane concentrations as low as 3 mol%. The remaining portion of pristane is built into regular bilayers and maintains the quadrupolar splitting of pristane that is characteristic of low concentrations in bilayers. In other words, samples in which pristane was added directly to the dry lipid are nonhomogeneous and are characterized by high local concentrations of the compound. The less ordered or isotropic spectral components in these samples resemble the structures obtained from chloroform preparations at higher concentrations of pristane. The differences in free energy of pristane between these structures are small. As a result, different forms of the incorporation of pristane coexist with low exchange rates of pristane molecules between them. The maximum quadrupolar splittings of pristane-d_{40} are somewhat smaller than those of *n*-alkanes of similar chain length [44], indicating a higher motional freedom and/or different orientations of the individual methyl and methylene segments of the molecule. This is supposedly caused by the influence of the bulky methyl groups of pristane. In contrast, the presence of pristane in a phospholipid bilayer does not seem to have any influence on the lipid order parameters in the headgroup and fatty acid regions; the mobility and conformation of phosphatidylcholine as measured by NMR are unchanged. Pristane molecules are unlikely to perturb intermolecular lipid interactions to any significant extent. In good agreement with this result is the observation that the quadrupolar splitting of pristane itself is not concentration dependent (up to 5 mol%). At low concentrations, the binding sites for pristane between the lipid fatty acid chains are independent of each other.

Incorporation of 5 mol% pristane into DPPC bilayers decreased the main phase transition temperature of DPPC only slightly. For the detection of these small changes it was critical to keep the samples for a longer time in the liquid crystalline phase and to perform the measurements in the cooling regime. The tailing of the DSC peaks down to a temperature of 36 °C was most likely connected with a redistribution of pristane in the dispersion. In previous studies it was observed that *n*-alkanes with a chain length of about 12 carbon atoms or less decrease the phase transition temperature of DPPC, while longer *n*-alkanes cause an increase [18,19,31]. The chain length of pristane corresponds to that of *n*-pentadecane. If even-odd effects of the number of carbon atoms can be neglected, *n*-pentadecane can be expected to increase the phase transition temperature slightly. But such an increase was not observed. We ascribe the deviation from the behavior of *n*-alkanes to the presence of bulky methyl groups along the chain in the pristane molecule. At temperatures below 36 °C pristane is either displaced from DPPC membranes or is incorporated in a way that results in isotropic motions of pristane molecules. The longer time necessary to achieve homogeneous incorporation of pristane molecules after heating the sample into the liquid crystalline phase suggests that diffusion controlled steps are involved in that process. This favors an interpretation that pristane formed microdroplets in the DPPC gel phase. Isotropic alkane signals in lipid gel phases have been observed for *n*-hexane [18,19].

Pristane, like other alkanes, increases the hydrophobic volume of bilayers. In lipid mixtures like DOPE/DOPC which are capable of transforming into nonlamellar phases, pristane helps to reduce the packing stress in the hydrocarbon region, making the formation of a hexagonal lipid phase more likely. The formation of particles with radii of curvature less than 1 μm after pristane addition could be caused by a similar effect. That behavior might suggest that the rate of pristane incorporation depends strongly on membrane curvature [15], but until now we have no direct evidence for this. ^{2}H-NMR of deuterated pristane in coexisting lamellar and hexagonal phases showed that pristane is located in both phases. Nonetheless, we can not rule out a slightly different uptake of pristane by one of the phases

When pristane solubilized in β-CyD was added to liposome preparations, a maximal uptake of about 2 mol% relative to the dry weight of DOPC was observed. This amount is considerably lower than the 5 mol% of pristane homogeneously incorporated after its prior mixing with matrix lipid in chloroform. The lower uptake of pristane in the liposome experiments is not surprising considering that at least four different phases that compete for pristane need to be taken into account; (i) the watery phase, (ii) the β-CyD phase, (iii) the plastic surface, and (iv) the lipid phase. The amount of pristane in these phases depends on the concentration of binding sites and on the differences between them in the free energy of pristane. Moreover, in the NMR experiments using multilamellar liposomes the lipid concentration was considerably higher (625 mM) than in the tritiated pristane experiments employing unilamellar liposomes (4 mM). It is aiso important to realize that increased solubility of pristane in the watery phase caused by the addition of β-CyD improves the establishment of an equilibrium distribution of pristane between all binding sites, while at the same time decreasing the amount of pristane in the membrane at thermodynamic equilibrium. The complexity of the thermodynamic picture is further illustrated by the effects of the vehicle on delivery of pristane to the liposome matrix. Solubilization of pristane in β-CyD, as compared to DMSO, led to significantly higher incorporation rates. This result is intriguing since these solubilization methods differ in principal. Whereas DMSO is an organic solvent that simply dissolves pristane, β-CyD disperses the compound at the molecular

level by wrapping individual molecules with ring-shaped oligomers of glucose that have a hydrophobic molecular core and a hydrophilic surface. Thus, the β-CyD method solubilizes pristane in an aqueous phase in the complete absence of organic solvents.

Biological significance of the incorporation of pristane into biomembranes

The finding of an enhancement of the uptake of pristane into unilamellar liposomes after its solubilization with β-CyD seems particularly important from the practical point of view; i.e., for devising experiments to test effects of pristane on cells in aqueous tissue culture media. In the past, such experiments have been hampered by insufficient bioavailability of pristane after its dissolution in DMSO. This situation is very similar to other systems where the solubilization of alkanes proved to be the limiting step for studying their biological effect [9,32]. Using β-CyD-solubilization we now obtain reproducible results when assaying toxicity, mitogenicity, and genotoxicity of pristane to bacteria and to various B lymphocyte cell lines [22,23].

During the uptake of pristane by a cell, this hydrophobic compound should partition into the membrane interior where it may act as a perturbant. Using a ^{51}Cr release short-term cytotoxicity assay, we determined that the critical concentration of pristane (solubilized in β-CyD) capable of disrupting cells during an 120 min incubation was about 100–200 μM, depending on the cell type used [23]. Yet, the exact nature of this membrane perturbing activity has not been clarified. At the molecular level as measured by NMR and DSC, the perturbation of membrane lipids after incorporation of pristane is minor. However, at the cellular level these small changes may add up to significant alterations which may be even sufficient to kill a cell. One possible mechanism for membrane lysis after addition of pristane may be derived from the observation of the lamellar-hexagonal phase transition in DOPE/DOPC lipid mixtures. If induced in a plasma membrane, this phase transition would be lethal for a cell. At sublytic concentrations, pristane may alter physiologic membrane activities such as cell fusion, pore formation, vesiculation, ion channel activities, and the activity of integral membrane proteins [6,16,24].

The generalization has been made that non-genotoxic carcinogens are primarily hydrophobic. It has further been proposed that in the absence of specific membrane receptor mediated effects, some compounds derive their activity solely from their hydrophobicity [30]. We speculate this may be the case for pristane. Along these lines, C_{10} to C_{18} *n*-alkanes are tumor promoters in murine skin carcinogenesis [2]. A strong correlation has been observed between the length of the alkyl chains and promotional activity of organic peroxides and hydroperoxides in murine skin carcinogenesis [42]. We still do not know how hydrophobicity alters cell functions. Further studies are warranted to see to which extent the concentration of pristane in membranes, the alterations of membrane properties, and the biological effects of this compound are correlated.

Acknowledgements

We thank the 'In vivo NMR Research Center' at NIH for providing the Bruker MSL 300 spectrometer. We are indebted to Dr. Michael Potter from the Lab. Genetics, NCI, NIH for suggesting these studies. We are grateful to Dr. Emily Shacter, also from the Lab. Genetics, for critical reading of the manuscript. The authors are supported through the NIH's Fogarty International Program.

References

1 Anderson, P.N. and Potter, M. (1969) Nature 222, 994–995.
2 Baxter, C.S. and Miller, M.L. (1987) Carcinogenesis 8, 1787–1790.
3 Bly, J.E., Garrett, L.R. and Cuchens, M.A. (1990) Cancer Biochem. Biophys. 11, 145–154.
4 Bost, K.L., Garrett, L.R. and Cuchens, M.A. (1986) Carcinogenesis 7, 1257–1265.
5 Burnell, E.E., Cullis, P.R. and De Kruijff, B. (1980) Biochim. Biophys. Acta 603, 63–69.
6 Cossins, A.R. and Sinensky, M. (1984) in Physiology of Membrane Fluidity, Vol. 2 (Shinitzky, M., ed.), pp. 1–20, CRC Press, Boca Raton.
7 Davis, H.D. (1979) Biophys. J. 27, 339–358.
8 Davis, J.H., Jeffrey, K.R., Bloom, M., Valic, M.I. and Higgs, T.P. (1976) Chem. Phys. Lett. 42, 390–394.
9 Delraso, N.J., Mattie, D.R. and Godin, C.S. (1989) In vitro Cell. Devel. Biol. 2, 1031–1038.
10 Freund, Y.R. and Blair, P.B. (1982) J. Immunol. 129, 2826–2830.
11 Garrett, L.R., Bost, K.L., Buttke, T.M. and Cuchens, M.A. (1987) Agents Actions 20, 104–112.
12 Garrett, L.R., Pascual, D.W., Clem, L.W. and Cuchens, M.A. (1987) Chem.-Biol. Interact. 61, 249–263.
13 Gawrisch, K., Richter, W., Möps, A., Balgavy, P., Arnold, K. and Klose, G. (1985) Stud. Biophys. 108, 5–16.
14 Gawrisch, K., Stibenz, D., Möps, A., Arnold, K., Linsz, W. and Halbhuber, K.-J. (1986) Biochim. Biophys. Acta 856, 443–447.
15 Gruen, D.W.R. and Haydon, D.A. (1980) Biophys. J. 30, 129–136.
16 Henry, G.D. and Sykes, B.D. (1990) Biochem. Cell Biol. 68, 318–329.
17 Ho, F.C.S. and Fu, K.H. (1987) Br. J. Exp. Pathol. 68, 413–420.
18 Jacobs, R.E. and White, S.H. (1984) J. Am. Chem. Soc. 106, 915–920.
19 Jacobs, R.E. and White, S.H. (1984) J. Am. Chem. Soc. 106, 6909–6912.
20 Horton, A.W., Bolewicz, L.C., Barstad, A.W. and Butts, C.K. (1981) Biochim. Biophys. Acta 648, 107–112.
21 Janz, S., Birkenfeld, T., Herzschuh, R. and Storch, H. (1989) Arch. Geschwulstforsch. 59, 415–422.
22 Janz, S., Brede, O. and Müller, J. (1991) Carcinogenesis 7, 1241–1246.
23 Janz, S. and Shacter, E. (1991) Toxicology, in press.
24 Jensen, J.W. and Schutzbach, J.S. (1989) Biochemistry 28, 851–855.

25 Klose, G., König, B., Meyer, H.W., Schulze, G. and Degovics, G. (1988) Chem. Phys. Lipids 47, 225–234.
26 Kripke, M.C. and Weiss, D.W. (1970) Int. J. Cancer 6, 422–430.
27 Le Bon, A.M., Cravedi, J.-P. and Tulliez, J.E. (1988) Lipids 23, 424–429.
28 Luquet, P., Cravedi, J.-P., Tulliez, J. and Bories, G. (1984) Ecotoxicol. Environ. Saf. 8, 219–226.
29 Mabrey, S. and Sturtevant, J.M. (1976) Proc. Natl. Acad. Sci. USA 373, 3862–3866.
30 McCoy, G.D., Rosenkranz, H.S. and Klopman, G. (1990) Carcinogenesis 11, 1111–1117.
31 McIntosh, T.J., Simon, S.A. and MacDonald, R.C. (1980) Biochim. Biophys. Acta 597, 445–463.
32 Miller, R.M. and Bartha, R. (1989) Appl. Environ. Microbiol. 55, 269–274.
33 Nobis, P. and Löhler, J. (1983) Haematol. Blood Transfus. 28, 280–281.
34 Nordan, R.P. and Potter, M. (1986) Science 233, 566–569.
35 Platica, M., Bojko, C., Steiner, G. and Hollander, V.P. (1980) Cancer Res. 40, 2229–2233.
36 Pope, J.M., Littlemore, L.A. and Westerman, P.W. (1989) Biochim. Biophys. Acta 980, 69–76.
37 Potter, M. (1987) Adv. Viral Oncol. 7, 99–122.
38 Potter, M. (1990) Carcinogenesis 11, 1–13.
39 Rance, M. and Byrd, R.A. (1983) J. Magn. Res. 52, 221–240.
40 Rand, R.P., Fuller, N.L., Gruner, S.M. and Parsegian, V.A. (1990) Biochemistry 29, 76–87.
41 Shacter, E., Beecham, E.J., Covey, J.M., Kohn, K.W. and Potter, M. (1988) Carcinogenesis 9, 2297–2304.
42 Slaga, T.J., Solanki, V. and Logani, M. (1983) in Radioprotectors and Anticarcinogens (Nygaard, O.F. and Simic, M.G., eds.), pp. 471–485, Academic Press, Orlando.
43 Tokumura, T., Ueda, H., Tsushima, Y., Kasai, M., Kayano, M., Amada, I., Machida, Y. and Nagai, T. (1984) J. Incl. Phenom. 2, 511–521.
44 Ward, A.J.I., Friberg, S.E., Larsen, D.W. and Rananavare, S.B. (1984) J. Phys. Chem. 88, 826–827.
45 White, S.H. (1977) Ann. N.Y. Acad. Sci. 303, 243–265.
46 White, S.H., King, G.I. and Cain, J.E. (1981) Nature 290, 161–163.
47 Wooley, P.H., Seibold, J.R., Whalen, J.D. and Chapdelaine, J.M. (1989) Arthritis Rheum. 32, 1022–1030.
48 Yerganian, G., Paika, I., Gagnon, H.J. and Battaglino, A. (1979) Prog. Exp. Tumor Res. 24, 424–434.