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Electron spin resonance study of the interactions of retinoids with a phospholipid model membrane

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The effects of up to 20 mol% incorporation of all-*trans*-retinol (vitamin A), retinal (vitamin A aldehyde) and retinoic acid (vitamin A acid) on acyl chain order and dynamics in liquid crystalline dipalmitoylphosphatidylcholine membranes at pH 7.5 were studied by electron spin resonance (ESR) of 5-, 7-, 10-, 12- and 16-doxyl spin-labelled stearic acids intercalated into the membrane. Order parameters S and correlation times τ_c determined from the ESR spectra demonstrate that the influence of retinoic acid differs from retinol or retinal. Whereas the latter two retinoids have negligible effect (less than 1%) on acyl chain order towards the membrane surface (5 position), retinoic acid reduces the order parameter by as much as 8% at 20 mol% incorporation. All three retinoids restrict acyl chain motion to a similar extent approaching the center of membrane (10, 12 and 16 positions), where up to 22% increases in order parameter and correlation time were observed. Complementary osmotic swelling and carboxyfluorescein release measurements show that the enhancement in permeability of egg phosphatidylcholine membranes to erythritol and carboxyfluorescein is greater with all-*trans*-retinoic acid than all-*trans*-retinol or retinal.

Introduction

The retinoids (vitamin A and derivatives) – retinol (vitamin A), retinal (vitamin A aldehyde) and retinoic acid (vitamin A acid) – form a homologous series of lipid-soluble molecules that are essential for the maintenance of health of an organism [1]. Although they vary in structure only at the polar end, their biological functions differ markedly. Retinol promotes reproduction, retinal is necessary for vision while retinoic acid, which does not support either of the two aforementioned processes, is active in growth promotion. The amphiphilic nature of the retinoids suggests that they will locate in membranes and that the membrane may constitute a site of action. Indeed, retinal is, together with phospholipid and protein, a major component in visual receptor membranes. More pertinently to the present investigation, high doses of retinoid are toxic and membrane disruption may be responsible. Elucidation of this point is important, since toxicity is a major drawback to the reported therapeutic use of retinoids in treating several cancers [2].

Several studies have shown that retinoids modify the properties of membranes. Decreased mem-

Abbreviations: DPH, diphenylhexatriene; PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; CF, carboxyfluorescein.

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brane microviscosities due to retinoids were seen by fluorescence polarization of 1,6-diphenylhexatriene (DPH) in rat erythrocyte ghosts and mouse fibroblasts [3,4]. All-*trans*-retinol and retinal were found to exhibit a concentration-dependent influence on order within phospholipid model membranes as monitored by electron spin resonance (ESR) of cholestane [5]. At low concentrations (less than 20 wt.% for phosphatidylcholine membranes) increased order was observed, while at high concentrations order was decreased. In contrast, 13-*cis*-retinal only led to reduced order. Stillwell and co-workers have reported extensively on retinoid-associated increases in membrane permeability to non-electrolytes and ions [6–8]. All-*trans*-retinol enhancement of phosphatidylcholine (PC) membrane permeability to urea, erythritol and water, to K^+, Cl^-, Br^- and I^-, to glucose, glycine and lysine and to [^{14}C]glucose were measured by osmotic swelling, ion-specific electrodes, colorimetric assay and radiotracer methods, respectively; while all-*trans*-retinoic acid enhancement of PC membrane permeability to water, to K^+ and I^- and to glucose were measured by osmotic swelling, ion-specific electrodes and colorimetric tests, respectively. These researchers also saw that all-*trans*-retinol and retinoic acid decrease the electrical resistance of black lipid membranes prepared from egg PC and that the temperature of the gel to liquid crystalline phase transition, as monitored by the variations with temperature of permeability to water and of liposome size, was lowered for dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) membranes. Greater effects were obtained with retinoic acid. More recently, differential scanning calorimetry (DSC) studies employing 0–20 mol% incorporation of all-*trans*-retinol, retinal and retinoic acid into DPPC membranes have been performed [9]. The studies demonstrated that all three retinoids abolish the pretransition at 5 mol% or less, and that the main gel-liquid crystalline phase transition is broadened and its onset temperature lowered with increasing retinoid concentration.

In the present communication we compare the effects of all-*trans*-retinol, retinal and retinoic acid on acyl chain motion and permeability in PC membranes. ESR of nitroxide spin labelled (5-, 7-, 10-, 12- and 16-doxyl) stearic acids intercalated at low concentration into DPPC multilamellar liposomes was employed to determine the influence each retinoid has on acyl chain order and mobility throughout the membrane interior. Free fatty acids have been shown to be reliable membrane probes [10] and the utility of the ESR spin label approach has been demonstrated extensively [11]. In the upper portion of the fatty acid chain (the 5, 7 and 10 position), where molecular motion is sufficiently anisotropic to produce spectra in which outer and inner hyperfine extrema are defined, order parameters S may be calculated according to

$$S = \frac{A_{\parallel} - A_{\perp} - C}{A_{\parallel} + 2A_{\perp} + 2C}(1.66) \tag{1}$$

where $A_{\parallel}$ and $A_{\perp}$ are the apparent parallel and perpendicular hyperfine splitting parameters of the spectra, the constant $C = 1.4 - 0.053\ (A_{\parallel} - A_{\perp})$ is an empirical correction for the difference between the true and apparent values of $A_{\perp}$, and the factor 1.66 is a solvent polarity correction factor [12]. In the lower portion of the chain (the 12 and 16 position) correlation times τ_c may be estimated from spectra characteristic of high disorder on the assumption of isotropic motion according to

$$\tau_c = 6.5 \cdot 10^{-10} W_0 \left[\left(\frac{h_0}{h_{-1}} \right)^{1/2} - 1 \right] \tag{2}$$

where W_0 is the peak-to-peak width of the central line, and h_0/h_{-1} is the ratio of the heights of the central and high field lines, respectively [13]. As the molecular motions are not truly isotropic, the τ_c values calculated are not true correlation times. They are determined by the degree of anisotropy of motion as well as by the rate.

The effects of all-*trans*-retinol, retinal and retinoic acid on membrane permeability were investigated to complement the ESR work. The permeability to erythritol of egg PC and egg PC/retinoid multilamellar liposomes was measured by an osmotic swelling technique [14]. In this method, liposomes, prepared in a glucose solution, are introduced into an isotonic erythritol solution and their subsequent initial rate of swelling is controlled by erythritol leakage. Liposome swelling

was followed by optical absorbance A [15], where the initial rate of change of absorbance with time $\Delta A/\Delta t$ is proportional to the rate of change of liposome volume [16] and the initial swelling velocity $d(1/A)/dt$ % has previously been shown to be proportional to permeability [17].

The release of sequestered carboxyfluorescein (CF) from large unilamellar vesicles was also observed to compare retinoid-induced changes in the permeability of egg PC bilayers [18]. High concentrations of CF trapped within vesicles are essentially self quenched [19], enabling the rate of release and hence permeation of CF to be monitored from the increase in fluorescent signal with time.

Materials and Methods

Materials

DPPC and egg PC were purchased from Avanti Polar Lipids or Sigma Chemical Co. All-*trans*-retinol, retinal and retinoic acid were obtained from Aldrich Chemical Co. or Sigma. Molecular Probes was the source of 5-, 7-, 10-, 12- and 16-doxyl spin-labelled stearic acids (β-5-, 7-, 10-, 12- and 16-(4′,4′-dimethyloxazolidinyl-*N*-oxyl)stearic acids). Erythritol and decanoic acid were bought from Sigma. Eastman Kodak supplied CF and decanol.

Preparation of samples

Multilamellar liposomes of phospholipid in the presence or absence of retinoid, decanoic acid or decanol were prepared in aqueous dispersion by vortex mixing at temperatures at least 10 °C above the gel to liquid crystalline phase transition. Prior to hydration the lipid mixtures were codissolved in chloroform and the solvent was then removed under a stream of nitrogen followed by vacuum pumping overnight.

ESR experiments

An IBM/Bruker ER 200D X-band ESR spectrometer with variable temperature capability was employed. The spectrometer is interfaced to and controlled by a Hewlett Packard 9816 computer system with graphics display unit and Winchester disk drive. Order parameters S and correlation times τ_c were obtained, where possible, by direct computer calculation from the spectra according to Eqns. 1 and 2. Uncertainties in S and τ_c are less than $\pm 1\%$ and $\pm 2\%$ or less, respectively. The phospholipid concentration of the liposomes was 1% w/v DPPC in 20 mM phosphate/1 mM ethylenediaminetetraacetic acid (EDTA) buffer (pH 7.5). They contained 1 mol% spin label, which was dispensed as a 5 mM solution in ethanol and mixed with the lipids in chloroform before solvent removal and subsequent hydration. This choice of sample and spin label concentrations in general provided an excellent spectral signal to noise ratio. Occasionally, a noise reduction scheme [20] was applied selectively to poorly defined regions of spectra if crucial to parameter calculation. The sample temperature was 50 ± 0.5 °C, which ensures that the membranes were in the biologically relevant liquid crystalline state [21].

Osmotic swelling experiments

Liposome swelling in isotonic erythritol was monitored from the rate of change with time t in optical absorbance A at 500 nm using a Beckmann DU-8 Computing Spectrophotometer. The liposomes (13 mM phospholipid) were prepared from egg PC and egg PC/retinoid in 40 mM glucose/10 mM phosphate (pH 7.0) solution. Small aliquots were then rapidly injected into the 40 mM erythritol/10 mM phosphate (pH 7.0) swelling buffer and the initial swelling velocity $d(1/A)/dt$ % measured. Each determination was repeated at least five times and the averages, standard errors of $\pm 4\%$ or less, are reported. The temperature was controlled within ± 0.1 C°.

Carboxyfluorescein release experiments

Large unilamellar vesicles of egg PC or egg PC/20 mol% retinoid were prepared from multilamellar liposomes by first passing the liposome dispersion 500 times through a Liposor Liposome Cylinder (Lidex Technologies, Inc., Bedford, MA) and then sequentially extruding the resultant dispersion through 1.0 and 0.2 μm Nucleopore filters [22]. This procedure produced consistently sized vesicles, as demonstrated by light scattering at 775 nm, with similar trapping efficiencies (8% for a lipid concentration of 22 mM). The sequestered volume was determined by the method of trapping water-soluble markers, where Methylene blue was

the dye used [23]. The efficiency quoted represents the average of 16 determinations which indicated that trapped volume was slightly higher in the presence of retinoid.

The vesicles initially contained 100 mM CF/25 mM phosphate (pH 7.0), at which concentration the fluorescence of CF is almost totally self quenched [19]. After passing through a Sephadex G-50 column to remove non sequestered CF, the permeation of CF from within vesicles, expressed as a percentage of initial trapped CF, was monitored as a function of time at 45 °C by a Turner Model 110 fluorimeter. Excitation was at 470 nm while emission was at 515 nm. Total trapped CF was ascertained by release in Triton X-100 [24].

Results

ESR

The effect of 20 mol% incorporation of all-*trans*-retinol and retinal on ESR spectra recorded for 5-doxylstearic acid intercalated (1 mol%) into multilamellar liposomes of 1% w/v DPPC in 20 mM phosphate/1 mM EDTA buffer (pH 7.5) at 50 °C is shown in Fig. 1. The spectra are characteristic of the anisotropic motion anticipated in the upper portion of the acyl chain, enabling calculation of an order parameter S via Eqn. 1, and demonstrate that both retinoids have a negligible influence on acyl chain ordering at the 5 position. The situation differs from that illustrated by Fig. 2 where the effects of 10 and 20 mol% incorporation of all-*trans*-retinoic acid are shown. As can be seen, higher concentrations of this retinoid progressively decrease order within the membrane at the 5 position.

Fig. 3 shows the effect of 20 mol% incorporation of all-*trans*-retinol, retinal and retinoic acid on ESR recorded for 16-doxylstearic acid intercalated (1 mol%) into multilamellar liposomes of 1% w/v DPPC in 20 mM phosphate/1 mM EDTA buffer (pH 7.5) at 50 °C. Acyl chain motion in the lower portion of the chain is highly disordered, so that the spectra correspond to approximately isotropic motion and correlation times τ_c may be calculated by Eqn. 2. The spectra establish that, irrespective of the particular retinoid, correlation times at the 16 position are increased to a similar extent by incorporation of all three retinoids into

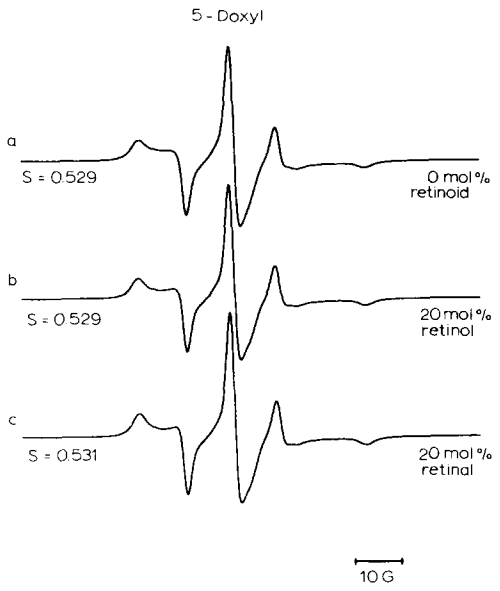

Fig. 1. The effect of all-*trans*-retinol and retinal incorporation of ESR spectra for 5-doxylstearic acid (1 mol%) intercalated into 1% w/v multilamellar dispersions of DPPC in 20 mM phosphate/1 mM EDTA buffer (pH 7.5) at 50 °C: (a) 0 mol% retinoid; (b) 20 mol% retinol; and (c) 20 mol% retinal.

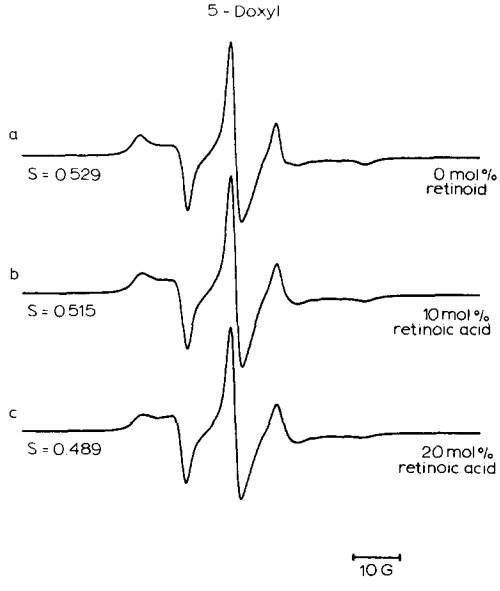

Fig. 2. The effect of all-*trans*-retinoic acid incorporation on ESR spectra for 5-doxylstearic acid (1 mol%) intercalated into 1% w/v multilamellar dispersions of DPPC in 20 mM phosphate/1 mM EDTA buffer (pH 7.5) at 50 °C: (a) 0 mol% retinoid; (b) 10 mol% retinoic acid; and (c) 20 mol% retinoic acid.

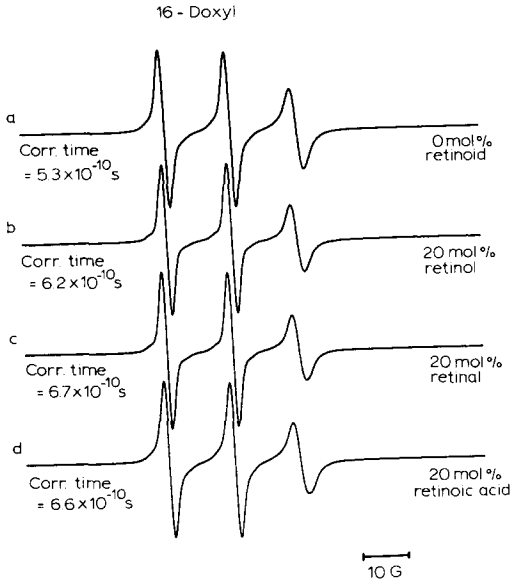

Fig. 3. The effect of all-*trans*-retinoid incorporation on ESR spectra for 16-doxylstearic acid (1 mol%) intercalated into 1% w/v multilamellar dispersions of DPPC in 20 mM phosphate/1 mM EDTA buffer (pH 7.5) at 50 °C: (a) 0 mol% retinoid; (b) 20 mol% retinol; (c) 20 mol% retinal; (d) 20 mol% retinoic acid.

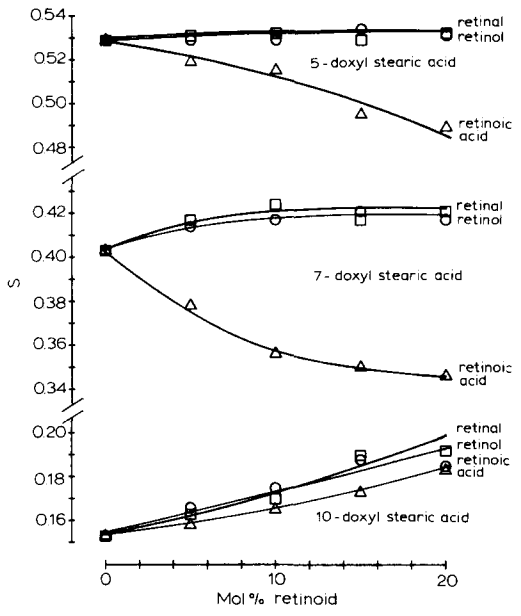

Fig. 4. All-*trans*-retinoid concentration dependence of order parameters S for (a) 5-, (b) 7- and (c) 10-doxylstearic acids (1 mol%) intercalated into 1% w/v multilamellar dispersions of DPPC in 20 mM phosphate/1 mM EDTA buffer (pH 7.5) at 50 °C. ○, Retinol; □, retinal and △, retinoic acid.

the bilayer. Thus, the difference in effect on acyl chain motion of all-*trans*-retinoic acid vs. all-*trans*-retinol or retinal seen in the upper portion of the chain is not apparent in the lower portion.

More details of the changes in molecular dynamics within the membrane interior produced by incorporation of all-*trans*-retinol, retinal and retinoic acid are given in Figs. 4 and 5. Order parameters and correlation times are plotted as a function of retinoid concentration for 5-, 7- and 10-doxylstearic acids (Fig. 4) and for 12- and 16-doxylstearic acids (Fig. 5), respectively, in DPPC multilamellar liposomes at 50 °C (pH 7.5). The concentration dependence depicted at the 5 position confirms that retinol and retinal have very little effect (less than 1%) on acyl chain order, whereas retinoic acid causes disorder. At 20 mol% incorporation of retinoic acid the reduction in order parameter is 8%. The behaviour at the 7 position is similar. Retinol and retinal slightly order the membrane (at least 4%), while retinoic acid reduces the order parameter by as much as 14% at 20 mol% incorporation. In contrast, at the 10 position all three retinoids have an essentially

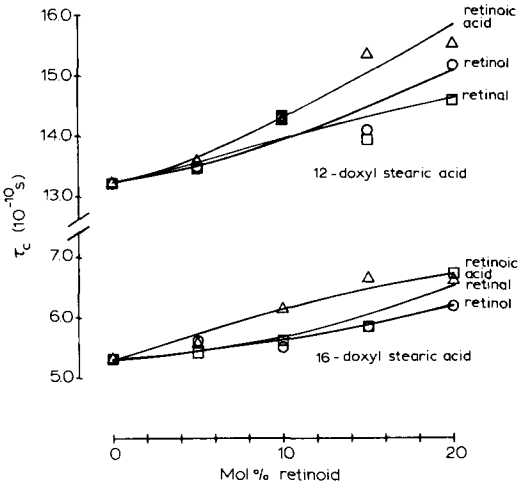

Fig. 5. All-*trans*-retinoid concentration dependence of correlation times τ_c for (a) 12- and (b) 16-doxylstearic acids (1 mol%) intercalated into 1% w/v multilamellar dispersions of DPPC in 20 mM phosphate/1 mM EDTA buffer (pH 7.5) at 50 °C. ○, Retinol; □, retinal and △, retinoic acid.

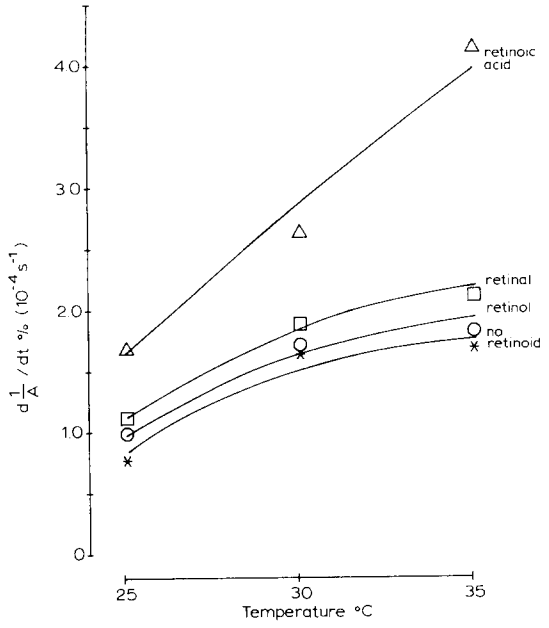

Fig. 6. Initial osmotic velocities $d(1/A)/dt$ % in isotonic erythritol vs. temperature (pH 7.0) for multilamellar liposomes of egg PC (13 mM) in the absence and presence of 20 mol% all-*trans*-retinoid. ×, No retinoid; ○, retinol; □, retinal; △, retinoic acid.

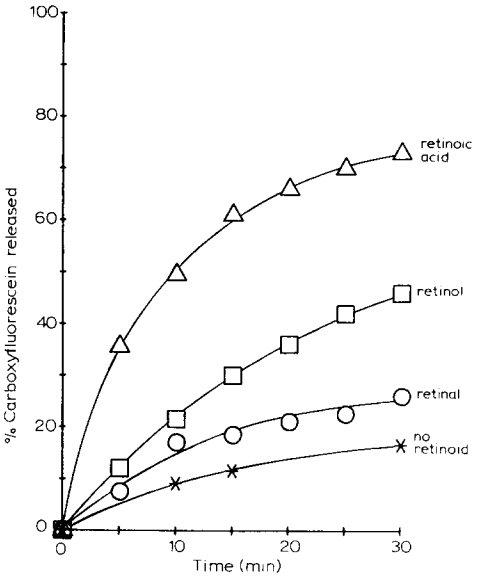

Fig. 7. Time dependence of the leakage of CF from large unilamellar vesicles of egg PC with and without all-*trans*-retinoid at 45 °C (pH 7.0). ×, No retinoid; □, retinol; ○, retinal; △, retinoic acid.

identical effect and lead to an approximately 22% greater order in the presence of 20 mol%. The same trend is qualitatively exhibited at the 12 and 16 positions. Correlation times are increased to a similar degree by all three retinoids, approximately 14 and 22% increases being measured with 12- and 16-doxylstearic acids, respectively, when 20 mol% retinoid is introduced.

Osmotic swelling

Initial osmotic swelling velocities $d(1/A)/dt$ % in isotonic erythritol for multilamellar liposomes of egg PC in the absence and presence of 20 mol% all-*trans*-retinol, retinal or retinoic acid (pH 7.0) are plotted against temperature in Fig. 6. As the quantity $d(1/A)/dt$ % is proportional to the rate of membrane permeability to erythritol [17], the graph displays the effect each retinoid has on permeability to a small non-electrolyte molecule. In agreement with the ESR data, the influence of retinoic acid contrasts with that of retinol or retinal. Specifically, the latter two retinoids cause little change in permeability, whereas retinoic acid substantially enhances permeability. The enhancement becomes greater with increasing temperature.

Carboxyfluorescein release

The time dependence of the release of CF from large unilamellar vesicles of egg PC with and without 20 mol% all-*trans*-retinol, retinal or retinoic acid (pH 7.0, 45 °C) is depicted in Fig. 7. The behaviour monitored for the fluorescent ion is qualitatively consistent with the osmotic swelling results, since CF leakage clearly occurs most quickly in the presence of retinoic acid. Relative enhancements in permeability with respect to a control reference of pure egg PC bilayers, as estimated by comparison of initial release rates (i.e., initial slopes in graph), are 2-, 3- and 11-fold for retinal, retinol and retinoic acid, respectively.

Discussion

The ESR results indicate that all-*trans*-retinol and retinal have essentially the same effect on acyl chain ordering and dynamics within DPPC bilayers. Order in the upper portion of the chain, as

monitored at the 5 position, is virtually unaffected, while increased order parameters and correlation times are produced in the lower region (Figs. 4 and 5). The observation of increased order is consistent with earlier ESR work in which the spin label cholestane detected that low concentrations (less than 20 mol%) of all-*trans*-retinol and retinal cause higher order in phospholipid bilayers [5]. Corey-Pauling-Koltun space filling molecular models suggest that the profile of effect along the acyl chain described here is physically plausible. The bulky hydrophobic cyclohexene group would be expected to locate, and hence restrict acyl chain motion, in the lower region of the lipid chain. Location of the hydrophilic alcohol or aldehyde group near the aqueous interface would not be expected to have a dramatic steric and/or motional influence on acyl chain motion in the upper portion of the chain.

Order parameters measured at the 5 and 7 position, in contrast, indicate that all-*trans*-retinoic acid disorders the upper region of the acyl chain (Fig. 4). In the lower portion, as monitored at the 10, 12 and 16 position, molecular motion is restricted to a similar extent to that seen with retinol or retinal (Figs. 4 and 5). These apparently contradictory effects on molecular motion in different parts of the acyl chain appear to be specific to retinoic acid. Incorporation of a lipophilic compound into a model membrane usually seems to have the same general effect throughout the chain, e.g., the motional restriction imposed by cholesterol [25]. Previous fluorescence polarization studies have reported that incorporation of all-*trans*-retinoic acid into erythrocyte ghosts and mouse fibroblasts increases membrane fluidity [3,4]. Given the uncertainty in te precise transverse location of the DPH probe employed [26], it is impossible to decide whether agreement exists between the fluorescence work on biological membranes and the current study of a phospholipid model membrane. The presence of protein, alternatively, could be influencing the behaviour seen.

An explanation for the different profile of effect on acyl chain motion produced by retinoic acid, as opposed to by retinol or retinal, is proposed in terms of the strongly hydrophilic nature of the carboxylic group. This, we contend, would place retinoic acid higher within the membrane than retinol or retinal and tend to force phospholipid molecules apart at the aqueous interface. Such a disruption would loosen acyl chain packing in the upper region of the chain and so lead to increased disorder. In the lower region, however, the bulky cyclohexene group would, as with retinol and retinal, hinder motion.

A schematic representation of our explanation is presented in Fig. 8 for retinol and retinoic acid. The hydroxyl group of retinol is placed near the carbonyl group of the phospholipid, which would facilitate the assumed hydrogen bonding betwen the two groups. Support for this approximate depth within the membrane for the hydroxyl group is additionally derived from a recent deuterium nuclear magnetic resonance (^{2}H-NMR) study of selectively deuterated 1-decanol in DPPC bilayers [27]. From a comparison of quadrupole splittings recorded for alkanol and phospholipid, it was concluded that the hydroxyl group of 1-decanol is at the level of C-2 (*sn*-1 chain) of the phospholipid. The carboxylic group of retinoic acid, which would be ionized at pH 7.5, is assumed to project into the head group region of the membrane because it is strongly hydrophilic. A location in the vicinity of phospholipid phosphate is depicted in Fig. 8. Spin label-induced carbon-13 nuclear magnetic resonance (^{13}C-NMR) relaxation rates measured for egg PC vesicles following the incorporation of doxylstearic acids suggest that the carboxyl group of the fatty acid is located at approximately this position [28]. The relative depths of penetration illustrated for the two retinoids thus indicate that, while retinol would produce minimal perturbation to molecular packing at the membrane surface, since its polar group resides below the surface, because retinoic acid protrudes through the aqueous interface it would tend to push phospholipid molecules apart and disrupt molecular packing towards the membrane surface. The cyclohexene group at the opposite end of both retinoids would, nevertheless, similarly restrict acyl chain motion in the membrane interior.

Experimental evidence in favour of the proposal that the carboxyl group of retinoic acid leads to membrane disordering in the upper portion of the acyl chain is provided by the effects of decanol and decanoic acid on ESR spectra for 5-doxylstearic acid intercalated (1 mol%) into mul-

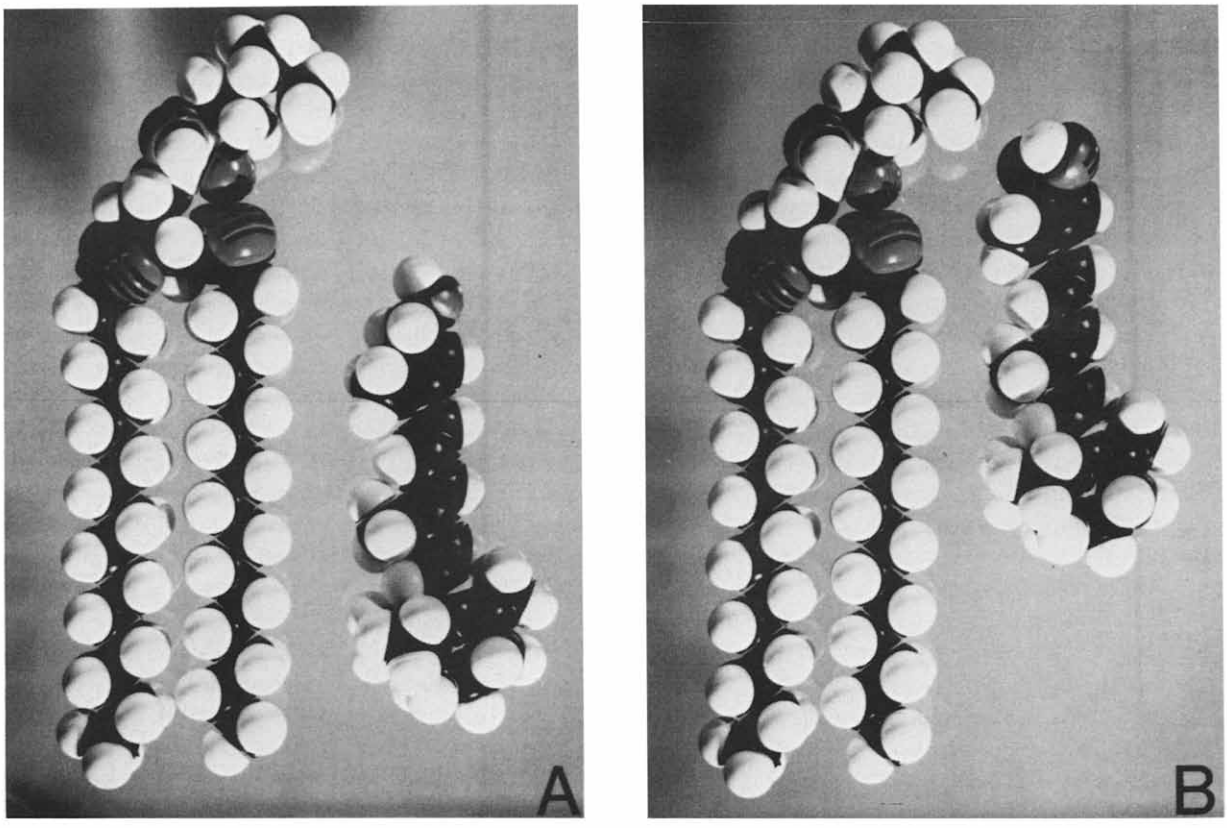

Fig. 8. Schematic representation of the positions relative to DPPC within the membrane of retinol (A) and retinoic acid (B).

tilamellar liposomes of DPPC (1% w/v) in 20 mM phosphate/1 mM EDTA buffer (pH 7.5) at 50 °C. These two lipids were chosen for comparison as their chain length is comparable to that of retinol and retinoic acid, and they possess the same hydroxyl vs. carboxyl head group difference. Fig. 9 shows a graph of order parameter against decanol and decanoic acid concentration. In agreement with our proposal, the alcohol has negligible effect (1% or less increase) on order at the 5 position, whereas the fatty acid causes disordering. However, unlike the retinoids which restrict acyl chain motion with their cyclohexene group towards the centre of the membrane, ESR spin label measurements lower down the chain (positions 10, 12 and 16) show that decanoic acid increases disorder/fluidity throughout the membrane, while decanol continues to exert minor influence. The small increase in membrane order seen here in the presence of decanol is consistent with earlier ^{2}H-NMR reports [29,30].

A difference in behaviour following incorporation of retinoic acid as opposed to retinol or retinal is also manifested by osmotic swelling measurements of the permeability of egg PC membranes to erythritol. All-*trans*-retinoic acid markedly enhances permeability, while all-*trans*-retinol or retinal produce slight increases (Fig. 6). Qualitatively, the same trend is exhibited by the CF leakage experiments, which showed a greater enhancement of the permeability of egg PC bilayers to the fluorescent ion with retinoic acid than either retinol or retinal (Fig. 7), and was observed previously in a study of the influence of retinoic acid and retinol on the permeability of various PC membranes to K^+, I^-, glucose and water [7]. It is possible, moreover, to reconcile the ESR and permeability data, since a greater in-

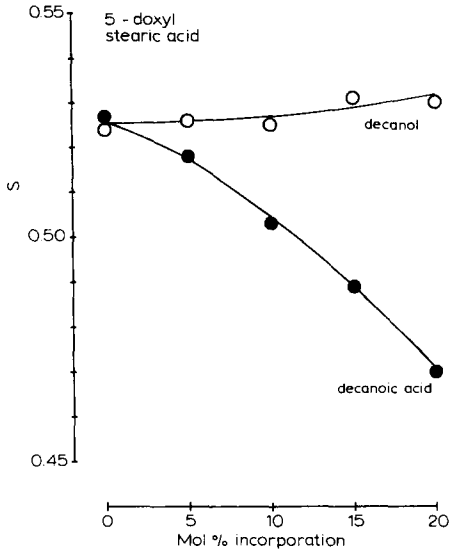

Fig. 9. Dependence on decanol (○) and decanoic acid (●) concentration for the order parameter S of 5-doxylstearic acid (1 mol%) intercalated into 1% (w/v) multilamellar dispersions of DPPC in 20 mM phosphate/1 mM EDTA buffer (pH 7.5) at 50 °C.

crease in permeability would be expected to occur as a consequence of the increased membrane disorder associated with retinoic acid.

To conclude, the present study establishes that all-*trans*-retinoic acid modifies the properties of phospholipid model membranes in a different manner to all-*trans*-retinol and retinal. The latter two retinoids restrict acyl chain motion in the lower portion of the chain and have little effect in the upper portion. Retinoic acid similarly restricts acyl chain motion in the lower portion of the chain but, in contrast, increases disorder in the upper portion. Enhancement of membrane permeability is also greater with retinoic acid than retinol or retinal. In the context of retinoid toxicity, it is thus interesting to note reports that retinoic acid induces hypervitaminosis A more effectively than retinol [31]. Future experiments will address the dependence of retinoid interactions on membrane composition.

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