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EFFECT OF RETINOL AND RETINOIC ACID ON PERMEABILITY, ELECTRICAL RESISTANCE AND PHASE TRANSITION OF LIPID BILAYERS

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Retinol and retinoic acid have been incorporated into the artificial membrane systems, planar bimolecular lipid membranes and liposomes, and their effects on several membrane parameters have been measured. 1. Retinol and retinoic acid increased the permeability of egg lecithin liposomes to K^+, I^- and glucose when incorporated into the membranes at levels as low as 0.5 membrane mol%. Retinoic acid influenced permeability more than did retinol for each of the solutes tested. 2. Retinol and retinoic acid both decreased the electrical resistance of egg lecithin-planar bimolecular lipid membranes from 0.5 to 8 membrane mol%. Retinoic acid effected a larger change than did retinol. 3. Retinol and retinoic acid increased the permeability of dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine liposomes to water at 1.0 and 3.0 membrane mol%. A larger effect on water permeability was measured for retinoic acid than for retinol. 4. Retinol and retinoic acid at 1.0 and 3.0 membrane mol% were shown to lower the phase-transition temperature of liposomes composed of dimyristoylphosphatidylcholine or dipalmitoylphosphatidylcholine. Phase-transition temperatures were monitored by abrupt changes in water permeability and liposome size associated with the transition. Retinoic acid lowered the phase-transition temperature of dimyristoylphosphatidylcholine liposomes more than did retinol, while both retinoids had almost the same effect on dipalmitoylphosphatidylcholine liposomes.

Introduction

Despite intense research efforts, the molecular mode of action of vitamin A in nonvisual processes remains a mystery [1]. One possible site of action for this very hydrophobic vitamin is on membranes. Although some time ago vitamin A was found to affect the structure and permeability of erythrocytes [2], lysosomes [3] and mitochondria [4], the nature of this interaction has yet to be deduced.

To circumvent interpretational problems stemming from the study of very complex natural membranes, vitamin A and derivatives (collectively known as retinoids) have been added to the simple, protein-free artificial membrane systems, lipid monolayers, planar bimolecular lipid membranes and liposomes, and their perturbations on membrane physical properties measured.

Retinoid-phospholipid interactions with lipid monolayers were first described by Bangham [5] and later extended by Roels and co-workers [6–8] and Brody [9,10]. Strong ion-dipole interactions between the polar head groups of retinol and lecithin were detected. Leslie and Chapman [11] first incorporated β-carotene and *trans*-retinal into planar bimolecular lipid membranes and established the membrane location of these chromophores spectrophotometrically. Pant and Rosen-

Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

berg [12], Tien [13–15] and later Bordy [16] incorporated *trans*-retinal into bimolecular lipid membranes in an attempt to mimic the visual receptor membrane. These workers all measured large increases in retinal-dependent photoconductivities. Tien also reported a membrane dark potential resulting from a retinal-induced proton conductivity.

Using liposomes, several workers have demonstrated that retinoids can increase membrane permeability. Bonting [17,18] reported a large increase in cation permeability with phosphatidylethanolamine (but not phosphatidylcholine) membranes when exposed to retinal (but not retinol). Phosphatidylethanolamine-retinal Schiff base (retinylidinephosphatidylethanolamine) was responsible for the permeability changes. Using galactocerebroside-containing liposomes, Conrad [19] found retinol-dependent increases in permeability. Recently we [20,21] demonstrated increases in the permeability of egg lecithin liposomes to H^+, Na^+, K^+, Cl^-, Br^-, I^-, glucose, glycine and lysine with retinol and retinal. A relationship was found between solute size and the level of retinoid required to affect permeability.

The major retinoid forms involved in the nonvisual processes are retinol and retinoic acid [22]. Since most of the artificial membrane studies have concentrated on retinal (to mimic the visual receptor) and have all but neglected retinoic acid, we decided to compare the relative affects of retinol and retinoic acid on the permeability of membranes to a cation (K^+), an anion (I^-), a neutral solute (glucose) and water. Also described here are the relative effects of retinol and retinoic acid on the electrical resistance of planar bimolecular lipid membranes and on the phase-transition temperature of dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) liposomes. From these experiments we conclude that retinol and retinoic acid both perturb membrane structure at low levels and that retinoic acid exerts a larger effect than does retinol.

Materials and Methods

Diffusion methods. The diffusion methods used in this study have previously been described in detail [20]. 300 mg egg lecithin (Sigma-Type IX-E) were dissolved in 15 ml diethyl ether with 0–7 membrane mol% retinol (Sigma-Type X) or retinoic acid (Sigma-Type XX). Liposomes were made by slowly injecting the diethyl ether solution under aspiration at 60°C [23] into 15 ml of an aqueous buffer composed of 10 mM Tris-HCl (pH 9.5) and either 250 mM KCl, 250 mM NaI or 500 mM glucose. The nonsequestered solutes were removed by three preliminary dialysis steps followed by chromatographic separation on Sephadex G-50 (Pharmacia) using osmotically balanced buffers to prevent liposome breakage. Liposomes were transferred to dialysis bags which were than put into osmotically balanced buffers, free of the species, the diffusion rate of which was sought. The diffusing species was detected by ion-specific electrodes for K^+ (Markeson Scientific) and I^- (Lazer Research Laboratories) or a colorimetric assay for glucose (glucose oxidase test [24]). Solute diffusion was measured for several hours and a final measurement was taken after all diffusion had ceased (3 days). The initial diffusion rate per minute is expressed as the percentage of the total solute that was initially sequestered and is the average of two or three determinations.

Electrical resistance. Planar bimolecular lipid membranes were made by the method of Tien [25]. The membrane forming solution consisted of 15 mM egg lecithin (Sigma-Type VI-E) in *n*-octane to which 0–10 membrane mol% retinol or retinoic acid were added. Thinning of the membrane to the bimolecular state was followed by 90° reflected light. Electrical resistance was measured as described in Huemoeler and Tien [26] using a Keithley Model 616 High-Speed Digital Electrometer, Beckman Fiber Junction Electrodes and appropriate circuitry. All measurements were made in a Faraday cage. Each point represents the average of five determinations.

Water permeability. Water permeability was measured by the method of Blok et al. [27]. Multilayered liposomes [28] were made from synthetic dimyristoylphosphatidylcholine(Sigma Chemical Co.) or dipalmitoylphosphatidylcholine (Sigma Chemical Co.) at temperatures well above the phase-transition temperature (60°C). A stock liposome solution was made daily from 13.1 mM phospholipid in 80 mM glucose/2 mM histidine/2 mM Tris-HCl buffered at pH 7.4. 100 μl of an

ethanolic solution of retinol or retinoic acid were then mixed with 2.5 ml of the liposomes. The retinoid/phospholipid ratio was 0%, 1.0% or 3.0%. 100 μl of the high ionic strength retinoid-liposomes were then rapidly injected into 2.0 ml of a low-ionic-strength buffer (2 mM histidine/2 mM Tris, pH 7.4) which had been preequilibrated for 5 min at the appropriate temperature. The initial rapid absorbance change associated with liposome swelling as followed at 450 nm on a Beckman DU-8 Computing Spectrophotometer. Cuvette temperatures were carefully controlled at ± 0.1°C from 10°C to 50°C with the sample transport mechanism. The initial absorbance change rate, $\Delta A/\Delta t$, which is known to be proportional to the liposome volume change [29], was determined for each liposome preparation. The initial swelling velocity $dl/A/dt\%$ [27] was then calculated and plotted against the temperature. From these curves the phase-transition temperature was determined. Each osmotic swelling was performed in duplicate.

Liposome size. The size of liposomes is known to be affected by temperature, with the most rapid change in size occurring at the phase transition [27,30,31]. DMPC and DPPC liposomes were made as described above and their relative sizes were measured as absorbance at 450 nm. Slightly different amounts of liposomes were used for each of the three retinoid concentrations tested to offset the curves and allow for easier determination of the phase-transition temperature. Phase-transition temperatures were determined from the midpoint of the rapid absorbance change portion of the curve.

Results

The effect of increasing amounts of retinol (Fig. 1) and retinoic acid (Fig. 2) incorporated into egg lecithin liposomes on solute permeability is shown for K^+, glucose, and I^-. In all cases solute permeability is significantly higher with retinoic acid than with retinol. The order of solute permeability is $K^+ >$ glucose $> I^-$ at any retinol (Fig. 1) or retinoic acid (Fig. 2) concentration. The retinoids affect permeability of the smaller solute, K^+, at levels lower than needed to change the permeability of the larger solutes. By these retinoic levels of 0.5 membrane mol% are enough to increase permeability measurably.

Fig. 3 present data for the effect of retinol and retinoic acid on the electrical resistance of egg

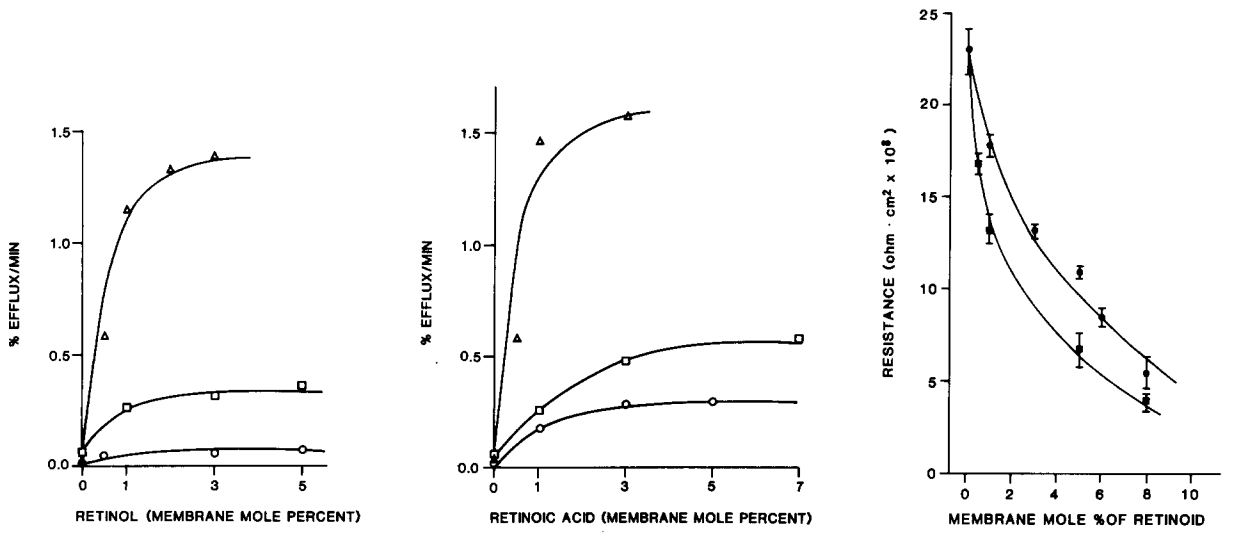

Fig. 1. (Left.) Efflux rate for K^+ (△), glucose (□) and I^- (○) from egg lecithin liposomes as a function of incorporated retinol.

Fig. 2. (Center.) Efflux rate for K^+ (△), glucose (□) and I^- (○) from egg lecithin liposomes as a function of incorporated retinoic acid.

Fig. 3. (Right.) Electrical resistance of egg lecithin-retinoid planar bimolecular lipid membranes (retinol, ●; retinoic acid, ■).

lecithin-planar bimolecular lipid membranes. Both retinoids decrease the electrical resistance from the initial value of $23 \cdot 10^8\ \Omega \cdot cm^2$ for the unperturbed membrane. Retinoic acid affected a larged decrease in resistance than did retinol for all concentrations ranging from 0.1 to 8 membrane mol%.

Retinol and retinoic acid were shown to increase the water permeability of DMPC and DPPC liposomes as measured by rapid osmotic swelling (Fig. 4, Table I). Liposomes behave as almost ideal osmometers [29] and the initial osmotic volume change (water permeability) has been shown to be in direct proportion to the change in absorbance [27]. Since water permeability changes abruptly at the phase-transition temperature, we used this simple spectrophotometric technique to measure the

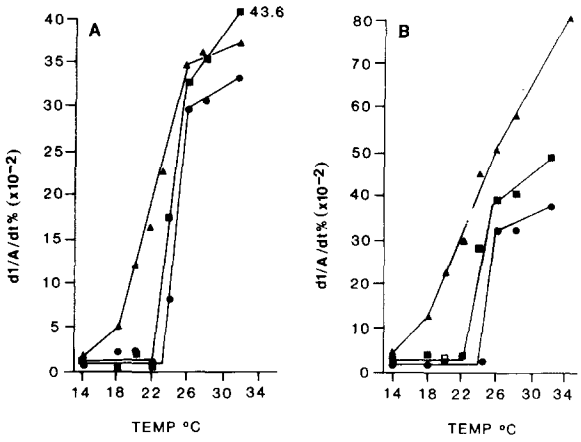

Fig. 4. Effect of retinol (A) and retinoic acid (B) on the water permeability of DMPC liposomes as a function of temperature (no retinoid, ●; 1.0 membrane mol% retinoid, ■; 3.0 membrane mol% retinoid, ▲).

TABLE I

EFFECT OF RETINOL AND RETINOIC ACID ON THE PHASE-TRANSITION TEMPERATURE OF DMPC AND DPPC LIPOSOMES

Published phase-transition temperatures for DMPC and DPPC are 23.1 and 41.3°C, respectively.

Experiment	Phospholipid	Retinoid	Retinoid membrane mol%	Measured phase-transition temperature (°C)	Decrease in phase-transition temperature (°C)
Water diffusion	DMPC	retinol	0	23.2	0
			1.0	22.0	1.2
			3.0	17.0	6.2
		retinoic	0	23.7	0
		acid	1.0	21.9	1.8
			3.0	15.9	7.8
	DPPC	retinol	0	41.5	0
			1.0	39.8	1.7
			3.0	38.3	3.2
		retinoic	0	41.6	0
		acid	1.0	41.3	0.3
			3.0	39.4	2.2
Liposome size	DMPC	retinol	0	23.5	0
			1.0	22.3	1.2
			3.0	21.6	1.9
		retinoic	0	23.5	0
		acid	1.0	23.0	0.5
			3.0	22.0	1.5
	DPPC	retinol	0	42.0	0
			1.0	41.6	0.4
			3.0	40.9	1.1
		retinoic	0	42.0	0
		acid	1.0	41.7	0.3
			3.0	40.4	1.6

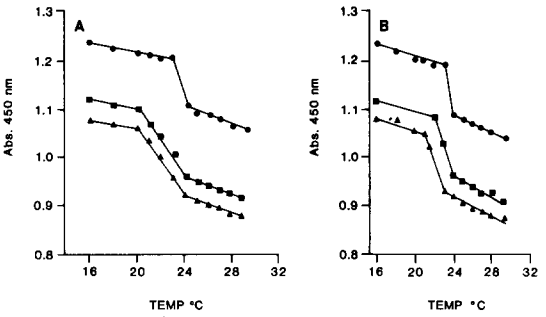

Fig. 5. Effect of retinol (A) and retinoic acid (B) on liposome size (absorbance at 450 nm) of DMPC liposomes as a function of temperature (no retinoid, ●; 1.0 membrane mol% retinoid, ■; 3.0 membrane mol% retinoid, ▲).

effect of retinol and retinoic acid on the phase-transition temperature of liposomes made from the synthetic phosphatidylcholines.

The effect of retinol and retinoic acid on the initial liposome swelling rate ($d(1/A)/dt$ (%)) is plotted as a function of temperature for DMPC liposomes (Fig. 5) and for DPPC liposomes (data in Table I). A retinoid-induced decrease in the phase transition temperature is clearly evident with both liposome populations for 1.0 and 3.0 membrane mol% retinoid.

Phase transition temperatures can also be monitored by abrupt changes in liposome size associated with the transition [27,30,31]. By this method retinol and retinoic acid were also shown to decrease the phase-transition temperature of DMPC (Fig. 5) and DPPC (data in Table I) liposomes. This method is not as sensitive as the water permeability method and the smaller measured phase-transition shifts were similar to both retinoids.

The phase-transition temperatures determined for both water permeability and liposome size for DMPC and DPPC liposomes as affected by 0, 1.0 and 3.0% retinol and retinoic acid are summarized in Table I.

Discussion

Retinol is the major form of vitamin A found in the body and is involved in the systemic, nonvisual functions [22]. Retinoic acid, a rapidly metabolized oxidation product of retinol, is found only in minute amounts in organisms and supports many of the same functions as retinol, including normal body growth and epithelial differentiation. However, unlike retinol, retinoic acid does not participate in the visual or reproductive functions [32]. Both forms of the vitamin possess many similar properties, including 'detergent shape' and participation in glycoprotein synthesis [33]. However, the transport, storage, metabolism, pharmacology, toxicology and probably the as-yet-unknown biochemical functions of these two retinoids differ [34]. Although both forms probably affect membranes, there has not been any comparison of their effects on membrane properties. Here we report the comparative effects of retinol and retinoic acid on several properties of lipid bilayers.

Previously we have demonstrated large increases in liposome permeability to cations (K^+, Na^+, H^+), anions (Cl^-, Br^-, I^-), zwitterions (lysine and glycine) and a neutral solute (glucose) with retinol [20] and increases in H^+ and glucose permeability with retinal [21]. Retinol's effect on solute permeability was shown to be related more to solute size than to solute charge. Small solutes diffused at lower retinoid levels than did larger solutes.

In the experiments reported here retinoic acid was also shown to affect membrane permeability severely at low levels (0.5 membrane mol% or less). Large increases in retinoic acid-dependent permeabilities for K^+, glucose and I^- are evident in Fig. 1 and 2, respectively. The liposomes were buffered at pH 9.5 and so we expect that the retinoic acid was dissociated. Anionic liposomes should be more permeable to cations (K^+) and neutral solutes (glucose) than to anions (I^-). If the charge on the polar head group of the retinoid were the determining factor in permeability control we would expect I^- permeability to be greater for retinol-liposomes than for retinoic acid-liposomes. In fact the permeability of retinoic acid-liposomes to all solutes including I^- is greater than for retinol-liposomes. From these obsevations we conclude that the difference between retinoid-induced solute permeability is related more to the size and shape of the vitamin than to the charge on the polar head group.

For the smallest solute tested, K^+, the dif-

ference between retinol and retioic acid-induced permeability was minimal (Fig. 1). However, for the larger solutes, glucose and I^-, retinoic acid was clearly superior at stimulating solute permeability (Figs. 1 and 2). From these observations and also from the previously reported experiments [20,21], we conclude that retinoids at low levels can severely affect the permeability of lipid bilayers. Retinol and retinoic acid can perturb membrane structure enough to increase to a similar extent the membrane permeability of small solutes like H^+ and K^+. With larger solutes, retinoic acid clearly increases permeability more than does retinol and so retinoic acid likely disrupts the lipid bilayer to a greater extent than does retinol.

To further test this hypothesis, we decide to compare the effect of retinol and retinoic acid on the electrical resistance of planar bimolecular lipid membranes. Since these retinoids increase membrane permeability we expected that they would also lower membrane electrical resistance. In fact, both retinol and retinoic acid at concentrations of 0.5 to 8 membrane mol% were shown to lower electrical resistance substantially, with retinoic acid once again exerting the larger effect.

An additional indication than retinoids perturb lipid bilayers, with retinoic acid exerting a larger effect than retinol, is provided by the water-permeability studies. A retinol-induced increase in water permeability was noted for both DMPC and DPPC liposomes over a range of temperatures ± 10°C from the phase transition (Fig. 4 and Table I). A decrease in the phase-transition temperature was evident for both types of liposome at 1.0 and 3.0 membrane mol% retinol or retinoic acid. Retinoic acid exerted a larger effect with the DMPC liposomes than did retinol; however, both retinoids displayed similar effects with the DPPC liposomes.

Retinoid-induced decreases of the phase transition were also evident by measuring the size of liposomes as a function of temperature with and without retinol or retinoic acid (Fig. 5 and Table I). The gel-to-liquid crystalline phase transition is known to be associated with an abrupt change in absorbance [30,31]. Table I presents a summary of the effect of retinol and retinoic acid on the phase transition temperatures of synthetic phosphatidylcholine liposomes as measured by changes in water permeability and liposome size. Both types of experiment clarly indicate that retinol and retinoic acid lower phase transitions. The largest effect measured, that of retinoic acid on DMPC liposomes, resulted in a decrease in the phase-transition temperature of 7.8°C for 3.0 mol% retinoid. Hill [35] previously demonstrated that long-chain alcohols (up to C_8) decreased the phase-transition temperature of DPPC lipid bilayers. He reported a 1°C temperature decrease for 4.4 membrane mol% *n*-octanol in lecithin. Retinoids therefore appear to affect phase transition more than does *n*-octanol.

Because of their effect on the phase transition, retinol and retinoic acid would be expected to also increase membrane fluidity. Recently Meeks and co-workers [36,37] have found that several retinoids decrease the membrane microviscosity of erythrocyte ghosts and embryonal carcinoma and fibroblast cells at levels of $1 \cdot 10^{-5}$ M or less in the external solution. They suggest that this effect may be related to the vitamin's toxicity. Detergents such as SDS, Triton X-100 and lysophosphatidylcholine produce similar effects at $1 \cdot 10^{-3}$ M. However, these workers could detect no effect of retinoic acid on DMPC liposomes at $1 \cdot 10^{-4}$ M. In our experiments we report the retinoid as membrane mole percent to show more clearly the molecular ratio between phospholipid and retinoid concentration. Our 3.0 membrane mol% experiments are the equivalent of about $5 \cdot 10^{-4}$ M found in the external solution. At this level and less, retinol and retinoic acid clearly lower the phase transition of DMPC and DPPC liposomes. We predict therefore that retinoid levels of about $1 \cdot 10^{-4}$ M should affect the fluidity of phosphatidylcholine liposomes.

From these experiments we conclude that retinol and retinoic acid do severely affect several physical parameters of lipid bilayers at levels between 0.5 and 8 membrane mol%. We also conclude that retinoic acid perturbs membranes to larger extent than does retinol. Both retinoids also lower the phase transition of phosphatidylcholine liposomes and probably exert their bilayer effects by increasing membrane fluidity. Perhaps these effects on membrane fluidity and permeability may in part explain the toxicity of vitamin A.

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