Interactions of retinol with lipid bilayers: studies with vesicles of different radii

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Abstract The interactions of retinol with vesicles of dioleoylphosphatidylcholine of varying radii were studied. The rate constants of dissociation of retinol from bilayers (k_{off}) and the equilibrium partition constants (K_{eq}) of retinol into bilayers of different sized vesicles were measured. The rate constants for association of retinol with vesicles were calculated from the expression $K_{eq} = k_{on}/k_{off}$. k_{off} was 10-fold faster in the smallest versus the largest vesicles tested. K_{on} was also somewhat faster in vesicles with small radii, but the effect on k_{off} was more pronounced, leading to an overall higher affinity for retinol of bilayers in large vesicles. The thermodynamic parameters of the dissociation reaction were studied in vesicles with 0.025, 0.1, and 0.4 μm diameter. The enthalpy of activation decreased while the entropy of activation of the dissociation of retinol from bilayers increased as the vesicles become larger. It is suggested that restructuring of lipid–lipid interactions within the bilayer play a role in determining the rate by which retinol is solvated off bilayers. Overall, the data indicate that the rates by which retinol moves between different cell types in vivo may depend on the geometry of cellular surfaces.—**Noy, N., D. J. Kelleher, and A. W. Scotto.** Interactions of retinol with lipid bilayers: studies with vesicles of different radii. *J. Lipid Res.* 1995. **36:** 375–382.

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Vitamin A derivatives, retinoids, are poorly soluble in water and are found in vivo mainly bound to specialized retinoid-binding proteins or associated with cellular membranes. The interactions of retinoids with biological membranes, therefore, have significant consequences for the processes by which these compounds are distributed between different cells and subcellular organelles. Knowledge of the kinetic parameters governing the movement of retinoids into, across, and out of membranes is especially important for understanding the factors that may limit the rates by which retinoids transfer between different compartments in vivo. Previous studies of these issues have led to the conclusions that retinol spontaneously and rapidly transverses lipid bilayers and membranes (1–5). Transfer of retinol between unilamellar vesicles of phospholipids proceeds by hydration from donor vesicles, followed by diffusion through the aqueous phase and association with acceptor vesicles. The slowest step in this process is the hydration from the donor bilayer, a step that has a first order rate constant of about 0.6 s^{-1} ($t_{1/2}$ on the order of 1 s). The $t_{1/2}$ for transfer of retinol across lipid bilayers is less than 0.1 s, and is not expected to constitute a rate-limiting step in the transport of retinol (5). The lipid composition as well as the packing properties of bilayers were found to be important determinants for the affinity of a membrane for retinol as well as for the kinetics by which retinol interacts with membranes (5).

The information regarding the interactions of retinol with lipid bilayers summarized above was obtained by utilizing membrane model systems comprised of unilamellar vesicles of phospholipids prepared by sonication, vesicles that have an average diameter of about 0.025 μm (6). The small radius of curvature of these vesicles imposes a strain on the outer leaflet of their bilayers resulting in a larger distance between the phospholipid head groups as compared to membranes with larger radii in which the bilayer will be more closely packed (7). As packing properties of lipid bilayers have been shown to affect the interactions of retinol with membranes, the present study was undertaken to investigate the effect of the radius of curvature on the interactions of retinol with lipid bilayers. One advantage of this system is that the effect of the packing properties of the lipids on the rates by which retinol interacts with bilayers can be studied without varying the chemical composition or the physical state of the lipids. The effects of radius of curvature on the interactions of retinol with bilayers may also have important implications for movement of retinol between different cell types in vivo. For example, continuous movement of retinoids between retinal pigment epithelium cells and photoreceptor

Abbreviations: PC, phosphatidylcholine; DOPC, dioleoyl PC; DOPE, dioleoylphosphatidylethanolamine; FaT MLVs, multilamellar vesicles made by the freeze and thaw technique.

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cells in the eye is crucial for regeneration of rhodopsin in the visual cycle (8, 9). Neither of these cell types is spherical and different areas of their plasma membranes have different radii of curvature. This is mainly apparent in the geometry of the retinal pigment epithelium cells which have villous processes protruding into the interphotoreceptor matrix. While the radius of the body of these cells is on the order of several microns, the radius of curvature of the villi can be as small as 0.05 μm (10). The studies described below show that the rate of dissociation of retinol from bilayers strongly depends on the bilayers' radius of curvature. These data thus imply that retinoid fluxes may vary at different locations on cellular surfaces.

MATERIALS AND METHODS

Lipids were obtained from Avanti Polar Lipids. All-*trans* retinol was obtained from Kodak. [^{3}H]retinol was from New England Nuclear. All other chemicals were from Sigma Chemical Co. Scintillation liquid (Liquiscint) was from National Diagnostics. Male Wistar rats weighing about 300 g were obtained from Charles River Breeding Laboratories.

Rat liver plasma membranes were isolated on a discontinuous sucrose gradient as in described by Swislocki, Magnuson, and Tierney (11).

Concentrations of phospholipids were determined by the phosphorus content (12).

Lipid vesicles

Multilamellar vesicles of DOPC and vesicles of DOPC containing DOPE were prepared essentially as previously described (13). The phospholipids were co-mixed in chloroform and the organic solvent was removed in a rotary evaporator at 37°C. The lipid residue was further dried under vacuum for 2–4 h, and the lipids were hydrated by rotating the dried lipid film with several glass beads for 1 h in a buffer containing 50 mM HEPES (pH 7.5) and 100 mM KCl at 5–10 mg/ml. Lipids were frozen and thawed 10 times to obtain a suspension of hydrated and fused vesicles (FaT MLVs). The combination of freeze–thaw with extrusion allows the preparation of vesicles with well-defined and homogenous size distributions in the 0.03–0.4 μm range (14–17). A series of "sized vesicles" were prepared from the frozen and thawed lipid suspension by extrusion through polycarbonate filters with defined pore sizes. Suspensions were extruded 10 times for each preparation through two stacked membranes in a Lipex extruder essentially as described by Hope et al. (14) and by Mayer, Hope, and Cullis (15).

Several modifications were incorporated into the protocol to ensure that the vesicles yielded a consistent progression of smaller vesicles with decreasing membrane pore size. All FaT MLVs were pre-sized three times through a 1.0-μm membrane prior to preparation of smaller vesicles. For preparation of vesicles smaller than 0.1 μm, 1.0 μm vesicle suspension was further pre-sized by three passages through membranes with 0.1-μm pores. Standardized pressure settings ranging from 50 psi for 1.0 μm vesicles to 550 psi for 0.03 μm vesicles were used in all preparations. Buffers used to hydrate the lipids and/or rinse the membranes were filtered through a 0.22-μm membrane and subsequently through a PM-30 Amicon ultrafiltration membrane to assure particulate free solutions.

Measurements from a number of laboratories have indicated that vesicle size may be somewhat smaller than the pore size of the polycarbonate membrane but closely related to that size (14–17). Recently, quasi-elastic light scattering studies have demonstrated that the size distribution of 0.1–0.4 μm vesicles prepared by extrusion have a narrow unimodal distribution of sizes independent of lipid concentration between 0.1 and 10 mg/ml with vesicle size being slightly influenced by changes in applied pressure (18). The size distribution of vesicles produced by extruding vesicles through membranes with pore sizes larger than 1 μm was examined by electron microscopy after negative staining with uranyl acetate on carbon-coated formvar grids. Vesicles extruded through membranes with pore sizes of 5, 8, and 10 μm were multimodal and significantly smaller than the pore size. Consequently, these vesicles were not used in the present study. Electron micrographs of vesicles prepared using a 1.0-μm membrane indicated that these vesicles had a diameter of 1.04 $\pm$ 0.37 μm (n = 20).

The smallest vesicles used were prepared by sonication of FaT MLVs that have been sized 10 times using a 0.1-μm membrane. Five ml of a lipid suspension (5 mg/ml) was placed in a 30-ml polycarbonate tube flushed with nitrogen and sealed with a silicone stopper. This sample was sonicated at 5°C using a W350 cup horn attached to a Heat-System sonicator at 50% pulse for 20 min. Samples appeared clearer at the end of sonication which was confirmed by a decrease in light scattering at 350 nm. Vesicles were allowed to anneal overnight at 37°C.

Number of bilayers in the different populations of vesicles

To determine the number of bilayers in vesicles populations with diameters larger than 0.1 μM, DOPC vesicles containing dioleoylphosphatidylethanolamine (DOPE) (5–9 mol%) were prepared. The amount of DOPE in the outer leaflet at the vesicle surface and the total DOPE content were determined using 2,4,6-trinitrobenzensulfonic acid (TNBS) as described by Norlund et al. (19). The distribution of DOPE between the outer and the inner leaflets of lipid bilayers may be asymmetrical (e.g., Norlund et al., 19). To account for such asymmetry, this distribution was measured in large extruded PC vesicles with a diameter of 0.1 μm that are known to be unilamel-

lar (15). Of the total DOPE, 46.05% ± 0.9 (n = 4) was found to be in the outer leaflet of the bilayers in 0.1-μm vesicles (**Table 1**). This value was used to standardize the DOPE distribution between outer and inner leaflets in subsequent calculations. To obtain the number of bilayers in vesicles with diameters of 0.2 μm or larger, the amounts of DOPE in the outer leaflets of the outermost bilayers of these vesicles were measured. Using the above value for the distribution of DOPE between outer and inner leaflets, the amount of DOPE in the outermost bilayer in each vesicle population was calculated. The number of bilayers in vesicles of each population was calculated by two methods: *1)* by assuming that all bilayers in a specific vesicle contained the same amount of DOPE as the outermost bilayer (i.e., that the sizes of all bilayers were the same); and *2)* by assuming that the radii of bilayers within multilamellar vesicles are successively smaller by steps of 15 nm. This value was obtained by taking the thickness of each bilayer to be 7.5 nm and by assuming that a hydration layer of 7.5 nm thickness exists between bilayers. The lipid content of each bilayer was then computed based on their respective surface areas as compared to the surface area of the outermost bilayer.

Partitioning constants of retinol between the vesicles and the aqueous phase

These were measured as previously described by partitioning of [^{3}H]retinol between three phases: vesicles, plasma membranes from rat liver, and an aqueous phase (5). Vesicles were mixed with plasma membranes isolated from rat liver. Radioactively labeled retinol was added and the mixture was incubated for 20 min which constitutes over 500 half-lives for transfer of retinol between vesicles (5). Plasma membranes were separated from vesicles by centrifugation in an Eppendorf centrifuge for 30 min. Supernatants and pellets were counted for the presence of [^{3}H]retinol. The amount of retinol in the supernatant, in this system, is comprised of retinol that is associated with the vesicles and retinol in the aqueous phase. To obtain the partitioning constant of retinol between vesicles and water, the amount of retinol in water was calculated from the equilibrium partition constant of retinol between plasma membranes and water, and the amount of retinol associated with plasma membranes (in the pellet) in each experiment. The amount of retinol associated with the vesicles was obtained by subtracting the amount in water from total retinol found in the supernatant.

TABLE 1. Number of lamellae in sized DOPC vesicles

Diameter	% DOPE in Outer Leaflet	Number of Bilayers A	Number of Bilayers B
μm			
0.1	46.0 ± 0.9[a]	1.0	
0.2	36.8	1.3	1.4
0.4	29.4 ± 0.9[b]	1.6	1.7
0.6	25.9 ± 0.6[b]	1.8	1.9
1.0	22.4	2.1	2.2
FaT MLVs	8.1	5.8	ND

DOPC vesicles containing dioleoylphosphatidylethanolamine (DOPE) (5–9 mol%) were prepared and the amount of DOPE in the outermost leaflet and the total DOPE content were determined as described in Methods. The distribution of DOPE between outer and inner leaflets of 0.1 μm vesicles was used to obtain the amount of lipids in the outermost bilayer of larger vesicles. The number of bilayers in each population was estimated by assuming either that all bilayers of a given vesicle have the same diameter (A) or that bilayers are successively smaller (B). See Methods for details.

Values represent means of two experiments except those marked [a] n = 4 and [b] n = 3.

Rate of dissociation of retinol from vesicles

The rate of transfer of retinol from donor to acceptor vesicles of phospholipids represents the rate of dissociation of this ligand from the donor vesicles (4, 5). The rate of transfer was measured as previously described (5) using the fluorescent lipid probe (N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine) (NBD-DPPE). The excitation spectrum of NBD overlaps extensively with the emission spectrum of retinol. When NBD-DPPE and retinol are embedded in the same bilayer, excitation of retinol (at 325 nm) results in energy transfer between the two fluorophores and emission from NBD-DPPE is observed. NBD fluorescence was followed at 600 nm where the fluorescence of retinol itself is negligible. Acceptor vesicles with a diameter of 0.1 μM containing NBD-DPPE (4 mole%) were made by co-extrusion of DOPC and NBD-DPPE. Movement of retinol was monitored by the increase in the fluorescence intensity of NBD as retinol moved from donor to acceptor vesicles upon mixing the two vesicle populations. Mixing was done in a High-Tech (Salisbury, United Kingdom) rapid mixing apparatus in conjunction with a Spex (Metachen, NJ) Fluorolog 2 fluorometer. Data analysis was performed using Origin software (Microcal, Inc.). Excitation and emission wavelengths were 325 and 600 nm, respectively.

RESULTS

Sized unilamellar vesicles with diameters of 0.03, 0.05, 0.08, 0.1, 0.4, and 1.0μm were prepared by extrusion of a suspension of multilamellar vesicles of DOPC through membranes with pores of a uniform size (see Methods). Two additional populations of vesicles were made: *1)* by extensive sonication, a procedure that results in vesicles of an average diameter of about 0.025 μm (6) and are the vesicles with the smallest diameter tested; and *2)* lipids in an aqueous buffer were frozen and thawed 10 times to produce a suspension of multilamellar vesicles (FaT MLVs) which had the largest diameter tested.

Number of bilayers in each vesicle population

DOPC vesicles of different sizes containing 5–9 mol% of DOPE were prepared and the number of bilayers in each vesicle population was measured as detailed in the Methods. The results presented in Table 1 indicate that vesicles with a diameter larger than 0.1 μm gradually acquired an additional lamella in agreement with previous characterizations of extruded vesicles (14, 15). The estimated number of bilayers in the largest vesicles used in this study (FaT MLVs) was about six. The largest sized vesicles used (1 μm) had an average of two bilayers per vesicle.

Rate of dissociation of retinol from vesicles of different sizes

The rate of dissociation of retinol from unilamellar vesicles can be directly obtained from the rate by which retinol transfers from donor to acceptor vesicles. This is so because the rate of dissociation from donor vesicles is the rate-determining step for the transfer process (4, 5). Retinol was added to the vesicles from a concentrated solution in ethanol, and these donor vesicles were mixed in the stopped-flow spectrofluorometer with acceptor vesicles containing NBD-DPPE (see Methods). Transfer of retinol from donor to acceptor vesicles was monitored by the time-dependent increase in fluorescence intensity. **Figure 1** shows typical traces obtained with donor vesicles obtained by sonication (diameter of ca. 0.025 μm) (Fig. 1A), and with donor vesicles with diameters of 0.1, or 1 μm (Fig. 1B, and 1C, respectively). The transfer reaction with all vesicle populations followed first order kinetics and the rate constants for the transfer of retinol (representing the rate constants for dissociation from the donor vesicles) as a function of donor vesicles size are shown in **Fig. 2.** The data shown are the means of the values obtained from three independent experimental sets using different vesicle preparations. In each set, the rate constant for dissociation was obtained by averaging 7–10 separate measurements. SEMs never exceeded 11% of the values for measurements with small vesicles, i.e., 0.025–0.1 μM. Measurements with larger vesicles showed better consistency and had SEMs of 3–5% of the values. The rate constants for dissociation of retinol from the smallest (sonicated) and the largest (FaT) vesicles studied were

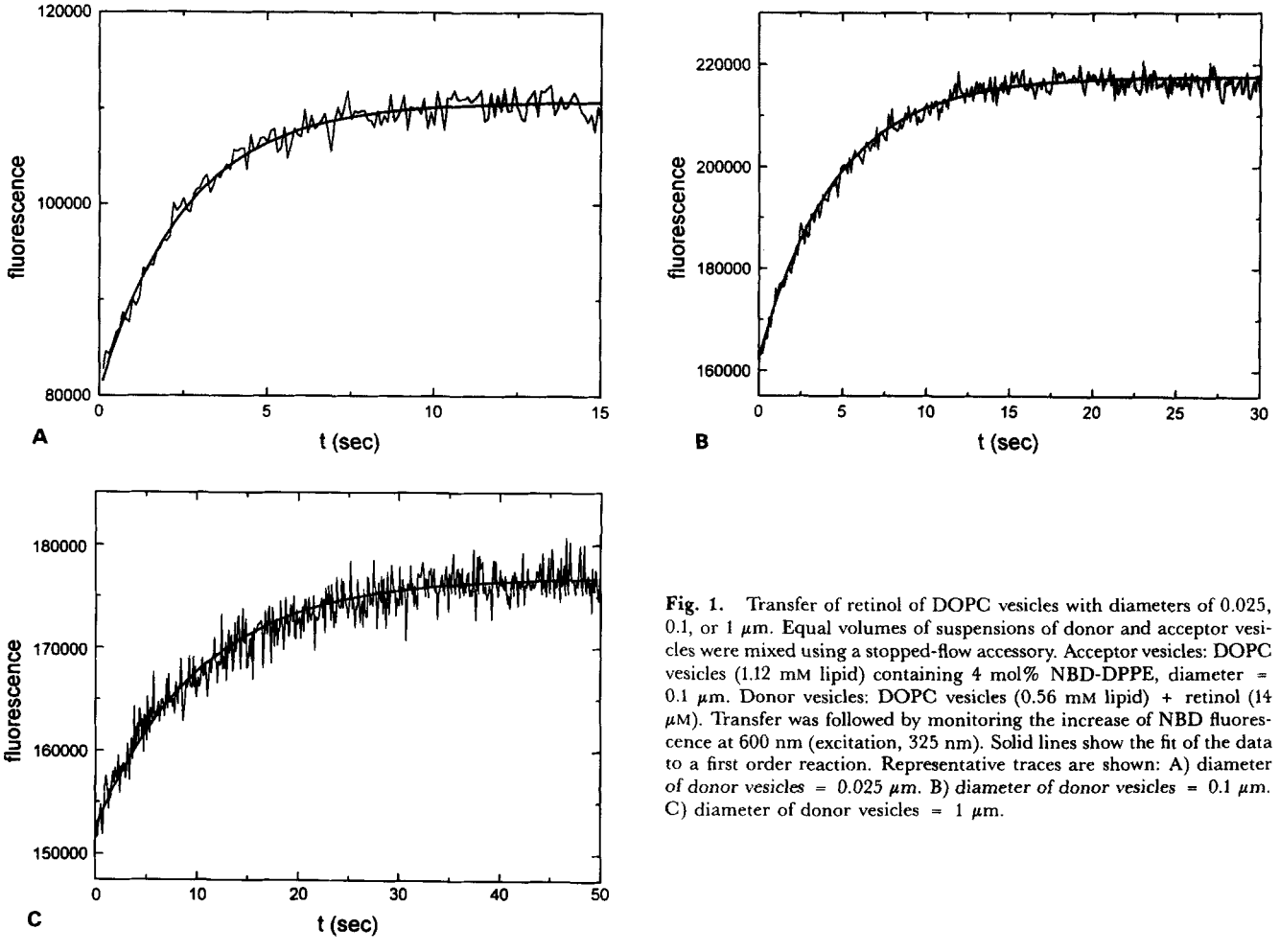

Fig. 1. Transfer of retinol of DOPC vesicles with diameters of 0.025, 0.1, or 1 μm. Equal volumes of suspensions of donor and acceptor vesicles were mixed using a stopped-flow accessory. Acceptor vesicles: DOPC vesicles (1.12 mM lipid) containing 4 mol% NBD-DPPE, diameter = 0.1 μm. Donor vesicles: DOPC vesicles (0.56 mM lipid) + retinol (14 μM). Transfer was followed by monitoring the increase of NBD fluorescence at 600 nm (excitation, 325 nm). Solid lines show the fit of the data to a first order reaction. Representative traces are shown: A) diameter of donor vesicles = 0.025 μm. B) diameter of donor vesicles = 0.1 μm. C) diameter of donor vesicles = 1 μm.

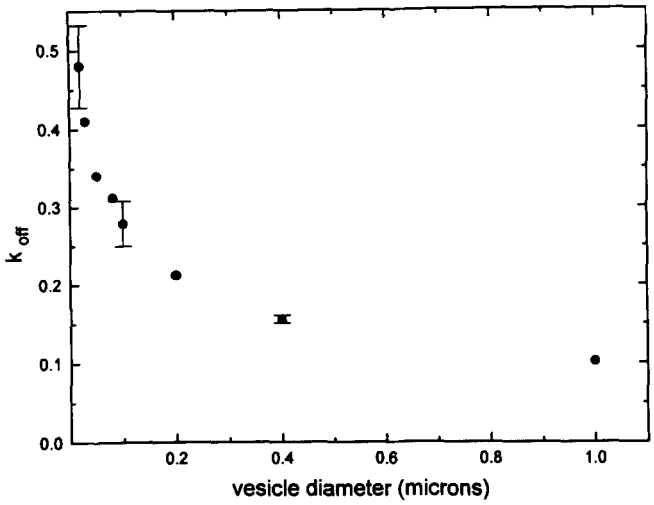

Fig. 2. Effect of vesicle diameter on the rate constant of transfer of retinol off DOPC vesicles. Stopped-flow experiments were carried out as described in the legend to Fig. 1. Vesicles of the designated diameters were used as donor vesicles. Values shown are mean of the values obtained from three independent experimental sets using different vesicle preparations. In each of the sets, the rate constant for dissociation was obtained by averaging 7–10 separate measurements. SEMs were 11% of the value or smaller as shown by the representative error bars (and see text).

0.48 ± 0.05 and 0.048 s^{-1}, respectively. Retinol thus dissociated from large vesicles at a significantly slower rate versus small vesicles. We considered the possibility that in vesicles containing more than one lamellae (i.e., vesicles that are larger than 0.1 μm), the rate of transfer of retinol between the different lamellae in the donor vesicles may be rate limiting for transfer to acceptor vesicles. However, the data (Fig. 1) show that the rate of dissociation of retinol from lipid bilayers approached a finite value as larger vesicles were tested, though the number of lamellae in these vesicles continued to increase, e.g., only a 2-fold difference was found between the rate of transfer of retinol from donor vesicles of 1 μm that contained an average of two lamellae and from multilamellar vesicles made by the freeze–thaw technique, that contained about six lamellae. This difference is small as compared to the difference in k_{off} found between vesicles of 0.025 and 1 μm which was about 5-fold though only one additional lamellae was present in the latter versus the former vesicles. In addition, the transfer reaction fit a first order reaction for all vesicle populations tested and there was no indication of a second process under any condition. These observations verified that transfer of retinol between bilayers within the donor vesicles was faster than the solvation of retinol into the bulk aqueous phase and did not become rate limiting for transfer in large vesicles. We concluded, therefore, that the observed differences between the kinetic parameters of the interactions of retinol with the different vesicle populations originated from the differences in their radii of curvature and were indicative of the interactions of retinol with the outer leaflets of the bilayers, i.e., the leaflet in direct contact with the bulk aqueous phase.

Partitioning constant of retinol between vesicles and the aqueous phase (K_{eq})

K_{eq} between the aqueous medium and plasma membranes isolated from rat liver was determined as detailed in Methods. $K_{eq}^{\text{plasma membrane}}$ was expressed as a molal fraction: K_{eq} = [(mole ret)$_m$/(mole lipid)$_m$]/[(mole ret)$_{aq}$/(mole H_2O)], and was found to be 0.7 (± 0.08) × 10^6 (n = 5). K_{eq}s for the partitioning of retinol between a specific population of vesicles and the aqueous phase were determined in an experiment that included plasma membranes, vesicles, and retinol as detailed in the Methods. The values of K_{eq}s (expressed as the ratio of molal fractions) for vesicles with diameters of 0.025 and 0.1 μm are shown in **Table 2.** These particular vesicle populations were used for K_{eq} measurements as they represent the smallest and the largest unilamellar vesicles

TABLE 2. Equilibrium distribution and rate constants for the interactions of retinol with DOPC vesicles with diameters of 0.025 and 0.1 μm

Diameter	K_{eq}[a]	ΔG°	k_{off}	k_{on}
μm		*kcal/mol*		
0.025	1.03(± 0.07) × 10^6	8.17	0.48	4.94 × 10^5
0.1	1.41(± 0.10) × 10^6	8.36	0.28	3.95 × 10^5

The partitioning constants of retinol between vesicles and the aqueous phase (K_{eq}) were measured in a system containing vesicles (75 nmol lipids), rat liver plasma membranes liver (75 nmol of lipid), and ^{3}H-labeled retinol (1 nmol) as detailed in the Methods. The final volume was 0.5 ml. The mixture was incubated for 20 min. Membranes (pellet) were separated from vesicles (supernatant) by centrifugation, and pellets and supernatants were counted for [^{3}H]retinol. Equilibrium distribution constants were calculated from the counts and from K_{eq} between plasma membranes and water (see Methods) and are expressed as the ratio of mol fraction of retinol in the lipids of the vesicles and the water. ΔG° was calculated by the expression: $\Delta G^0 = -RT \ln K_{eq}$. Rate constants for dissociation of retinol from the vesicles (k_{off}) were taken from Fig. 2. Rate constants for association (k_{on}) were calculated from the expression $K_{eq} = k_{on}/k_{off}$. The units of k_{off} and k_{on} are s^{-1} and ([ROH]$_{lipids}$/[lipids])/([ROH]$_{water}$/[water])*s^{-1}, respectively.

[a] *P* value for the differences < 0.003 (n = 7).

in this study. Thus, the interpretation of results of affinity measurements will not be complicated by distribution of retinol into additional bilayers with varying sizes in each structure. The data show that the affinity of 0.1 μm vesicles for retinol is 37% larger versus the affinity of 0.025 μm vesicles.

Rate constant for association of retinol with vesicles

The rate constants for association of retinol with vesicles of 0.025 and 0.1 μm diameter were calculated from the expression: $K_{eq} = k_{on}/k_{off}$. Values of k_{off} and K_{eq} were taken from the data in Fig. 2 and Table 2, respectively, and the values obtained are shown in Table 2. The data show that retinol interacts faster with the small versus the large vesicles. Thus, it was found that both k_{off} and k_{on} are larger for vesicles with a diameter of 0.025 μm versus vesicles with a diameter of 0.1 μm. However, the effect of the difference between the radii of curvature on k_{off} is larger than its effect on k_{on}, resulting in the overall stronger affinity for retinol displayed by the larger vesicles.

Thermodynamic parameters for the solvation of retinol off vesicles

To obtain better insight into the factors that contribute to the observed difference in k_{off} between small and large vesicles, the effect of temperature on this parameter was studied. Arrhenius plots for the rate of dissociation of retinol from vesicle populations with diameters of 0.025, 0.1, and 0.4 μM were constructed. The thermodynamic parameters obtained from these data are shown in **Table 3.** The data indicate that the enthalpy of activation decreases while the entropic contribution to the free energy of activation of dissociation of retinol from bilayers increases as the vesicles become larger.

DISCUSSION

The present work was undertaken to investigate the effect of the radius of curvature of phospholipid bilayers on their interactions of retinol. To do so, two types of reactions were studied: the dissociation of retinol from lipid bilayers into an aqueous phase, and the reverse reaction leading to association of the ligand with lipid bilayers. Both the association and the dissociation reactions were faster in small than in large vesicles (Table 2). The effect of bilayer radius on the rate of retinol dissociation was, however, more pronounced than the effect on the rate of association, resulting in an overall higher affinity for retinol in large as compared to small vesicles.

To better clarify the basis for the difference in the rate of dissociation of retinol from small and large vesicles, the thermodynamic parameters of the dissociation reaction in vesicles of 0.025, 0.1, and 0.4 μM diameter were measured. The data (Table 3) show that the free energy for activation of the dissociation reaction ($\Delta G^{\#}$, Table 3) is much larger than the energy for aqueous solvation of retinol off bilayers (ΔG^{0}, Table 2). This indicates the existence of a rate-determining step that precedes the solvation of retinol in the bulk aqueous phase. Such a step could be the formation of a transient, energetically unfavorable, intermediate involving the interactions of retinol with either the acyl chains or the head groups of the lipids. It could also involve restructuring of lipid-lipid interactions within the bilayer after the removal of retinol. Another potential rate-limiting step is the solvation of retinol in bound water adjacent to the bilayer surface which retinol must pass before entering the bulk aqueous phase (20). No information is available regarding this point but it is unlikely that the nature of the bound water layer will dramatically vary between small and large vesicles.

Examination of the energetics of dissociation of retinol from vesicles with different sizes (Table 3) revealed that the observed faster dissociation of retinol from small vesicles stemmed from a lower entropy of the activation energy; thus, at 25°C, $\Delta S^{\#}$ constituted a 2.3 kcal/mol smaller barrier for dissociation of retinol from 0.025 versus 0.4 μm vesicles. The larger $\Delta S^{\#}$ accompanying dissociation of retinol from the larger vesicles can be understood in terms of the better packing that these bilayers assume after removal of the ligand. In such a scenario, the activated intermediate of the retinol-bilayer complex that

TABLE 3. Thermodynamic parameters for the dissociation of retinol from DOPC vesicles with diameters of 0.025, 0.1, and 0.4 μm

Diameter	E_a	$\Delta H^{\#}$	$\Delta S^{\#}$	$T\Delta S^{\#}$	$\Delta G^{\#}$
μm	*kcal/mol*		*cal/mol* × deg	kcal/mol	
0.025	10.01	9.42	−31.92	−9.51	18.93
0.1	8.96	8.37	−36.57	−10.90	19.27
0.4	8.38	7.79	−39.75	−11.84	19.64

Values of the energy of activation (E_a) were calculated from the slopes of Arrhenius plots of the effect of temperature on the rate of transfer of retinol off DOPC vesicles with diameters of 0.025, 0.1, or 0.4 μm. $\Delta H^{\#}$ at 25°C was calculated by the expression: $\Delta H^{\#} = E_a - RT$. $\Delta S^{\#}$ at 25°C was calculated using the expression: $k_{off} = RT/Nh \exp(\Delta S^{\#}/R) \exp(-\Delta H^{\#}/RT)$, where R, N, and h are the gas constant, Avogadro's number, and the Planck constant, respectively. $\Delta G^{\#}$ was calculated from the expression: $\Delta G^{\#} = \Delta H^{\#} - T\Delta S^{\#}$.

precedes complete solvation of retinol will constitute a state in which retinol is partially removed from the hydrophobic environment of the bilayer interior into the aqueous phase. As a result, in the activated state, some lipid–lipid interactions will take place, i.e., lipid acyl chains will pack behind the ligand, and will cause a decrease in entropy. Such an effect will be more significant in larger vesicles which are better packed.

$\Delta H^{\#}$ for the dissociation reaction can also originate either from dissociation of bonds between retinol and bilayer lipids, or from restructuring of lipid–lipid bonds within the bilayer which may accompany the formation of the activated state of retinol prior to its complete solvation. The data in Table 3 show that $\Delta H^{\#}$ is smaller as the vesicles grow larger. This observation again indicates that the differences in the energetics of the process by which retinol leaves large versus small vesicles stem from differences in lipid–lipid interactions rather than from differential interactions of retinol with these bilayers. This conclusion is based on the consideration that bonds between retinol and lipids within bilayers (e.g., hydrogen bonds of the respective head groups, van der Waals interactions between the hydrophobic chains) will be stronger in the more closely packed bilayers of large vesicles. A larger energy of activation will thus be required to break such bonds. The data show that the reverse is true, i.e., that the energy of activation required for formation of the activated intermediate is smaller in large vesicles. Other parameters in the system, i.e., lipid–lipid bonds forming behind retinol as it leaves the bilayers, most likely compensate for these differences. Hence, the thermodynamic parameters of the reaction by which retinol leaves vesicles of different sizes suggest that the activated state is constituted of retinol that is partially removed to the aqueous phase, and that restructuring of lipid–lipid interactions within the bilayer play a role in determining the rate by which this ligand is solvated.

It is not clear whether the effects of a membrane radius of curvature on the rate of dissociation of retinol observed here reflect a general phenomenon relating to the interactions of small hydrophobic ligands with membranes. Similar to the finding of the present study, it was reported that the off rate of the fluorescent fatty acid derivative anthroloxy stearic acid from large vesicles is 18-fold slower as compared to small vesicles (21); and that the desorption of dimyristoyl PC from large gel-phase vesicles has a much lower rate than from gel-phase small vesicles (22). On the other hand, desorption of liquid-crystalline dimyristoyl PC was independent of the size of the donor vesicles (22). Studies of the transfer of cholesterol between vesicles and the effect of vesicle size on this process resulted in conflicting conclusions. It was reported that cholesterol moves between bilayers by a diffusion-dependent mechanism and that desorption from large vesicles is significantly slower than from small vesicles (23, 24). However, it was also reported that the rate of transfer of cholesterol between vesicles is independent of the donor vesicle size (25) and that cholesterol moves between bilayers by a collision-mediated process rather than by diffusion (26).

Many cells, especially those that are involved in substantial uptake or secretion of compounds, contain villi with radii of curvatures that are much smaller than the radius of the cell body. One such example is retinal pigment epithelium cells that supply retinoids for regeneration of rhodopsin in the eye. The radius of curvature found in different areas of the plasma membranes of pigment epithelium cells vary in the range of 0.05–5 μm (10). The data presented above indicate that the rate by which retinol is solvated from membranes will strongly depend on the radius of curvature in this range and may deviate by 5- to 10-fold. If the effects of the radius of curvature on the rate of interactions of retinoids with biological membranes are as significant as was found here with synthetic lipid bilayers, it is possible that the rate by which retinoids enter or leave cells will be significantly faster at the tips of villi as compared to other areas of the plasma membrane. The geometry of cellular surfaces hence may play a role in determining the rates as well as the direction by which amphipathic compounds such as retinol are secreted or taken up by cells.

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