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Kinetic Parameters of the Interactions of Retinol with Lipid Bilayers†

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ABSTRACT: The process of transfer of vitamin A alcohol (retinol) between unilamellar vesicles of phosphatidylcholine was studied. The transfer was found to proceed spontaneously by hydration from the bilayer and diffusion through the aqueous phase. The rate-limiting step for transfer was the dissociation from the bilayer, a step that was characterized in bilayers of egg phosphatidylcholine (PC) by a rate constant k_{off} = 0.64 s^{-1}. The rate constant for association of retinol with bilayers of egg PC was also determined: k_{on} = 2.9 × 10^6 s^{-1}. The relative avidities for retinol of vesicles comprised of PC lipids with the various fatty acyl chains were measured. It was found that the binding affinity was determined by the composition of the lipids, such that PC with symmetric acyl chains had a lower affinity for retinol vs those with mixed chains. To clarify the mechanism underlying this observation, the rates of dissociation and association of retinol bound to vesicles of dioleoyl-PC were determined. The rate of association of retinol with bilayers strongly depended on the composition of the fatty acyl chains of the lipids. The rate of dissociation of retinol from the bilayers of PC was found to be independent of that composition. The implications of the observations for the interactions of hydrophobic ligands with lipid bilayers are discussed.

Retinol (vitamin A alcohol), which is poorly soluble in water, circulates in blood bound to a water-soluble transport protein (Goodman, 1984). To explain how it is transported from blood to cells, it is usually proposed that the vitamin moves directly between several types of carrier proteins. Thus, it is hypothesized that transfer of retinol from serum retinol binding protein (RBP) to cells is mediated by specific receptors that are imbedded in plasma membranes (Chen & Heller, 1977; Rask & Peterson, 1976; McGuire et al., 1981; Heller, 1975; Ottonello & Maraini, 1981; Pfeffer et al., 1986) and that retinol transfers from these receptors directly to a cellular retinol binding protein (CRBP). CRBP, in turn, is proposed to be involved in carrying the vitamin to its sites of action (Chytil & Ong, 1984; Pfeffer et al., 1986).

It has been recently shown, however, that retinol spontaneously dissociates from RBP in vitro in model systems that do not contain any receptors (Fex & Johannesson, 1987; Noy & Xu, 1990). Free retinol will rapidly associate with lipid bilayers or with membranes if these components are present. Recent reports also indicate that retinol readily transfers across and between phospholipid bilayers (Fex & Johannesson, 1988; Noy, 1987, 1988). It is difficult to see, therefore, how association of retinol with the lipids of biological membranes and its movement between them can be prevented in vivo. The solubility of retinol in biological membranes then may have significant consequences for the mechanism by which this compound is distributed between different cells and different cellular compartments.

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It seems important, therefore, to quantitatively study the factors that define the association of retinol with membranes. In the present work, the relative avidities of various lipid bilayers and biological membranes for retinol were studied. In addition, the kinetic parameters of the interactions of retinol with membranes were determined. To enter cells via the lipids of plasma membranes, retinol in blood needs to associate with the plasma membrane, cross from the outer to the inner leaflet of the membrane, and dissociate into the cytosol. The rate constants of these steps were measured in order to estimate whether any of them may be rate limiting for uptake of retinol and to assess the importance of spontaneous transport of retinol via the lipids of plasma membranes for the process of uptake in vivo.

Materials and Methods

Lipids were obtained from Avanti Polar Lipids. *all-trans*-Retinol was obtained from Kodak. [^{3}H]Retinol and [^{14}C]dioleoylphosphatidylcholine were 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.

Membranes. Small unilamellar vesicles were prepared by sonication. The appropriate lipid in chloroform or hexane solution was pipetted into a stainless-steel cup, and the organic solvent was evaporated under a stream of argon or nitrogen. Buffer containing 100 mM NaCl, 10 mM HEPES (pH 7.0), and 1 mM ascorbate was added and the suspension sonicated in a Heat-System sonicator to clarity. Vesicles were centrifuged at 100000*g* for 15 min to pellet multilamellar vesicles. Concentrations of phospholipids were determined by the phosphorus content (Dittmer & Wells, 1969).

Rat liver plasma membranes were isolated on a discontinuous sucrose gradient as in Song et al. (1969). Total lipids were extracted from the plasma membranes according to the method outlined by Kates (1986).

Relative Avidities of Various Membranes for Retinol. These were measured by mixing small unilamellar vesicles 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-times for transfer of retinol between vesicles; see data below). Plasma membranes were than separated from vesicles by centrifugation in an Eppendorf centrifuge for 20 min. Supernatants and pellets were counted for retinol. To control for clean separation, the vesicles were, in some experiments, radiolabeled by cosonication with [^{14}C]dioleoylphosphatidylcholine ([^{14}C]PC DOPC)[1] and the pellet counted which ascertained that vesicles do not precipitate with the membranes. The presence of plasma membranes in the supernatant (incomplete precipitation) was monitored by determining the phosphorus content of the supernatant (indicating the amount of phospholipids). This control was done by centrifuging plasma membranes in the absence of vesicles and showed that better than 99% of plasma membrane lipids precipitated. An additional correction was made for the amount of retinol in the supernatant that is not associated with vesicles, i.e., retinol in water. This was done by measuring the equilibrium partition constant of retinol between plasma membranes and water. This allowed for the calculation of the amount of retinol in the water in each experiment, and the calculated value was subtracted from the amount of retinol found in the supernatant. To monitor the stability of retinol throughout the experiments, retinol was extracted into heptane (Goodman & Raz, 1972) after incubation under the experimental conditions. The absorption spectrum of the extracted retinol was found to be identical with the spectrum of fresh retinol.

Distribution constants of retinol between vesicles and membranes were calculated as the ratio of mole fractions of retinol in the lipids of the two phases:

$$K_{eq} = [(\text{mol of ret})_v/(\text{mol of lipid})_v]/[(\text{mol of ret})_m/(\text{mol of lipid})_m]$$

The subscripts v and m in the above expression denote the vesicles and the membranes, respectively.

K_{eq} is thus a measure for the relative affinity of bilayers for retinol.

Rate of Transfer of Retinol between Unilamellar Vesicles. To monitor the rate of transfer of retinol between two populations of vesicles, the fluorescent lipid probe *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (NBD-DPPE) was used. The excitation spectrum of NBD overlaps extensively with the emission spectrum of retinol. If 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. This was followed at 600 nm where the fluorescence of retinol itself is negligible. Movement of retinol thus was monitored by the increase in fluorescence intensity as retinol moved away from donor vesicles to acceptor vesicles which contained NBD upon mixing the two populations of vesicles. Mixing was done in a Durrum stopped-flow spectrophotometer connected to an On-Line Instrument System computer. Data analysis was performed by that computer. The detection system was operated in the fluorescence mode with excitation set at 325 nm. Emission was measured with a 600-nm-wide band-pass filter.

Results

Rate of Transfer of Retinol between Unilamellar Vesicles of Egg Phosphatidylcholine. The rate constant for transfer of retinol between two populations of unilamellar vesicles of egg phosphatidylcholine (egg PC) was determined. The transfer process can be summarized as

$$\text{ret–(mem)}_d + (\text{mem})_a \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \text{ret–(mem)}_a + (\text{mem})_d \quad (1)$$

$(\text{mem})_d$ and $(\text{mem})_a$ denote the acceptor and the donor vesicles, respectively. k_f and k_b are the rate constants for the forward and the backward reactions, respectively.

The fluorescent lipid probe NBD-DPPE was incorporated into the bilayer of unilamellar vesicles of egg PC by cosonication. These vesicles served as acceptor vesicles. When retinol and NBD-DPPE are in the same bilayer, energy transfer upon excitation of retinol (at 325 nm) results in fluorescence of NBD (at 600 nm). The fluorescence of retinol itself, which peaks at 480 nm, is negligible at 600 nm. Figure 1 shows that the fluorescence of NBD-DPPE, incorporated in unilamellar vesicles, increases linearly, upon addition of retinol up to an retinol/NBD mole ratio of 0.45. The fluorescence intensity can thus be used directly to monitor the presence of retinol in the vicinity of the probe. Retinol was incorporated into donor vesicles. Donor and acceptor vesicles were mixed in a stopped-flow spectrophotometer, and transfer of retinol was monitored by the time-dependent increase in fluorescence intensity. Figure 2A shows a typical trace. Data analysis was performed by an On-Line computer and showed that the best

[1] Abbreviations: egg PC, egg yolk phosphatidylcholine; DOPC, dioleoylphosphatidylcholine.

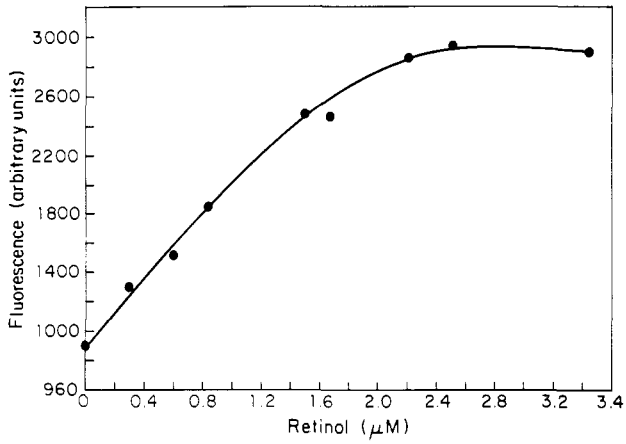

FIGURE 1: Intensity of the fluorescence of NBD-DPPE incorporated in vesicles of egg PC vs the concentration of retinol. Vesicles containing 312 nmol of egg PC and 6 nmol of NBD-DPPE were suspended in 1.5 mL of buffer (30 mM sodium phosphate + 0.2 M sodium chloride). Fluorescence intensity was monitored at 600 nm (excitation wavelength, 325 nm). Retinol was added from a concentrated solution in ethanol.

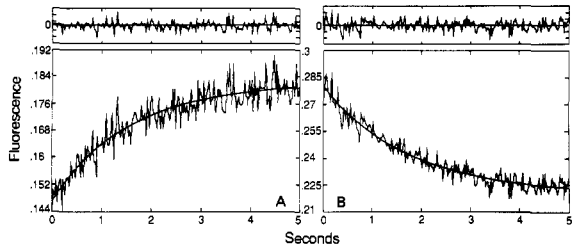

FIGURE 2: Transfer of retinol between unilamellar vesicles of egg PC. Equal volumes of suspensions of donor and acceptor vesicles were mixed in the stopped-flow apparatus. (A) Acceptor vesicles: 0.55 mM lipid of egg PC vesicles into which 2 mol % NBD-DPPE was incorporated. Donor vesicles: 0.825 mM lipid of egg PC vesicles + 5.5 nmol/mL retinol. (B) Acceptor vesicles: 0.825 mM lipid of egg PC vesicles. Donor vesicles: 0.55 mM lipid of egg PC vesicles into which 2 mol % NBD-DPPE was incorporated + 5.5 nmol/mL retinol. Transfer was followed by monitoring the increase (A) or decrease (B) of NBD fluorescence using a 600-nm band-pass filter. Excitation was at 325 nm. The curves drawn through the data points were obtained by nonlinear least-squares analyses, and the upper panels show the residuals.

fit for the transfer was a single first-order reaction. The apparent rate constant obtained by least-squares analysis was 0.57 s^{-1}. Transfer was also followed in the reverse direction. Retinol and NBD-DPPE were incorporated in the same vesicles, and these were mixed with "empty" vesicles of egg PC. Figure 2B shows a trace in which decrease in fluorescence is seen representing movement of retinol away from the NBD-containing vesicles. The rate constant of this reaction was found to be identical with the rate constant found for the forward direction, indicating that the inclusion of 2 mol % NBD-DPPE in the bilayer does not affect the rate of transfer.

Rate of Transfer at Various Ratios of Donor/Acceptor Vesicles. There are two possible pathways for the transfer of retinol from donor to acceptor vesicles. The simplest potential pathway is via the aqueous phase such that the first step would be solvation of retinol from the donor bilayer to the aqueous phase, as is presented in eq 2. Retinol would then diffuse to

$$\text{ret–(mem)} + n(H_2O) \underset{k_{on}}{\overset{k_{off}}{\rightleftharpoons}} \text{ret–}(H_2O)_n + \text{(mem)} \quad (2)$$

the acceptor bilayer and associate with it. Alternatively, retinol could transfer directly from one bilayer to the next during collisions of vesicles. In the later case, an increase in the acceptor/donor ratio will increase the probability of collisions between them which would result in enhanced transfer rates. The rate constants for transfer of retinol were determined at various ratios of donor/acceptor vesicles. The data, shown in Table I, indicate that the rate of transfer was independent of the donor/acceptor ratio. This points at the conclusion that transfer proceeds via the aqueous phase and not by collision. To test whether the vesicles fused or aggregated with each other during the experiment, the turbidity of the mixture was monitored by measuring its absorption at 400 nm over a period of 60 min. No increase in optical density was observed so that transfer of retinol by fusion or aggregation could be ruled out.

Table I: Effect of Varying the Ratio of Lipid Concentration in Donor and Acceptor Vesicles on the Rate of Transfer of Retinol[a]

[donor]	[acceptor]	[d/a]	k (s^{-1})	$t_{1/2}$ (s)
139	417	0.3	0.63 ± 0.03[b]	1.1
277	417	0.67	0.56 ± 0.03[c]	1.24
417	277	1.5	0.59 ± 0.04[c]	1.17

[a] A suspension of donor (+retinol) egg PC vesicles was mixed in the stopped-flow apparatus with acceptor (+NBD-DPPE) vesicles. Buffer included 30 mM sodium phosphate, pH 7.0, and 0.2 M sodium chloride. Final concentrations of lipids are given in nanomoles per milliliter. Final retinol concentration was 2.75 nmol/mL in all cases. The concentration of NBD-DPPE in the lipids of the acceptor vesicles was 2 mol %. Rate constants for transfer (±SEM) were determined from the time-dependent increase in NBD fluorescence (excitation, 325 nm; emission, 600 nm). [b] $n = 6$. [c] $n = 4$.

Rate of "Flip-Flop" of Retinol across Lipid Bilayers. Retinol is incorporated in both the inner and outer leaflets of the vesicles. To determine whether traversing the bilayer is rate limiting for transfer of retinol between vesicles, the following experiment was performed: retinol was incorporated into donor vesicles either by addition from a solution in ethanol or by cosonication with the lipids. The latter procedure was used to ensure the incorporation into both the inner and the outer leaflets of the bilayer. Donor vesicles were mixed with acceptor vesicles to give a donor/acceptor mole ratio of lipids of 1/9; i.e., the experiment was designed to drive 90% of the retinol to acceptor vesicles at equilibrium. Under these conditions, the transfer from both types of donor vesicles behaved like a single first-order reaction characterized by the same rate constant as in Figure 2 and Table I (data not shown). It can be concluded then that the rate by which retinol "flip-flops" from one leaflet of the bilayer to the other is not limiting for the overall transfer process. This follows from the consideration that a slow rate at this step would have resulted in a biphasic reaction in which a fraction of the retinol (corresponding to the amount in the outer leaflet of the donor vesicles) would have been transferred faster than a second fraction (corresponding to the amount in the inner leaflet). The rate of "flip-flop" or diffusion through the bilayer is thus faster than the time resolution of the stopped-flow apparatus, i.e., several milliseconds.

Rate Constants for Association and Dissociation of Retinol Bound to Unilamellar Vesicles of Egg Yolk Phosphatidylcholine. Retinol is a hydrophobic compound, which means that its partition constant between water and lipid bilayers ($K_{eq} = k_{on}/k_{off}$) considerably favors the latter; i.e., k_{on} is much larger than k_{off}. The rate-limiting step for the transfer of retinol between unilamellar vesicles is, most likely, the solvation of retinol from the donor bilayer into water (k_{off}). The apparent rate of the observed transfer reaction represents, therefore, the rate of the dissociation of retinol from egg PC bilayers and $k_{off}^{egg\ PC} = 0.64\ s^{-1}$.

K_{eq} was determined between water and plasma membranes as detailed under Materials and Methods. Radiolabeled retinol was mixed with plasma membranes and incubated to equi-

Table II: Equilibrium Distribution of Retinol between Synthetic Lipid Vesicles and Plasma Membranes from Rat Liver[a]

lipid composition of vesicles	K_{eq}
egg PC	4.8 ± 0.25[b]
1-oleoyl-2-palmitoyl-PC	4.4[c]
1-palmitoyl-2-oleoyl-PC	4.3[c]
dimyristoyl-PC	3.0[c]
dioleoyl-PC	2.2 ± 0.2[d]
dioleoyl-PC + 15 mol % cholesterol	2.0[c]
lipid extract from plasma membranes	1.2 ± 0.15[b]

[a] Vesicles (100 nmol of lipids) were mixed with plasma membranes isolated from rat liver (containing 100 nmol of lipid). ^{3}H-Labeled retinol (1–3 nmol) was added from a concentrated solution of ethanol. The final volume was 0.5 mL, and the buffer composition was the same as in Table I. The mixture was incubated at room temperature for 15 min and then centrifuged for 10 min in an Eppendorf centrifuge to separate membranes (pellet) from vesicles (supernatant). Pellets and supernatants were counted for [^{3}H]retinol. Equilibrium distribution constants are expressed as the ratio of the mole fraction of retinol in the lipids of the vesicles and the plasma membranes. [b] $n = 4$. [c] Average of two experiments. [d] $n = 3$.

librium, and plasma membranes were centrifuged to a pellet. Supernatant and pellet were counted for [^{3}H]retinol, and K_{eq} was calculated as the ratio of the mole fraction of retinol in the lipids of the membranes and in the water. $K_{eq}^{\text{plasma membrane}}$ expressed as molal fractions (see Materials and Methods) was found to be $(0.6 \pm 0.1) \times 10^6$ ($n = 5$). To obtain the rate constant for association of retinol with vesicles of egg PC, K_{eq} between water and egg PC vesicles is needed. This was determined in an experiment that included plasma membranes, egg PC vesicles, and retinol. Vesicles were separated from membranes by centrifugation, and the amounts of retinol in the supernatant and pellet were determined. The amount of retinol in the water was calculated from the amount in the plasma membranes and the K_{eq} between water and plasma membranes. The amount of retinol associated with the vesicles could thus be calculated by subtracting the amount in water from the total retinol found in the supernatant. $K_{eq}^{\text{egg PC}}$ was found to be 4.5×10^6.

The association constant can now be calculated from the expression $k_{on} = K_{eq}k_{off} = 2.9 \times 10^6$ s^{-1}.

Relative Avidities of Various Bilayers for Retinol. These were determined by using plasma membranes isolated from rat liver as a reference phase. The distribution of retinol between synthetic unilamellar vesicles of phospholipids and plasma membranes was measured. Equilibrium distribution constants were calculated as the ratio of mole fractions of retinol in the lipids of the vesicles and the membranes at equilibrium (see Materials and Methods). The data are shown in Table II. It was found that of the vesicles studied, those comprised of phospholipids with one saturated and one unsaturated fatty acid chain, namely, egg PC, 1-palmitoyl-2-oleoyl-PC, and 1-oleoyl-2-palmitoyl-PC, had the highest affinity for retinol. Vesicles that were made of lipids with symmetric chains, whether saturated or not, namely, dimyristoyl-PC and dioleoyl-PC, showed lower affinity for retinol. The lowest affinity found was displayed by vesicles that were comprised of total lipid extracted from the plasma membranes. The results suggest that the lipid composition of a bilayer is an important determining factor for the affinity of specific membranes for retinol.

The role of lipids in determining the affinity of membranes for retinol is emphasized further by the measurement of the distribution of retinol between whole plasma membranes and vesicles comprised of total lipids extracted from these membranes. The distribution constant for retinol between plasma membranes and the vesicles was 1.25 (Table II), indicating that the affinity of the lipids extracted from plasma membranes for retinol is slightly higher than that of the intact membranes. This seems to indicate that the presence of proteins is much less important for the overall affinity of the plasma membranes for retinol than the lipid composition.

Table III: Kinetic Parameters of the Interactions of Retinol with Unilamellar Vesicles of Egg PC and DOPC[a]

lipid composition	affinity	k_{off}	k_{on}
egg PC	1	0.64 ± 0.025[b]	2.9×10^6
dioleoyl PC	0.46	0.72 ± 0.40[c]	1.5×10^6

[a] The avidities of retinol for the bilayers were taken from the data in Table II and normalized to egg PC. The dissociation rate constants (k_{off}, s^{-1}) were determined from the stopped-flow measurements as described under Materials and Methods. The final reaction mixtures contained 445 μM either egg PC or DOPC in donor vesicles (that included 2 mol % NBD-DPPE and 3 μM retinol) and a 445 μM sample of the same phospholipids as acceptor vesicles. Buffer composition was 30 mM sodium phosphate and 200 mM sodium chloride, pH 7.0. Fluorescence was followed with an excitation wavelength of 330 nm; emission was monitored through a 600-nm wide band-pass filter. Rate constants for association (k_{on}) were calculated from k_{off}, and the partition constants between bilayers and water and are expressed as s^{-1} (see text). [b] $n = 9$. [c] $n = 5$.

Rate Constants for Association and Dissociation of Retinol Bound to Unilamellar Vesicles of Dioleoylphosphatidylcholine. To gain further insight into the parameters that regulate the affinity of bilayers for retinol, unilamellar vesicles of DOPC were chosen to analyze the mechanism underlying their lower affinity for retinol vs vesicles of egg PC. This was done by determining the rate constant for dissociation of retinol from vesicles of DOPC by the stopped-flow spectrofluorometric technique as described above for vesicles of egg PC. k_{off} from these vesicles was found to be 0.72 s^{-1} (Table III). The rate constant for association of retinol with bilayers of DOPC was calculated from the differences of affinity for retinol between egg PC and DOPC (Table II), the measured k_{off}'s from the two lipids, and k_{on} for egg PC (see above) by using the expression:

$$K_{eq}^{\text{egg PC}}/K_{eq}^{\text{DOPC}} = (k_{on}^{\text{egg PC}}/k_{off}^{\text{egg PC}})/(k_{on}^{\text{DOPC}}/k_{off}^{\text{DOPC}})$$

The results are shown in Table III. It was found that the dissociation constants of retinol from egg PC and from DOPC are very similar. The difference in affinity of these bilayers for retinol largely stemmed from differences in the rate of association of retinol with them that was 2-fold slower with DOPC than with egg PC.

The effect of temperature on k_{off} was also studied. Figure 3 shows an Arrhenius plot for the rate of dissociation of retinol bound to vesicles of DOPC. The energy of activation was found to be 9.24 kcal/mol. $\Delta H^{\ddagger}$ was ($\Delta H^{\ddagger} = E_a - RT$) 8.66 kcal/mol. $\Delta S^{\ddagger}$ at 20 °C was calculated from the expression: $\Delta S^{\ddagger} = R \ln(NhX/RT)$, in which $X = k_{off}/e^{-\Delta H/RT}$. $\Delta S^{\ddagger}$ was −29.5 cal mol^{-1} deg^{-1} and $T\Delta S^{\ddagger} = -8.4$ kcal/mol. The contributions of entropic and enthalpic components to the formation of the activated species are thus, almost identical.

Discussion

Transfer of retinol between unilamellar vesicles was found, in this study, to proceed by hydration from a bilayer, followed by diffusion through water and association with another bilayer. The slowest step of this transfer was the hydration from the bilayer, a step that had a rate constant of 0.64 s^{-1} ($t_{1/2}$ = 1.1 s). In addition, retinol was found to traverse the bilayers at a rate that is faster than the time resolution capability of the stopped-flow methodology, i.e., several milliseconds. These

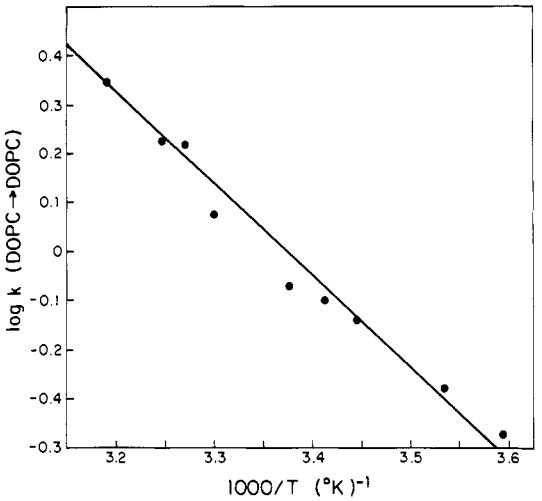

FIGURE 3: Effect of temperature on the rate of transfer of retinol between vesicles of DOPC. Stopped-flow experiments were carried out as in Figure 2 at the indicated temperatures. Acquisition of data and calculation of the rate constants were performed by the On-Line computer.

conclusions are compatible with qualitative data reported by Fex and Johannesson (1988) showing spontaneous transfer of retinol between unilamellar vesicles of phospholipids. In addition, following our initial report of these results (Noy, 1987, 1988), another group has reconfirmed them (Ho et al., 1989).

The relative avidities for retinol of unilamellar vesicles comprised of several types of phospholipids were determined (Table II). All of the phospholipids chosen for this study were at a liquid-crystal state at the temperature employed. This was done in order to simplify interpretation of the data. The data indicate that the lipid composition of a bilayer is an important determinant for its affinity for retinol. For example, a 4-fold difference was found between the affinity of vesicles of egg PC vs vesicles made from a total lipid extract from plasma membranes of rat liver. In addition, it was found that the presence of proteins in a bilayer decreases its affinity for retinol as compared to a bilayer comprised of the lipids alone. Interestingly, studies of the binding of free fatty acids to bilayers have also shown that the presence of proteins causes a decrease of the affinity of bilayers for these ligands by about 20% (Cooper et al., 1989). Since we have not studied this phenomenon further, the mechanism underlying it is not clear. It could, potentially, represent hindrance for accommodation of ligands in the hydrophobic core of the bilayers by integral membrane proteins. Alternatively, the presence of proteins could affect the system by masking the lipid–water interface, thereby retarding the approach of the ligands to the bilayers.

To further clarify the mechanism underlying the differences in affinity of phospholipid bilayers for retinol, the kinetic constants of the interactions of retinol with egg PC and with DOPC were compared (Table III). The origin of the difference between the interactions of retinol with these bilayers was found to lie with the rate constant for association. Thus, k_{on} for DOPC was 2-fold slower than k_{on} for egg PC, accounting for the major part of the observed difference in affinity which was 2.18-fold. The rate constants for dissociation of retinol from the two bilayers were very similar. It seems, then, that the energy barrier for association is distinct from the one controlling the dissociation. It is likely that the rate-limiting step for dissociation of retinol from the bilayer is the solvation into the water, which involves breaking the hydrogen bond that probably exists between the hydroxyl group of retinol and the PC head group. Such a step was found, for example, to be rate limiting for dissociation of cholesterol from bilayers (Ramsammy et al., 1984) and suggested to also limit the rate of dissociation of free fatty acids from bilayers (Daniels et al., 1985; Storch & Kleinfeld, 1986). Thus, since the head groups of the two bilayers studied are the same, no large differences should be expected between their respective k_{off}'s. The rate of association, on the other hand, may be determined by the tightness of packing of the bilayers. Thus, it was found (Table II) that bilayers comprised of mixed-chain fatty acids have a higher affinity for retinol (i.e., the rate of association is faster) than bilayers with symmetric chains. Not enough is known to quantitatively predict differences between these bilayers, but it is reasonable to assume that the ability, in vesicles comprised of symmetric lipids, to stack the fatty acid chains results in bilayers that are packed more tightly than those of inhomogeneous composition. This would also result in larger distances between the head groups at the lipid–water interface in the latter type of bilayers, explaining the faster rates by which retinol (and possibly other ligands) associates with them.

Comparing the data regarding the way by which retinol interacts with lipid bilayers with the interactions of free fatty acids with the same bilayers shows interesting differences. Our studies have shown previously that the rate constant for dissociation of palmitic acid from vesicles of egg PC ($k_{off,palm}$) is 5.3 s^{-1} (Daniels et al., 1985; Noy et al., 1986), a value that is 8-fold faster than the rate of dissociation of retinol. The rate constant for association of palmitate with bilayers ($k_{on,palm}$) can be calculated from this and from the partition constant between bilayers and water ($K_{eq,palm}$) which is 5×10^6 (molal fractions) (Noy & Zakim, 1985). Thus, $k_{on,palm} = K_{eq,palm}k_{off,palm} = 2.65 \times 10^7$ s^{-1}. The rate constant for association of retinol with bilayers of egg PC, reported in the present study, is 2.9×10^6 s^{-1}. These values probably represent a larger energy barrier for the penetration of the bulky ring of retinol than for the fatty acid chain, accounting for the slower rate of association of retinol vs palmitate with bilayers. The slower rate of solvation of retinol vs palmitate may reflect several components of the interactions of these ligands with the bilayers or with water adjacent to the bilayer. It is noteworthy that both the association and the dissociation of retinol with bilayers are an order of magnitude slower than the analogous reactions of palmitate, resulting in an overall similar affinity of egg PC bilayers for the two ligands. The data show that entropy and enthalpy contribute equally to the free energy of retinol activation. The rate of solvation of palmitate, on the other hand, is mainly determined by the enthalpy of activation (Daniels et al., 1985). This probably reflects the (predictable) introduction of greater disorder in the hydrophobic core of the bilayer by the inclusion of the ring of retinol than by the chain of palmitate.

A study of the solvation of 9-(3-pyrenyl)nonanoic acid from lipid bilayers has been reported (Doody et al., 1980). The carboxyl head group, in this fluorescent fatty acid, is separated from a pyrene group by nine carbons, so that it is similar to retinol. The values of the thermodynamic parameters reported for the activation of this fatty acid derivative were very similar to those found here for retinol. Thus, $\Delta G^{\ddagger}$ was reported to be 16.3 kcal/mol, $\Delta H^{\ddagger}$ was 8.6 kcal/mol, and $T\Delta S^{\ddagger}$ was 7.8 kcal/mol (Doody et al., 1980). The values of $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $T\Delta S^{\ddagger}$ for solvation of retinol were 17.3, 8.66, and 8.64 kcal/mol, respectively. A large fraction of $\Delta H^{\ddagger}$ is probably related to the hydrogen bonding of the ligands to the head group of the lipid which is dissociated upon solvation (Parkes et al., 1982; Ramsammy et al., 1984; Daniels et al., 1985;

Storch & Kleinfeld, 1986). The observation that the values of $\Delta H^{\ddagger}$ for retinol and for the pyrenylnonanoic acid are identical may indicate similar energy of hydrogen bonding between these ligands and PC.

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Thermodynamic Parameters of the Binding of Retinol to Binding Proteins and to Membranes†

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ABSTRACT: Retinol (vitamin A alcohol) is a hydrophobic compound and distributes in vivo mainly between binding proteins and cellular membranes. To better clarify the nature of the interactions of retinol with these phases which have a high affinity for it, the thermodynamic parameters of these interactions were studied. The temperature-dependence profiles of the binding of retinol to bovine retinol binding protein, bovine serum albumin, unilamellar vesicles of dioleoylphosphatidylcholine, and plasma membranes from rat liver were determined. It was found that binding of retinol to retinol binding protein is characterized by a large increase in entropy ($T\Delta S^{\circ}$ = +10.32 kcal/mol) and no change in enthalpy. Binding to albumin is driven by enthalpy (ΔH° = −8.34 kcal/mol) and is accompanied by a decrease in entropy ($T\Delta S^{\circ}$ = −2.88 kcal/mol). Partitioning of retinal into unilamellar vesicles and into plasma membranes is stabilized both by enthalpic (ΔH° was −3.3 and −5.5 kcal/mol, respectively) and by entropic ($T\Delta S^{\circ}$ was +4.44 and +2.91 kcal/mol, respectively) components. The implications of these finding are discussed.

Retinol (vitamin A alcohol) is a hydrophobic compound that has a single polar group, a hydroxyl. The vitamin is thus poorly soluble in water. Several types of specific water-soluble binding proteins for retinol are known to exist in the cytosol of cells (Saari et al., 1978; Fex & Johannesson, 1982; Bonelli & De Luca, 1985; Wiggert et al., 1982). In blood, retinol binds to retinol binding protein (RBP) (Goodman, 1984) and is also capable of binding to serum albumin (Sani et al., 1978). It has been recently shown that retinol spontaneously dissociates from RBP, a reaction that has a half-time of 5 min (Noy & Xu, 1990b), that retinol easily dissolves in the lipids of biological membranes, and that it rapidly traverses lipid bilayers and transfers between them (Fex & Johannesson, 1988; Noy & Xu, 1990a). It seems reasonable, therefore, to assume that retinol binds in vivo mainly to binding proteins and to membranes. Characterizing the interactions of the vitamin with these compartments, which have high avidities for it, is important, therefore, for understanding the factors that determine the vitamin's distribution in tissues and in cells.

In the present work, we report on the thermodynamic properties of the interactions of retinol with membranes and

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