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

Noa Noy* and Zhi-Jun Xu
Department of Medicine, Cornell University Medical College, New York, New York 10021
Received September 7, 1989; Revised Manuscript Received December 29, 1989

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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* To whom correspondence should be addressed.

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with the two proteins that can bind it in blood—RBP and albumin.

Materials and Methods

Lipids were obtained from Avanti Polar Lipids. Chromatography gels were purchased from Pharmacia. *all-trans*-Retinol was obtained from Kodak. [^{3}H]Retinol and [^{14}C]-dioleoylphosphatidylcholine were from New England Nuclear. Bovine serum albumin was purchased from Calbiochem. 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.

Proteins. Bovine RBP was purified from bovine serum (Pel-Freeze Co.) by the procedure outlined in Berni et al. (1985) and was found to contain 0.85–1 mol of retinol/mol of protein. Concentrations of RBP and of retinol were determined from their extinction coefficients (Horrowitz & Heller, 1974).

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 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. The concentration of lipids was 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).

Partitioning of Retinol between Plasma Membranes and Vesicles or Binding Proteins. Partitioning was measured by mixing small unilamellar vesicles or binding proteins with plasma membranes isolated from rat liver. ^{3}H-Labeled retinol (30000 cpm/nmol) was added, and the mixture was incubated at the designated temperature for 30 min. Buffer contained 30 mM sodium phosphate, pH 7.0, and 0.2 M sodium chloride. To determine the distribution of retinol between RBP and plasma membranes, holo-RBP containing 0.85–1 mol of retinol/mol of protein was used. [^{3}H]Retinol was added to the protein and the mixture incubated for 1 h, to achieve equilibrium of the radioactive trace with the protein-bound ligand, before the addition of the membranes. Plasma membranes were separated from vesicles or proteins by centrifugation at 100000*g* for 15 min in a Beckman L5 ultracentrifuge. The centrifuge chamber and the fixed-angle (type 40TI) rotor were preequilibrated at the appropriate temperature. Supernatants and pellets were counted for retinol. To control for clean separation, the vesicles were, in some experiments, radiolabeled by cosonication with [^{14}C]dioleoyl-PC (DOPC) and the pellet counted, which ascertained that vesicles did 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 amount of phospholipids). This was done by centrifuging plasma membranes alone. Better than 99% of plasma membrane lipids were found to precipitate to the pellet. An additional correction was made for the amount of retinol in the supernatant that is not associated wtih vesicles or proteins, i.e., retinol in water. This was done by measuring the equilibrium partition constant of retinol between plasma membranes and water at the various temperatures. The amount of retinol in the water in each experiment was then calculated from this partition constant and the amount of retinol in the plasma membranes in the pellet. 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.

Results

Retinol, being poorly soluble in water, distributes in vivo between binding proteins and cellular membranes. To gain insight into the parameters that govern the interactions of retinol with these compartments, the effects of temperature on the binding of retinol to proteins and to unilamellar lipid vesicles were studied. Binding was not measured directly from an aqueous phase because such measurements are technically difficult. Equilibrium dialysis methods are not very efficient when hydrophobic compounds are involved because the ligands tend to adsorb to dialysis bags and because formation of micelles retards distribution of the ligand. Consequently, the rate of equilibration is very slow. Since retinol is a labile compound, a slow rate of equilibration renders the experiment infeasible. Binding constants could alternatively be measured upon separation of binding protein or vesicles from the aqueous phase. This, however, is difficult to achieve without disturbing the equilibrium. Distribution of retinol was therefore determined between binding proteins or unilamellar lipid vesicles and a reference phase. The requirements for such a phase were (1) that it has a high affinity for retinol and (2) that it can easily and cleanly be separated from the proteins or the vesicles. For that purpose, plasma membranes isolated from rat liver were used. These have an affinity for retinol that is comparable to that of albumin (Noy & Xu, 1990a) and are easily separated by centrifugation from the water-soluble proteins or from a suspension of unilamellar vesicles. The effects of temperature on the equilibrium distributions of retinol between unilamellar vesicles of dioleoylphosphatidylcholine (DOPC), bovine RBP, or bovine serum albumin and plasma membranes were thus studied. The partitioning of retinol between plasma membranes and water at various temperatures was determined directly. The thermodynamic parameters for retinol binding to all of the above components could then be calculated.

Effect of Temperature on the Transfer of Retinol from Plasma Membranes to an Aqueous Phase. This was determined by incubating plasma membranes with ^{3}H-labeled retinol at various temperatures, centrifuging the membranes at the indicated temperature, and counting pellets and supernatants for the presence of [^{3}H]retinol, as described under Materials and Methods. Equilibrium distribution constants were expressed as the ratio of mole fractions of retinol in the water and in the lipids of the membranes according to the expression:

$$K_{eq} = [(\text{mol of ret})_{aq}/(\text{mol of } H_2O)]/[(\text{mol of ret})_{mem}/(\text{mol of lipid})_{mem}]$$

In this expression, $(\text{ret})_{aq}$ and $(\text{ret})_{mem}$ represent the amount of retinol in the aqueous and in the membrane phases, respectively.

In Figure 1, the data are shown in the form of the van't Hoff plot: $\ln K_{eq}$ vs $1/T$. The enthalpy of the transfer of retinol from membranes to water was found from the slope of the plot to be $\Delta H^\circ = +3.3$ kcal/mol. At 20 °C, K_{eq} is 2×10^{-6}, and $\Delta G^\circ = -RT \ln K_{eq} = +7.6$ kcal/mol. The entropy of the transfer can then be calculated by using the expression ΔG°

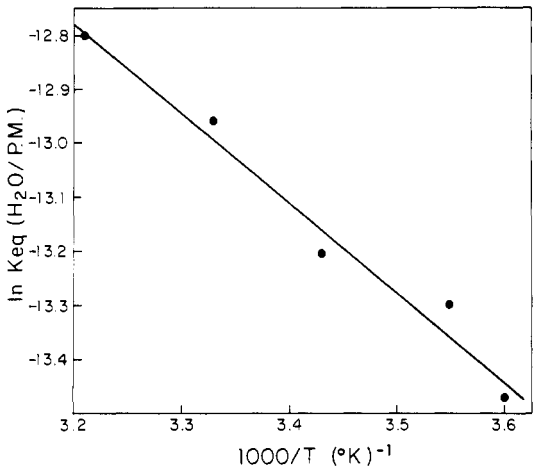

FIGURE 1: Temperature dependence of the partitioning of retinol between plasma membranes and an aqueous phase. Plasma membranes containing 100 nmol of phospholipids were mixed with 1 nmol of ^{3}H-labeled retinol. The mixture was incubated at the designated temperature for 30 min and then centrifuged as detailed under Materials and Methods. Supernatants and pellets were counted for [^{3}H]retinol. The data are presented in the form of the van't Hoff plot. K(H_2O/P.M.) is the partition constant calculated as the ratio of the mole fraction of retinol in the aqueous phase and in the lipids of the membranes.

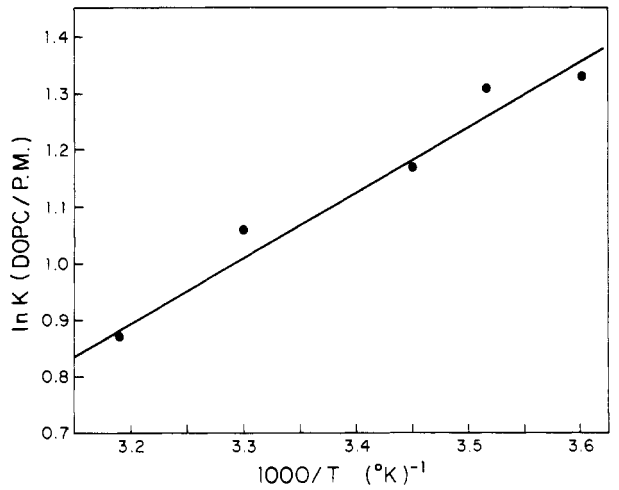

FIGURE 2: Temperature dependence of the partitioning of retinol between vesicles of DOPC and plasma membranes. Plasma membranes were mixed with vesicles of DOPC. Each phase contained 100 nmol of phospholipids. Following the addition of 1 nmol of retinol, the mixture was incubated and centrifuged as detailed under Materials and Methods. Data are presented in the form of the van't Hoff plot. K(DOPC/P.M.) is the partition constant calculated as the ratio of the mole fraction of retinol in the lipids of the vesicles and the membranes.

$= \Delta H° - T\Delta S°$. $\Delta S°$ thus calculated was −14.9 cal/(mol·deg)$^{-1}$.

Effect of Temperature on the Partitioning of Retinol between Unilamellar Vesicles of DOPC and Plasma Membranes from Rat Liver. The equilibrium distribution constants of retinol between DOPC vesicles and plasma membranes were measured at various temperatures. K_{eq} was expressed as the ratio of the mole fraction of retinol in the lipids of the vesicles and the membranes, respectively. The enthalpy of the partitioning was obtained from the van't Hoff plot (Figure 2). $\Delta H°$ was found from the slope of Figure 2 to be −2.17 kcal/mol. At 20 °C, K_{eq} was 3. $\Delta G°$ of the partitioning was −640.6 cal/mol. The entropy of the reaction (at 20 °C) calculated by using these parameters was $\Delta S° = -5.2$ cal/(mol·deg)$^{-1}$ ($T\Delta S° = -1.53$ kcal/mol). Transfer of retinol from plasma membranes to unilamellar vesicles is then driven by the decrease in the enthalpy of the system.

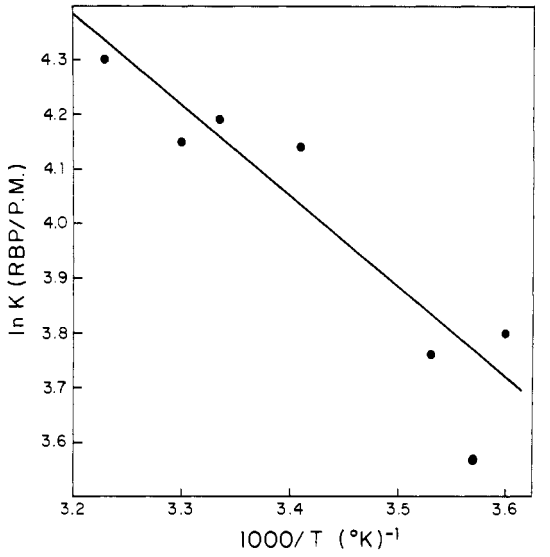

FIGURE 3: Temperature dependence of the distribution of retinol between RBP and plasma membranes. One nanomole of holo-RBP containing 0.85–1 mol of retinol/mol of protein was incubated with the radioactive label for 1 h to achieve equilibration of endogenic retinol with [^{3}H]retinol. Plasma membranes containing 100 nmol of lipids were then added, and the mixture was treated as detailed under Materials and Methods. The data are presented in the form of the van't Hoff plot. K(RBP/P.M.) is the distribution constant calculated as the ratio of the mole fraction of retinol bound to the protein and to the membranes.

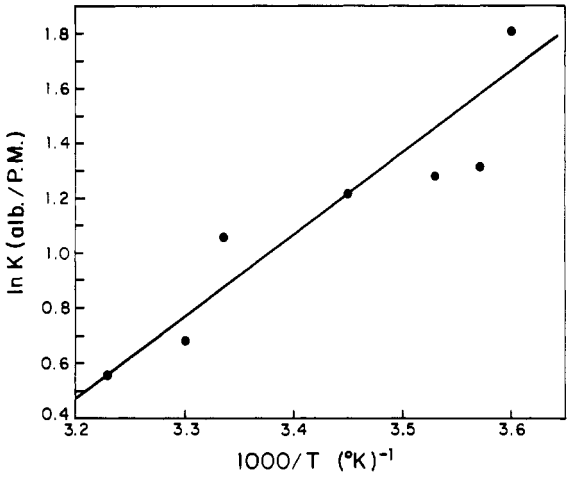

FIGURE 4: Temperature dependence of the distribution of retinol between serum albumin and plasma membranes. Five nanomoles of albumin was mixed with plasma membranes containing 100 nmol of lipids and 1 nmol of radioactively labeled retinol. The mixture was incubated at the various temperatures and treated as detailed under Materials and Methods. The data are presented in the form of the van't Hoff plot. K(alb/P.M.) is the distribution constant calculated as the ratio of the mole fraction of retinol bound to albumin and to the membranes.

Effect of Temperature on the Distribution of Retinol between Serum Retinol Binding Protein (RBP) or Serum Albumin and Plasma Membranes. Figures 3 and 4 show the van't Hoff plots of the effect of temperature on the distribution of retinol between bovine serum RBP (Figure 3) or between bovine serum albumin (Figure 4) and plasma membranes. The thermodynamic parameters obtained from these data are presented in Table I. It is clear from the data that different types of interactions drive the transfer of retinol from plasma membranes to RBP vs albumin. Transfer to albumin, similarly to binding to unilamellar vesicles, is driven by enthalpic com-

Table I: Thermodynamic Parameters for Transfer of Retinol from Liver Plasma Membranes to RBP or to Serum Albumin[a]

protein	$\Delta G°$	$\Delta H°$	$\Delta S°$	$T\Delta S°$
RBP	−2.48	+3.30	+19.70	+5.88
albumin	−0.64	−7.96	−24.56	−7.32

[a] $\Delta G°$, $\Delta H°$, and $T\Delta S°$ are presented as kilocalories per mole. $\Delta S°$ is in calories per mole per degrees centigrade. Values were calculated from the data in Figures 3 and 4. $\Delta H°$ was calculated from the slopes of the figures, using slope = $-\Delta H°/R$. $\Delta G°$ and $\Delta S°$ at 20 °C were calculated from the expressions $\Delta G°$ and $-RT \ln K_{eq}$ and $\Delta G°$ and $\Delta H° - T\Delta S°$. R in these expressions is the gas constant.

Table II: Thermodynamic Parameters for Binding of Retinol to RBP, Albumin, and Vesicles of DOPC[a]

binding phase	$\Delta G°$	$\Delta H°$	$\Delta S°$
RBP	−10.31	0	+34.6
albumin	−8.38	−11.26	−9.66
DOPC	−8.36	−5.47	+9.70

[a] Parameters for transfer of retinol from water to the various binding phases were calculated by subtracting the measured values for transfer from plasma membranes to water from the measured values for transfer from plasma membranes to the binding phases (Table I and see text).

ponents. Transfer to RBP, on the other hand, is characterized by a large increase in the entropy of the system.

Forces Contributing to Binding of Retinol to RBP, Albumin, and Lipid Bilayers. The thermodynamic parameters relating to the binding of retinol to these components from an aqueous phase can now be calculated. This was done by subtracting the values regarding transfer from plasma membranes to water from the appropriate values for the transfer from plasma membranes to proteins and vesicles. The results are shown in Table II. It is clear from these data that binding of retinol to RBP is driven purely by entropy while binding to albumin is stabilized by enthalpic forces that overcome the loss of the entropy of the system. Binding to unilamellar vesicles is driven both by bonding energy and by an entropy gain.

Discussion

The data presented above show that transfer of retinol from plasma membranes to an aqueous phase has thermodynamic parameters that are typical of solvation of hydrophobic compounds; i.e., it is characterized by a large decrease in entropy ($T\Delta S°$ = −4.44 kcal/mol) accompanied by a smaller increase in enthalpy ($\Delta H°$ = +3.3 kcal/mol) (Tanford, 1980). The situation is somewhat different when vesicles are involved; i.e., transfer of retinol from vesicles to water is characterized by a smaller decrease in entropy ($T\Delta S°$ = −2.91 kcal/mol) and a larger enthalpic component ($\Delta H°$ = +5.47 kcal/mol).

It is interesting to note, in regard to these data, the previously reported observation that the affinity of lipid vesicles comprised of total lipids of plasma membranes for retinol was 20% higher than the affinity of the whole plasma membranes (Noy & Xu, 1990a), which indicates that the presence of integral membrane proteins diminishes binding of retinol to membranes. A similar observation was made about differences in the affinity of plasma membranes vs vesicles comprised of plasma membrane lipids for free fatty acids (Cooper et al., 1989). The data presented here point at a higher interaction energy between retinol and the lipids of the vesicles vs the membranes. This could indicate that in membranes, hydrogen bonds between the head groups of phospholipids and integral membrane proteins limit the number of available groups for bonding with retinol or fatty acids as compared to vesicles comprised of lipids only. Hydrogen bonding in a low dielectric medium is accompanied, in addition to the decrease in enthalpy, by a decrease in entropy (Ross & Subramanian, 1981). The data show that, indeed, binding to vesicles results in a smaller change in entropy as compared to binding to the membranes, which supports the conclusion that the difference between the affinity for retinol of the membranes and the vesicles stems from the difference in hydrogen bonds.

The values of $\Delta H°$ and $\Delta S°$ for binding of retinol to vesicles of DOPC are comparable, both in sign and in magnitude, to the thermodynamic parameters for binding of the hydrophobic anions tetraphenylboron and dipicrylamine to bilayers (Flewelling & Hubbell, 1986). Binding of an analogous cation to bilayers has a positive enthalpy, but the entropic contribution to the binding is about the same as the anion (Flewelling & Hubbell, 1986). Specifically, $T\Delta S°$ for retinol was found to be 2 kcal smaller than $T\Delta S°$ for these ions. This is surprising because it is expected that the hydrophobic energies for binding of retinol with its one ring would be much lower than those of the tetraphenyl ions. A possible explanation is that not all of the hydrophobic groups of the tetraphenyl structure are expressed at their binding site in the bilayer because of steric hindrance.

The results concerning binding of retinol to RBP (Table II) reveal that it is driven purely by an increase in entropy and that the enthalpy of binding is approximately zero. This indicates that binding is mainly stabilized by hydrophobic interactions and that the hydroxyl of retinol does not play a role in binding to RBP. This conclusion is in agreement with a recent study in which it was qualitatively shown that binding of several types of retinoids to RBP displays a low specificity for the polar end group and a high specificity for the *all-trans*-retinyl moiety (Siegenthaler & Saurat, 1987). Binding of other retinoids to RBP was not studied here, but it can be predicted from these results that RBP will bind retinoic acid and retinal with the same affinity as binding of retinol. The dissociation constants of retinol and retinoic acid from human RBP were reported to be 1.9×10^{-7} and 2.1×10^{-7} M, respectively (Cogan et al., 1976).

Binding of retinol to serum albumin was found to be driven by enthalpy (Table II). Binding also resulted in a decrease in the entropy of the system. There are essentially four types of noncovalent interactions that could participate in binding of ligands to proteins. These are hydrophobic bonds, electrostatic interactions, van der Waals forces, and hydrogen bonds (Ross & Subarmanian, 1981; Klotz, 1973). Of these, only the last two types are accompanied by negative values of both $\Delta H°$ and $\Delta S°$. van der Waals forces are usually characterized by a large negative value of $\Delta H°$ relative to the entropic contribution (Klotz, 1973). The data in Table II show a value of −11.26 kcal/mol for the enthalpy change vs a value of −2.88 kcal/mol for the entropy term ($T\Delta S°$). These data, then, show that the interactions of retinol with albumin are mainly van der Waals interactions. We have found recently that binding of free fatty acids to albumin at a mole ratio of 1:1 does not alter the affinity of that protein for retinol and concluded that retinol and free fatty acids do not bind at the same site on albumin (Noy & Xu, 1990b). The data presented here seem to indicate that retinol does not bind at a specific site at all but that it interacts with albumin in a nonspecific manner.

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Spontaneous Interbilayer Transfer of Hexosylceramides between Phospholipid Bilayers†

Jeffrey D. Jones,‡ P. F. Almeida, and T. E. Thompson*
Department of Biochemistry, University of Virginia, Charlottesville, Virginia 22908
Received November 3, 1989; Revised Manuscript Received December 29, 1989

ABSTRACT: The kinetics of spontaneous transfer of various glucosyl- and galactosylceramides between 1-palmitoyl-2-oleoylphosphatidylcholine vesicles have been examined at 45 °C. Bovine brain galactosylceramides, kerasin and phrenosin, were found to transfer with biexponential kinetics. The kerasin fast pool is ~17% with a half-time of 29 h and the slow pool ~83% with a half-time of 2700 h. In contrast, semisynthetic *N*-palmitoylgalactosylceramide at the same temperature transfers with single-exponential kinetics with a half-time of 32 h. The half-time for *N*-lignoceroylgalactosylceramide under the same conditions proved to be greater than 3500 h. No concentration dependence for these half-times was found in the concentration range studied (0-10 mol %). Similar results were obtained for semisynthetic glucosylceramides. The biexponential kinetics observed for the two bovine brain ceramides, both of which are mixtures of short and long acyl chain molecules, are most probably a reflection of the strong dependence of transfer rate on acyl chain length. The very slow transfer rates of the long acyl chain hexosylceramides ensure that these molecules are lost very slowly, if at all, by spontaneous transfer from the external surface of plasma membranes; a result that is consistent with the putative biological role of glycosphingolipids.

Glycosphingolipids, a large class of lipid membrane components found almost exclusively in the external surface of the plasma membrane of eukaryotic cells (Hakomori, 1981; Barbosa & Pinto da Silva, 1983), play important rolls in cell-cell interactions, differentiation, and oncogenesis (Hakomori, 1983; Feizi, 1985). The localization of these molecules on the surface of the cell, while presumably critical for their cellular functions, would seem to make them susceptible to loss from the surface by spontaneous intermembrane transfer, a process well studied for phospholipids. Studies of the spontaneous interbilayer transfer of three glycosphingolipids between liquid-crystalline phosphatidylcholine vesicles have been reported. For Gaucher glucosylceramide the half-time of transfer at 37 and 45 °C was found to be greater than 30 days (Correa-Freire et al., 1982). In a study by Brown and co-workers (1985b) on asialo-GM_1 transfer, two kinetically distinguishable glycosphingolipid pools were observed. However, the bulk of the asialo-GM_1 transferred with a half-time of ~24 days at 45 °C. These results, which are generally consistent with the stable residence of these lipids in the external surface of plasma membranes, have been interpreted in terms of the presumptive phase structure of the glycosphingolipid/phosholipid bilayers (Thompson & Tillack, 1986; Curatolo, 1987a). In contrast to these very long half-times for transfer, the ganglioside GM_1 was observed to transfer with half-times of ~4 days (Masserini & Freire, 1986; Brown & Thompson, 1987). This observation is in agreement with other

† Supported by NIH Grant GM-23573.
‡ Present address: University of Texas Southwestern Medical Center, Department of Pharmacology, 5323 Harry Hines Blvd., Dallas, TX 75235.

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