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Retinol transfer across and between phospholipid bilayer membranes

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The transfer of retinol across and between bilayer membranes was studied in vitro using unilamellar liposomes and erythrocytes. Transmembrane movement of retinol in phospholipid bilayer membranes was a spontaneous and rapid process with a halflife of less than 30 s. Retinol transfer between liposomes and between liposomes and erythrocytes was also a spontaneous and rapid process with a halflife of less than 10 min. The results suggest that retinol transport in the cell might not need the participation of specific transfer proteins.

Introduction

Retinol (vitamin A alcohol) circulates in the plasma bound to a specific transport protein, the retinol-binding protein [1]. How retinol is transferred from its binding protein into cells is not known in detail. At least in some cells, cellular uptake of retinol is assumed to occur in conjunction with the interaction of the retinol–retinol-binding protein complex with a specific cell surface receptor [2–4]. Previously [5], we showed that retinol, like long-chain fatty acids [6–9], could transfer spontaneously in vitro from its binding protein to phospholipid bilayer membranes, and we suggested that this mechanism might also operate in vivo in addition to the receptor-mediated retinol uptake. To enter a cell, however, retinol must traverse the plasma membrane and also, from the inner leaflet of the membrane, transfer to intracellular compartments. Long-chain fatty acids [10], unesterified cholesterol [11,12] and diacylglycerol [13] are known to move rapidly and spontaneously across phospholipid bilayer membranes with halflives for the process in the order of seconds to minutes. The transfer of phospholipids across model membranes, on the other hand, is very slow, with halflives for the process in the order of days [14]. For phosphatidylcholine and aminophospholipids, specific transfer proteins have been described, which mediate the rapid transfer of these phospholipids across membranes [15,16]. Long-chain fatty acids [10] and unesterified cholesterol [17,18] move spontaneously between adjacent bilayer membranes with halflives of 1–2 min and 2–4 h, respectively. Phosphatidylcholine moves very slowly between membranes with halflives for the process in the order of 48 h [17]. In vivo, this transfer may be mediated by phospholipid transfer proteins [19]. The present experiments were performed to elucidate whether retinol transfers across and between phospholipid bilayer membranes in a way similar to long-chain fatty acids and unesterified cholesterol or whether it transfers similar to the phospholipids.

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Materials and Methods

[^{3}H]Retinol (specific radioactivity 29 mCi/mmol) and [^{4}C]triolein (specific radioactivity 111.8 mCi/mmol) were from New England Nuclear. Egg phosphatidylcholine, bovine brain ganglioside type II, wheat germ lectin, octyl-β-glucoside, retinol, retinal, retinyl acetate, α-tocopherol, butylated hydroxytoluene and horse liver alcohol dehydrogenase (lyophilized 1–2 U/mg at 25°C) were from Sigma. Hog muscle lactate dehydrogenase (solution containing 850 U/mg at 37°C) and nicotine adenine dinucleotide (NAD, free acid) were from Boehringer Mannheim and sodium pyruvate was from Fluka. Latex microspheres were from SERVA AG and Lumagel™ scintillation fluid from Lumac AG.

Preparation of liposomes. Unilamellar liposomes were prepared using octyl-β-glucoside (15 mol/mol phosphatidylcholine) by gel filtration on Sephadex G-25 as described [20], using deaerated, nitrogen-equilibrated buffers in all steps. All liposomes were prepared from egg phosphatidylcholine and contained 1 mol% α-tocopherol (relative to phosphatidylcholine). In some experiments, the liposomes contained one or more of the following additions: 0.1 mol% retinol (relative to phosphatidylcholine), 0.1–0.2 μCi [^{3}H]retinol per μmol phosphatidylcholine, bovine brain ganglioside (corresponding to 10% (w/w) of the amount of phosphatidylcholine) or 0.1–0.2 μCi [^{14}C]-triolein per μmol phosphatidylcholine (to label the phospholipid membrane). The liposomes were protected from light by the use of aluminum foil wherever practicable and kept under a nitrogen atmosphere whenever possible to minimize oxidation damage to the lipids.

Determination of liposome size. The size of the liposomes used for the experiments was determined by gel filtration on a 0.9 × 60 cm column of Sephacryl S-1000 (Pharmacia AB, Uppsala) operated at 20°C as described [21]. In short, the procedure was as follows. The column was first calibrated using latex microspheres with diameters of 1000, 170, 90 and 60 nm, respectively, in a buffer containing dodecyl sulphate. After washing with buffer without dodecyl sulphate, the column was saturated with sonicated phospholipids (10 μmol phosphatidylcholine sonicated for 3 × 5 min in 2 ml buffer at 20°C) overnight to prevent adsorption of liposomes during chromatography. The column was then washed again with buffer without dodecyl sulphate and 0.2 ml of a solution of liposomes (1.25 μmol phosphatidylcholine/ml) containing 0.1 mol% retinol and [^{14}C]triolein was applied. The elution volume of the liposome was determined by determining the ^{14}C radioactivity of the fractions. During the whole procedure, care was taken not to change the flow rate, fraction volume and column volume. From the elution volumes of the microspheres, a standard curve was constructed. The diameter of the labelled liposomes, as calculated from their elution volumes was 84 nm, which corresponds to an inner/outer surface ratio of 45/55 [22].

Encapsulation of ^{125}I-labelled peptide in liposomes. To detect possible leakage of the liposomes during exposure to alcohol dehydrogenase, an ^{125}I-labelled water-soluble synthetic undeca peptide was incorporated into the interior of the liposomes by adding it to the dried lipids together with the detergent and the buffer before the G-25 gel filtration step in the liposome preparation procedure (see above). In this way, about 1% of the labelled peptide was encapsulated. The liposomes with encapsulated ^{125}I-labelled peptide, which eluted in the void volume of the G-25 column, were then separated from remaining non-encapsulated labelled peptide by a second chromatography on a 0.9 × 25 cm column of Sepharose 4B equilibrated in the same buffer as the G-25 column. The liposomes with encapsulated ^{125}I-peptide eluted close to the void volume on Sepharose 4B with baseline separation from free labelled peptide.

Determination of the rate of transmembrane movement of retinol. The incubation conditions were adapted from Futterman and Heller [23] and Ong et al. [24]. Phosphatidylcholine liposomes (1.0 μmol phosphatidylcholine/ml) prepared as described above in 0.14 mol/l Tris-HCl (pH 8.50) and containing 1 mol% α-tocopherol and 0.1 mol% retinol (relative to phosphatidylcholine) were incubated in a total volume of 1 ml with alcohol dehydrogenase (15 U/ml), NAD (20 μmol/ml), lactate dehydrogenase (0.5 U/ml) and sodium pyruvate (20 μmol/ml). Care was taken to obtain the same final osmolality outside as inside the

liposomes (206 mosmol/kg). The final pH was 8.22. Parallel blank incubations were performed in the absence of alcohol dehydrogenase. All reagents were prewarmed at 37°C. The reaction was started by the addition of alcohol dehydrogenase. At intervals, 200 μl-aliquots were pipetted off and the enzyme reaction was terminated by freezing on dry ice. To the frozen samples was then added 180 μl absolute ethanol containing 0.23 mmol/l butylated hydroxytoluene, 20 μl retinyl acetate (10 nmol/ml in absolute ethanol with 0.23 mmol/l butylated hydroxytoluene) and 2 ml of hexane containing 0.23 mmol/l butylated hydroxytoluene. After mixing and subsequent centrifugation, the hexane layer was removed, taken to dryness under nitrogen and the residue was dissolved in 50 μl absolute ethanol containing 0.23 mmol/l butylated hydroxytoluene. The retinol concentration was determined by HPLC as described below.

Transfer of retinol between unilamellar liposomes. The methodology used was adapted from the work of Massey [25] and Backer and Dawidowicz [26] 'Donor' liposomes (containing phosphatidylcholine, bovine brain ganglioside, α-tocopherol, unlabelled retinol, [^{3}H]retinol and [^{14}C]triolein, as described above) were rapidly mixed with 'acceptor' liposomes (containing phosphatidylcholine, α-tocopherol and unlabelled retinol, as described above) at various ratios and incubated at 37°C for various times. Blank incubations containing only 'donor' liposomes were performed in parallel. The total phosphatidylcholine concentration was 1.25 μmol/ml. 'Donor' liposomes in 50 μl aliquots of incubation mixtures were precipitated by the addition of 88 μl ice-cold wheat germ lectin (5 mg/ml in distilled water) and centrifuged in the cold at 12000 × *g* for 2 min. In model experiments, this procedure precipitates more than 95% of [^{14}C]triolein-labelled liposomes. The ^{3}H and ^{14}C radioactivity of the supernatant and the whole incubation mixture was then determined. The percent transfer of retinol from 'donor' to 'acceptor' liposomes was calculated as follows: 'Percent transfer' = 100 [^{3}H in supernatant − ^{14}C in supernatant × ^{3}H/^{14}C in incubation mix] × 1/^{3}H in incubation mix. The figure obtained then was corrected for [^{3}H]retinol present in a non-lectin-precipitable form in the supernatant using the calculated 'percent transfer' in the blank incubations (i.e., incubations without 'acceptor' liposomes) = 100 − 100 × (^{3}H in 50 μl supernatant/^{3}H in 18 μl incubation mix). Non-lectin-precipitable ^{3}H-radioactivity was usually 2–5% of total ^{3}H radioactivity in the incubation mix.

Transfer of retinol from 'donor' liposomes to erythrocyte membranes. These experiments were performed in a way similar to those described above for liposome–liposome transfer, with the difference that phosphate-buffered saline was used for liposome preparation and that ganglioside was omitted in the 'donor' liposomes. Erythrocytes were isolated from fresh human blood from a healthy individual collected in EDTA-containing tubes. They were washed three times with phosphate-buffered saline and mixed with 'donor' liposomes at a 1:22 ratio based on phospholipid concentration (10^8 erythrocytes corresponded to 31 μg lipid phosphorus). The mixture was then incubated with shaking (to keep the erythrocytes in suspension) at 37°C for various times up to 60 min. At intervals, duplicate 150 μl samples were pipetted off and centrifuged in the cold at 12000 × *g* for 4 min. The ^{3}H/^{14}C radioactivity ratio was then determined in 50 μl of the supernatant and expressed as percent of the ^{3}H/^{14}C ratio of the whole incubation mixture. The percent decline in the ratio was considered equal to the percent of retinol transferred from the liposomes. The ^{3}H/^{14}C radioactivity ratio in the supernatant of similarly treated incubations without erythrocytes was used as a blank to correct for the retinol 'transfer' which would occur in the absence of erythocytes. There was no visible hemolysis of erythrocytes during incubation.

Other methods. Determination of retinol was performed by HPLC using a Waters model 400 liquid chromatograph. The column was a 15 cm × 5 μm C-18 reversed-phase column operated at a flow rate of 2 ml/min. The mobile phase was acetonitrile: 0.13 mol/l aqueous ammonium acetate, 75:25 v/v). Retinyl acetate was used as internal standard. The concentrations of ethanolic solutions of the retinoids was determined using $\epsilon_{325} = 46000\ M^{-1} \cdot cm^{-1}$ for retinol [27] and $\epsilon_{325} = 51500\ M^{-1} \cdot cm^{-1}$ for retinyl acetate [27]. Phospholipid phosphorus was determined accord-

ing to Chen et al. [28] after extraction according to Folch et al. [29]). Radioactivity was determined using standard settings in a Packard Tri-Carb Model 3255 Liquid Scintillation Spectrometer.

Results

The alcohol dehydrogenase-catalyzed oxidation of retinol in liposomes is shown in Fig. 1. The disappearance of retinol was rapid, and less than 10% of the retinol originally present remained after 90 s. A second degree polynomial gave the best fit to the experimental points. The halflife calculated from the polynomial was 17.5 s. The disappearance curve looked biphasic with the first part of the curve having a steeper slope than the second part. 40% of the retinol was oxidized during the first 20 s, with an apparent halflife of 15 s and another 50% during the subsequent 70 s, with an apparent halflife of 28 s. Several different alcohol dehydrogenase concentrations were tried, of which the experiments with the highest concentration (15 U/ml) is shown.

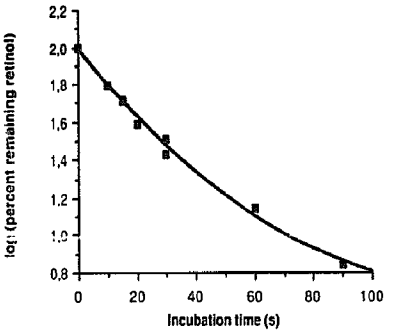

Fig. 1. Disappearance of retinol from liposomes during incubation with horse liver alcohol dehydrogenase. Liposomes (1.25 μmol phosphatidylcholine/ml) containing 1 mol% α-tocopherol and 0.1 mol% retinol were incubated with alcohol dehydrogenase as described in the text. At indicated times, aliquots were taken for retinol determination, as described in the text. The peak height ratio retinol/internal standard of the samples was expressed as percent of the mean ratio of five blank incubations (i.e., without alcohol dehydrogenase). The data represent the results of two different experiments. The curve fitted to the data was a second degree polynomial $Y = 1.9767 - X \times 0.0188 + X^2 \times 7.157 \times 10^{-5}$. The correlation coefficient was 1.00.

To ensure that alcohol dehydrogenase could oxidize retinol only on the external surface of the liposomes, control incubations were made with liposomes loaded with an ^{125}I-labelled synthetic peptide. If labelled peptide leaked out during incubation, this could mean that alcohol dehydrogenase might also oxidize retinol on the inside of the liposomes. As can be seen in Fig. 2A and B, respectively, the liposomes did not leak during incubation. The ^{125}I radioactivity eluted at a position close to the void volume of the Sepharose 4B, as expected for the liposomes, and only insignificant amounts of radioactivity were eluted at the position of free ^{125}I-labelled peptide (arrow).

The transfer of retinol between liposomes was also a rapid process (Fig. 3). Already during the time it took to mix and separate 'donor' and

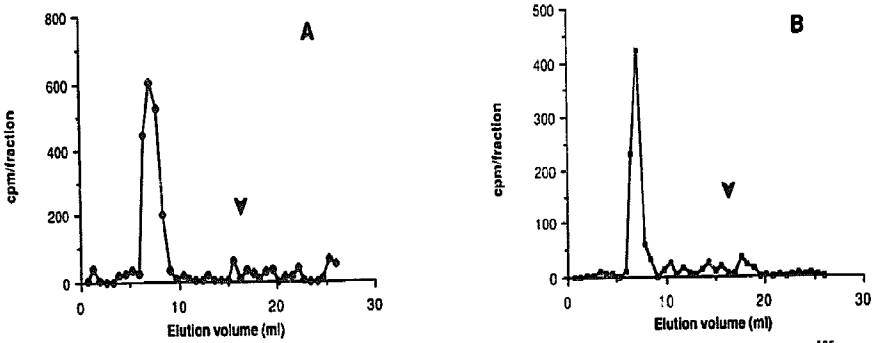

Fig. 2. Impenetrability of liposomes during incubation with alcohol dehydrogenase. Liposomes containing encapsulated ^{125}I-labelled peptide were treated as in Fig. 1. After 6 min incubation, the incubation mixture was subjected to Sepharose 4 B chromatography as described in the text. The liposomes containing ^{125}I-labelled peptide eluted with the void volume. The arrow shows the expected elution position for free ^{125}I-labelled peptide. A and B, incubations with and without alcohol dehydrogenase, respectively.

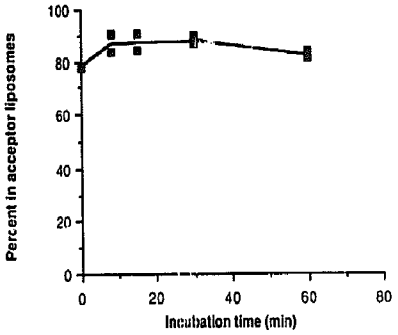

Fig. 3. Retinol transfer from 'donor' to 'acceptor' liposomes as a function of time. Liposomes (1.25 μmol phosphatidylcholine/ml) in a 'donor':'acceptor' phospholipid ratio of 1:21 were incubated at 37°C in 0.05 M Tris-HCl (pH 7.4) containing 0.05 M NaCl and 0.001 M EDTA. At the times indicated, dubplicate 50 μl samples of the incubation mixture were removed and the 'donor' lipsomes precipitated by the addition of 88 μl ice-cold wheat germ lectin (5 mg/ml) as described in the text. The zero-time incubations were precipitated immediately after mixing 'donor' and 'acceptor'. The whole precipitation procedure was complete within 10 min. Individual results are plotted.

'acceptor' liposomes (about 5–10 min), most of the retinol had transferred to the 'acceptor' liposomes. The transfer process seemed to have come to an equilibrium within 20–30 min. Increasing the 'acceptor'/'donor' phospholipid ratios increased the fraction of retinol radioactivity transferred to the 'acceptor' liposomes. However, the fraction of retinol transferred (not shown) was slightly less than expected purely from phospholipid ratios (expected transfer: 67–86–95% actual transfer: 61–65–75%. To determine the rate of transfer to a 'real' membrane, similar experiments were performed where erythrocytes were used as 'acceptors'. The rate of transfer of retinol from 'donor' liposomes to 'acceptor' erythrocytes seemed as rapid as the transfer to 'acceptor' liposomes. Based on the phospholipid ratio, the fraction of retinol transferred to erythrocytes (not shown) at equilibrium was slightly lower than with an 'acceptor' made from pure phospholipids (expected transfer: 95% actual transfer: 68%).

Discussion

The uptake of retinol into at least some cells is assumed to occur in conjunction with the binding of the retinol–retinol-binding protein complex to a specific cell surface receptor [2–4]. This receptor has so far not been characterized. Recently [5], we showed that retinol could transfer rapidly and spontaneously from the retinol–retinol-binding protein complex to phospholipid bilayer membranes. Based on these findings, we suggested retinol might be taken up into cells in vivo by a similar non-specific process, i.e., similar to that suggested for long-chain fatty acids bound to albumin [7,8,30]. However, to enter the cell, retinol must traverse the cell surface membrane and from its inner leaflet, transfer to intracellular compartments. Judging from its general physicochemical properties, it can be assumed that retinol in these respects might behave like unesterified cholesterol [11,12,17] and long-chain fatty acids [10], which move rapidly and spontaneously across and between membranes, rather than like phospholipids, which need the assistance of specific proteins for these transfers [15,16,19]. This does also seem to be the case. The experiments shown in Fig. 1 clearly demonstrate that retinol can indeed move rapidly and spontaneously across phospholipid bilayer membranes. It is also evident that the process was not first order. The explanation for this may be that the process shown consists of two rates – the oxidation of retinol initially present on the outside of the liposome, which would represent the first part of the curve, and oxidation of retinol gradually transferred from the inside to the outside of the liposome, which would represent the last part of the curve. The halflife corresponding to the last part of the curve would then correspond to the halflife of the inside–outside retinol transfer, i.e., at most 28 s. Lower enzyme concentrations did not show this biphasic pattern. Instead, they resulted in apparent first-order disappearance curves for retinol with halflives of several minutes, suggesting the enzyme concentration was limiting. That the results were not due to oxidation by alcohol dehydrogenase of retinol present on the inside of the liposomes is demonstrated by the negligible leakage of the liposomes during incubation with/without alcohol dehydrogenase (Fig. 2A and B). The halflife of the transfer process (up to 28 s) for retinol is similar to that reported for unesterified cholesterol [11,12], i.e., 3–60 s, long-chain fatty acids [10], i.e., 17–72 s and diacylglycerol [13], i.e., up to 15 s, but distinct

from the very long halflives for the transfer of phospholipids [14] all in similar experimental systems. These results are in agreement with the view [13] that the primary barrier to spontaneous transmembrane movement of amphiphilic lipids is the polar group and shows that fatty compounds with similar polar groups move at similar rates.

For the further transfer of retinol into the cell, retinol must transfer from the inner leaflet of the surface membrane to intracellular membranes or other cellular components capable of binding retinol. Our experiments show that retinol, similar to unesterified cholesterol [17,18] and long-chain fatty acids [19], but unlike phospholipids [17] can transfer rapidly and spontaneously between liposomal membranes (Fig. 3) and between liposomes and erythrocytes (not shown). The technique employed was not suited to measure such rapid rates, but it seems clear that the process had a halflife of less than 10 min.

The fraction of retinol transferred at equilibrium was roughly proportional to the 'donor': 'acceptor' phospholipid mass ratio. The apparent slightly lower affinity for retinol demonstrated by erythrocytes and 'acceptor' liposomes compared to 'donor' liposomes may be due to differences in the composition of membranes (ganglioside in 'donor' liposomes, proteins and several different phospholipids in the erythrocyte membrane). These results are in good agreement with the study of Rando and Bangerter [31]. These investigators, among other things, used 50-times higher retinol concentration in their liposomes than in the present investigation. Although such high retinol concentrations have profound effects on several properties of the liposomal membrane [32,33], the results of their experiments are qualitatively similar to ours.

Many cells contain a specific intracellular cytoplasmic retinol-binding protein which is distinct from the retinol-binding protein in plasma [34]. This cellular retinol-binding protein has been implicated in the transport of retinol within the cell [34]. The results of this and other investigations [31] do not prove that the cellular retinol-binding protein is not involved in intracellular retinol transport, but indicates that transfer of retinol between cellular membranes can occur rapidly without the participation of this protein.

Most of the retinol in the liver is stored in stellate cells as retinyl ester [35]. However, both hepatic uptake and mobilization of retinol occurs via the hepatocyte [36]. Thus, retinol is in some way transferred between the hepatocyte and the stellate cell, which are in close contact with each other [37]. How this transfer occurs is unknown, but retinol seems to be transferred in the alcohol form and not as an ester [37]. As retinol transfers readily between erythrocytes and bilayer membranes, one might speculate that retinol could transfer in a similar way between hepatocytes and stellate cells.

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