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THE RAPID INTERMEMBRANEOUS TRANSFER OF RETINOIDS

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SUMMARY: The intermembraneous rates of retinoid (all-trans-retinol(al), 11-cis-retinol and all-trans-retinol palmitate) transfer from vesicle to vesicle and vesicle to erythrocyte were studied. The rates of transfer of the retinols(al) were exceedingly rapid. The rates of transfer of the retinols(al) from egg phophatidyl choline based SUV's to bovine erythrocytes had a half-time of approximately 1-2 min. The vesicle to vesicle transfer rate was too rapid to measure by conventional techniques. By contrast, all-trans-retinol palmitate did not undergo transfer at an appreciable rate.

INTRODUCTION

As part of the visual cycle, retinol (vitamin A) and retinal (vitamin A aldehyde) undergo intermembraneous transfer within the retina (1). This transfer occurs mainly between the rod outer segment discs, the rod outer segment plasma membrane, and the pigment epithelium cell plasma membrane. It is not clear whether or not this transfer is mediated by carrier proteins although several retinol and retinal binding proteins have been found in the retina (2,3,4,5). The physiological functions of these proteins are unknown, and some of them may not be involved with retinol function at all but simply bind non-specifically to the hydrophobic vitamin.

It is generally assumed that intermembraneous hydrophobic ligand transfer proteins are reserved for compounds which undergo very slow transfer in the absence of a catalyst (6). For example, cholesterol undergoes fairly rapid transfer from membrane to membrane by itself, whereas phosphatidyl choline derivatives do not (7). Indeed, well characterized phospholipid transfer proteins have been reported in the literature, whereas none have been reported

Abbreviations used: SUV - small unilamellar vesicle
EYL-egg yolk lecithin

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for unmodified cholesterol (8). It was of some interest then to determine the basal rate of intermembraneous transfer of vitamin A and derivatives in the absence of putative transfer proteins. In this article it is shown that all-trans-retinol, all-trans-retinal and 11-cis-retinol undergo exceedingly facile intermembraneous transfer whereas the all-trans-retinol palmitate does not.

MATERIALS AND METHODS

All-trans-retinal, all-trans-retinol, and all-trans-retinal palmitate were products of the Sigma Chemical Company. 11-cis-retinal was a generous gift of Dr. William Scott of Hoffman-La Roche, Inc. [15-^{3}H]-all-trans-retinol was purchased from New England Nuclear, Inc. [15-^{3}H]-all-trans-retinal palmitate was synthesized from the alcohol by the published procedure (9). [15-^{3}H]-all-trans-retinal was prepared by oxidizing the alcohol with manganese dioxide by the published procedure (10). [15-^{3}H]-11-cis-retinol was prepared by the reduction of the aldehyde with [^{3}H]-sodium borohydride, also by the published procedure (10). The specific activities of the [^{3}H]-retinoids was in the 2 - 10 mCi/mmole range. [^{3}H]-sodium borohydride and [^{14}C]-cholesterol oleate were products of New England Nuclear, Inc. Lactosyl ceramide was a product of Miles Laboratories.

Ricin, the β-galactosyl-binding agglutinin from Ricinus communis, was obtained from Boehringer Mannheim, Inc. Egg yolk lecithin (EYL) was prepared and purified according to the method of Litman (11). Egg phosphatidyl ethanolamine was purchased from Sigma Chem. Co. The phospholipid concentration was determined as inorganic phosphate after ashing and acid hydrolysis (12). Bovine erythrocytes were collected in heparin NaCl/Tris or phosphate (140 mM NaCl, 50 mM Tis, pH 7.4) from a local slaughterhouse on the day that they were to be used. The erythrocytes were repeatedly washed with NaCl/Tris (phosphate) (pH = 7.4) and centrifuged (2000 x g). The cells were washed continuously until no more white layer appeared. At this point the cells were ready for the transfer measurements.

The SUV's used in these studies were prepared by both the sonication method and the ethanol injection technique (13,14).

Transfer Procedures

Vesicle-Vesicle Transfer

The transfer technique used here was adopted from the work of Dawidowicz (7). In these experiments, donor vesicles were made up containing the [^{3}H]-retinoid, and acceptor vesicles were made up containing approximately 10% lactosyl ceramide and trace quantities of [^{14}C]-cholesterol oleate. The donor to acceptor concentrations were varied from 1:1 to 1:10. The non-transferable lactosyl ceramide enables the acceptor vesicles to be precipitated by adding ricin agglutinin, and the non-exchangeable [^{14}C]-cholesterol oleate marker was added to determine the efficiency of precipitation. The donor vesicles (0.05-0.1 μmole/mL phospholipid) were incubated with the acceptor vesicles (0.1-1 μmole/mL phospholipid) in the Tris/NaCl buffer at various temperatures. At various times aliquots of the sample were removed and added to 1 mL, 200 μg/mL ricin agglutinin in the standard buffer at 10°. The sample was allowed to aggregate for 10 min and then centrifuged in a clinical centrifuge. The pellet was washed with cold buffer dissolved in ethanol and counted after removal of the precipitated protein. The amount of [^{3}H]-transferred to the acceptor vesicles as a function of time should theoretically yield a transfer rate (7).

Vesicle-Erythrocyte Transfer

In this procedure the donor vesicles containing the [^{3}H]-retinoid were mixed with freshly prepared bovine erythrocytes. With time, the erythrocytes were removed by centrifugation and counted. The vesicles, of

course, remain in the supernatent. These experiments were carried out similarly to other transfer experiments carried out in this laboratory (15).

Two mL of freshly obtained bovine erythrocytes (1 x 10^9 cells) in phosphate buffered saline and 5 mM lactose were treated with vesicles (0.5 μmole/mL phospholipid) containing 25 mole % cholesterol and 5 mole % [^{3}H]-retinoid at 25° with gentle shaking. At various times aliquots were removed, centrifuged and washed twice with cold buffer. At the end of 2 hours approximately 30% of the erythrocytes had lysed, a result not at odds with the known surface-active effects of the retinoids (16). The pellet containing the bovine erythrocytes was dissolved in Protosol and counted in Aquasol (New England Nuclear, Inc.) by standard techniques.

RESULTS AND DISCUSSION

Vesicle-Vesicle Transfer

Initial experiments on the intermembraneous transfer of the [^{3}H]-retinoids were done using SUV's. Donor vesicles were loaded with [^{3}H]-retinoid and incubated with acceptor vesicles containing non-transferable lactosyl ceramide and [^{14}C]-cholesterol oleate. With time, aliquots were removed and treated by the β-galactosyl binding aggluninin ricin to precipitate the acceptor vesicles. Surprisingly, under the conditions of the experiment, the transfer was so rapid that rates could not be measured by this technique (Table I). This technique is not very sensitive to rapid transfer because of the relatively long period of time (in minutes) required to fully precipitate the acceptor vesicles with ricin. Nevertheless, several interesting conclusions can be drawn from these experiments. First of all, the rate of transfer of the *cis* and *trans*-retinols and *trans*-retinal was exceedingly rapid. By way of comparison, the rate $t_{1/2}$ for [^{3}H]-cholesterol transfer under the same conditions was approximately 15'. The results with the free vitamin are to be contrasted with those obtained with retinol palmitate, where the transfer rate is negligible (Table I). This is expected due to the increased hydrophobicity of the ester.

The rapid rate of transfer of the retinoids appeared not to depend on the level of oxidation nor the stereochemistry about the 11-12 bond. Of special interest here are the experiments with all-*trans*-retinal and vesicles prepared from either phosphatidyl choline or phosphatidyl choline plus phosphatidyl ethanolamine. The Schiff base formed between the aldehyde and the amine did not appear to markedly slow the transfer rate, although of course the time scale of the assay employed exerts a leveling effect on the rates. In addition,

TABLE I

Intermembraneous Transfer of [^{3}H]-Retinoids

Mole % [^{3}H]-Retinoids in Donor Vesicle	Acceptor / Donor Rel. Conc.	Incubation Time (min)	% [^{3}H]-Retinoid Transfered to Acceptor Vesicles
5% [^{3}H]-all-trans-retinol/PC	1	2,5,10,30*	41 ± 3
5% [^{3}H]-all-trans-retinol/PC	2	2,5,10,30	47 ± 4
5% [^{3}H]-all-trans-retinol/PC	5	2,5,10,30	53 ± 4
5% [^{3}H]-all-trans-retinol/PC	10	2,5,10,30	83 ± 5
5% [^{3}H]-11-cis-retinol/PC	10	2,5,10,30	87 ± 5
5% [^{3}H]-all-trans-retinol/PC**	10	2,5,10,30	79 ± 5
5% [^{3}H]-all-trans-retinol/PC/PE-10%***	10	2,5,10,30	84 ± 6
5% [^{3}H]-all-trans-retinol palmitate/PC	10	2,5,10,30	5.7 ± 4

*Experiments performed at 5°,10°and 25° with identical results. Remaining experiments performed at 25°.
**PC - phosphatidyl choline; PE - phosphatidyl ethanolamine.
***In this experiment the acceptor vesicles also contained 10% PE.

In the above experiments small unilamellar donor vesicles were prepared in 50 mM Tris buffer (pH 7.5) + 140 mM NaCl with the [^{3}H]-labelled retinoid and pure egg phosphatidyl choline (PC) or PC plus 10 mole % pure egg phosphatidyl ethanolamine (PE). Acceptor vesicles were prepared from PC plus 10 % lactosyl ceramide plus 10% [^{14}C]-cholesterol oleate by procedures identical to those noted above. The non-exchangeable lactosyl ceramide enables the acceptor vesicles to be precipitated by adding ricin agglutinin and the non-exchangeable [^{14}C]-cholesterol oleate marker is added to determine the efficiency of precipitation. The donor vesicles (0.1 μmole/ml^{-1} phospholipid) are incubated with the acceptor vesicles (0.1 - 1.0 μmole/ml^{-1} phospholipid) in the Tris/NaCl buffer at various temperature. At various times aliquots of this sample were removed and added to 1 ml of 200 μg/ml^{-1} ricin agglutinin in the standard buffer at 10°. The sample was allowed to aggregate for 10 min then centrifuged in a clinical centrifuge. The pellet was washed with cold buffer and dissolved in ethanol and counted after removal of the precipitated protein. The amount of [^{3}H] transferred to these vesicles with time theoretically could give a transfer rate. Under the conditions of these experiments a time course could not be measured.

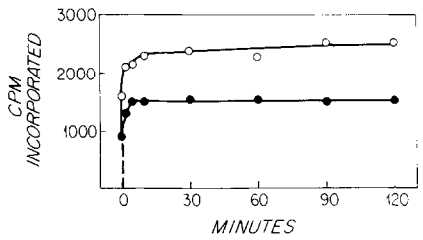
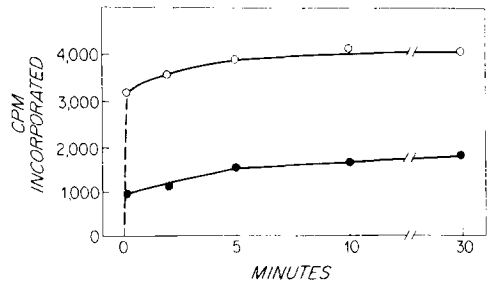


Fig. 1 Transfer of [^{3}H]-all-trans-retinol to Bovine Erythrocytes.
SUV's were prepared containing approximately 5% [^{3}H]-all-trans-retinol, 20% cholesterol and 75% EYL. The vesicles were mixed with the erythrocytes and the amount of [^{3}H] incorporation into these cells was measured with time according to the protocol described in the Methods section. In the above figure, (●) refers to an incubation in which the final EYL concentration was 0.5 μmole/mL, and in (○) the final concentration was 1.25 μmole/mL. The extent of transfer in (●) was 19% and in (○) it was 14%. The 0 time point in the experiment is actually in the neighborhood of 27".

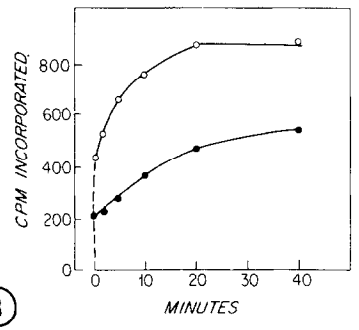
there was little or no effect of temperature on the transfer rate. In all cases, the retinoids could be completely transferred out of the donors to the acceptors, assuming a large enough excess of acceptor phospholipid was made available. However, it appeared that retinoid-lipid interactions were slightly more favorable in the donor vesicles relative to the acceptor vesicles (Table I).

Vesicle-Erythrocyte Transfer

The results from the vesicle-vesicle transfer experiments indicate that the rates of free retinoid transfer are exceedingly rapid and much too rapid to quantitate by that method. A direct centrifugation assay was required here without an intervening agglutination step. To these ends the transfer of the [^{3}H]-retinoids into bovine erythrocytes, which could be rapidly removed by centrifugation, was measured. In Fig. 1, the transfer of trans-[^{3}H]-retinol from EYL based SUV's at two different phospholipid concentrations into bovine erythrocytes is given. Even here the rates of transfer are barely measurable, with half-times of 1-2 min. It should be noted that the 0 time point in these figures is actually approximately 27". The rates of cholesterol transfer under similar conditions are 10-20 fold slower. At the lower concentration of phospholipid, approximately 20% of the total [^{3}H]-retinol is transferred to the erythrocytes. The erythrocyte is not limited in its capacity to accept retinol, as shown by increasing the [^{3}H]-retinol in the donor with a concomitant increase



(2)



(3)

Fig. 2 Transfer of [^{3}H]-11-cis-retinol to Bovine Erythrocytes.
SUV's were prepared by the ethanol injection technique containing 5 mole % [^{3}H]-all-trans-retinol (O) and 5 mole % [^{3}H]-11-cis-retinol (●) both with 20 mole % cholesterol. The transfer of the retinoids into the erythrocytes was measured in the usual way. There was 13% transfer in the case of all-trans-retinol (O) and 16% in the case of 11-cis-retinol (●).

Fig. 3 Transfer of [^{3}H]-all-trans-retinal to Bovine Erythrocytes.
SUV's prepared from EYL (O) or EYL + 10 % PE (●) both containing 5 mole % [^{3}H]-all-trans-retinol and 20 mole % of cholesterol were incubated with bovine erythrocytes by the procedure noted above. In the case of the EYL vesicles (O) there was 36% transfer and with the EYL + 10 % PE (●) there was 21% transfer.

in the amount of [^{3}H]-retinol transferred. However, at the higher concentrations significant lysis of the erythrocytes occurs. This has been observed before with the incorporation of all-trans-retinol into erythrocytes by injection into the buffer (16). All of the [^{3}H]-retinol could be removed from the vesicles by repeated incubations with fresh erythrocytes after centrifugation. To compare the rates of 11-cis-retinol and all-trans-retinol, transfer vesicles were made up by the ethanol injection technique. These rates were again approximately measured as before with the results shown in Fig. 2. As far as can be discerned, the two rates are very similar, if not identical. In a third set of experiments the rate of all-trans-[^{3}H]-retinal transfer was measured from vesicles prepared either from pure EYL or from mixtures of EYL choline and phosphatidyl ethanolamine (Fig. 3). The rates were again very rapid but there appears to be a retardation of transfer rate in the presence of the phosphatidyl ethanolamine. In addition, the amount of transfer appears to be less in the presence of phosphatidyl ethanolamine. Both phenomena could easily be ramifications of Schiff base formation between the membrane-bound retinal and the primary amino moiety of phosphatidyl ethanolamine (9).

In conclusion, we have shown that non-esterified retinoids undergo rapid intermembraneous transfer in the absence of catalytic transfer or exchange proteins. The rates of transfer are at least an order of magnitude faster than that for cholesterol. These results suggest that carrier protein assistance need not be required for bulk transfer of the retinoids in the retina although the putative carrier proteins might function by targeting the retinoids to specific membranes and protecting them from possible chemical degradation.

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