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Differential Transmembrane Diffusion of Triiodothyronine and Thyroxine in Liposomes: Regulation by Lipid Composition

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Abstract. How thyroid hormones move across biological or model membranes is a subject of controversy. The passage of the 3,5,3′triiodo L-thyronine and 3,5,3′,5′ tetraiodo L-thyronine across model membranes was evaluated by the addition of the hormones to liposomes containing 2,4,6-trinitrobenzene sulfonic acid. Results indicate that hormones can react with an amino-reactive compound pre-encapsulated into phosphatidylcholine liposomes. The transversal motions of thyroid hormones were characterized by using physiological concentration levels of (^{125}I) 3,5,3′triiodo L-thyronine and (^{125}I) 3,5,3′,5′ tetraiodo L-thyronine. The hormone distribution between the two monolayers was time-dependent and kinetic data were fitted to a single exponential. Results obtained show that 3,5,3′ triiodo L-thyronine can permeate phospholipid membranes and the diffusion time increases in the gel and liquid-ordered phase. On the contrary, 3,5,3′, 5′ tetraiodo L-thyronine could not diffuse the liposomal membrane from dimyristoyl and dipalmitoyl phosphatidylcholine in gel phase and egg yolk phosphatidylcholine:cholesterol in the liquid-ordered phase. Our results in the liquid-ordered phase suggest that diffusion movement of thyroid hormones across cell membranes depends on the amount of cholesterol in the bilayer.

Key words: Thyroid hormones — Liposomes — Membrane transport

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Abbreviations: PC, egg yolk phosphatidylcholine; DMPC, dimyristoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; L-T_3, 3,5,3′ triiodo L-thyronine; L-T_4, 3,5,3′,5′tetraiodo L-thyronine; Tb, Terbium; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

Introduction

The biological response to 3,5,3′ triiodo L-thyronine (L-T_3) is initiated by the binding of the hormone to nuclear receptors and followed by the subsequent transcriptional and post-transcriptional regulation of target genes (Tsai & O'Malley, 1994). 3,5,3′,5′ tetraiodo L-thyronine (L-T_4) is considered a prohormone that acquires biological activity only after the intracellular conversion into L-T_3. In order to reach the intracellular compartment, L-T_4 or L-T_3 has to cross the cell membrane. The mechanisms by which mammalian cells uptake the thyroid hormones remain poorly understood. It was postulated that the thyroid hormones get into the cell by endocytosis (Cheng et al., 1980). This process has been studied by fluorescence microscopy using rhodamine B-labeled L-T_3. However, interpretation of these experiments was confusing because the labeled hormone forms large molecular aggregates in aqueous solutions (Cheng & McPhie, 1989). Evidence of the existence of a saturable and specific membrane transport system for thyroid hormones was also reported (Krenning et al., 1978; Eckel et al., 1979; Holm & Jacqemin, 1979; Krenning et al., 1981; Pontecorvi & Robbins, 1986; Yamauchi, Horinchi & Takihawa, 1989; Osty et al., 1990; Mitchell, Manley & Mortimer, 1992), but the mechanism responsible for this saturability remains unknown. Furthermore, a nonsaturable transport was always found in the transport experiments (about 30–87% of total transport). Also this mechanism could be due, in part, to the L-T_3-binding to some nonspecific proteins and/or L-T_3-partition in the lipid components of membranes; it could suggest that a transmembrane diffusional uptake mechanism could be possible.

Using liposomes and electronic spin resonance techniques, the lateral diffusion of thyroid hormones were

studied (Lai & Cheng, 1982; Lai & Cheng, 1984). Reported data showed that once partitioned in the liposomal membrane, the spin labeled L-T_3 or L-T_4 derivatives can undergo a rapid lateral diffusion, similar to the lateral diffusion of spin-labeled fatty acids studied by Lai & Cheng, (1982, 1984). However, spin-labeled L-T_3 derivative remains in the outer half of the lipid bilayer and does not flip-flop at any appreciable rate in DPPC and PC liposomal membranes (Lai et al., 1985). These observations led them to propose that the movement of thyroid hormones across the cell plasma membranes requires the participation of proteins. Thus, the possibility of transmembrane diffusion as the cellular uptake mechanism for the thyroid hormones, raised objections (Lai et al., 1985). Previous reports from our laboratory showed that the lipophilic L-T_3 molecule readily partitioned in the liposomal membrane and perturbed its physical properties (Farías et al., 1995; Chehín et al., 1995).

In the present paper we show that the nonderivative L-T_3 can permeate and undergo transversal diffusion in liposomal phospholipid bilayers. Additionally, we have found that: (i) L-T_3 diffusion process depends on the lipid phase of the liposomal membranes and (ii) while L-T_4 can cross the lipid membrane in liquid crystalline state, it cannot cross the membrane in the gel and liquid-ordered states.

Materials and Methods

DMPC, DPPC and PC were obtained from Avanti Polar Lipids. Phospholipid preparations were dissolved in chloroform under nitrogen and stored at −20°C. The phospholipid concentrations of the stock solutions were determined by phosphate analysis according to Ames (1966). All lipids were chromatographically pure as tested by thin layer chromatography on silica gel. Cholesterol, L-T_3 and L-T_4 were purchased from Sigma Chemical (^{125}I) L-T_3 (specific activity, 3,300 μCi/μg) and (^{125}I) L-T_4 (specific activity 5,700 μCi/μg) were obtained from Du Pont-New England Nuclear.

Liposomes Preparation

Approximately 7 mg of PC, PC together with the appropriate amount of cholesterol, DMPC or DPPC, were dissolved in 100 μl of chloroform, dried under a nitrogen stream and suspended in 50 mM sodium phosphate buffer pH 7.4 to get multilamellar vesicles. The vesicles underwent five freeze-and-thaw cycles to obtain an increase of the vesicle-trapped volume (Mayer et al., 1985). Large unilamellar vesicles (400 nm) of PC were prepared by extrusion through a Nucleopore polycarbonate membrane (Hope et al., 1985). When DMPC or DPPC vesicles were prepared, the temperature was maintained 10°C above the phase transition temperature of each phospholipid. Total phospholipid concentrations were determined by phosphate analysis according to Stewart (1980). Although the last method is less sensitive than Ames (1966), the presence of inorganic phosphate in the buffer does not interfere with the assay.

Uptake of L-T_3 by Liposomes

The L-T_3 uptake by liposomes was determined by the ability of the hormone to cross the vesicle membrane and form a derivative with a hydrophilic reactive, such as TNBS, located inside of the liposomes. TNBS is a compound able to form a sulfonamide derivative with the amine groups. TNBS with high charged negative groups is unable to permeate the liposomal membrane (Nordlund et al., 1981) thus, if the sulfonamide derivative is formed during the incubation time, it would represent the hormone that is able to diffuse the liposomal membrane. Previously, Litman (1973) used this chemical labeling method for outer bilayer phosphatidylethanolamine determination.

To encapsulate TNBS into liposomes, large unilamellar vesicles were made by an extrusion technique (Hope et al., 1985) in the presence of 50 mM of phosphate buffer pH 7.4 containing 20 mM TNBS. The nontrapped TNBS was separated by gel filtration on Sephadex G-75 column (1 × 15 ml), equilibrated with 50 mM sodium phosphate buffer pH 7.4. The uptake of L-T_3 or L-T_4 by the liposomes was estimated by using ethyl acetate partition and HPLC chromatography.

Partition Analysis

To evaluate the sulfonamide compound, 1 ml of the mixture reaction containing about 10^{-10} M of (^{125}I) L-T_3 or (^{125}I) L-T_4 and 2 mM of TNBS or 100 μM of PC-liposomes precharged with TNBS were incubated at 25°C. At different times 1 ml of ethyl acetate was added. Most of the sulfonamide derivatives and the hormones partitioned in the organic and aqueous phase, respectively. Aliquots of 200 μl of the ethyl acetate phase were taken and the radioactivity was determined with Spectromatic Gamma Counter.

HPLC Analysis

The radioactive assay was carried out by mixing 0.5 ml of 100 μM of PC-liposomal precharged with TNBS with 1.8×10^{-10} M (^{125}I) L-T_3. After 2 hr at 25°C, the incubation was stopped by the addition of 0.02 ml HCL 1.5 M. The addition of HCl stops the reaction by protonation of TNBS. Before HPLC analysis an aliquot of the radioactive assay was mixed with the unlabeled derivative and unlabeled L-T_3. The unlabeled compounds were added as carrier and elution markers. Separation of L-T_3 and derivative were carried out by using a Water HPLC system with a reverse phase bondapak C_{18} column 250 × 4.6 mm. The column was eluted with acetonitrile/water (30:70 by volume) initially and changed to acetonitrile/water (40:60 by volume) for 10 min at a flow-rate of 1 ml/min and 1.5 ml/min, respectively. Both solvents contained 0.2% acetic acid. L-T_3 peak emerged with a retention time of 6 min whereas one minor peak of derivative product appeared with a retention time of 17 min. Fractions of 0.9 ml were collected and the radioactivity was determined. Unlabeled L-T_3-sulfonamide derivative was previously prepared by incubation of 0.02 mM of L-T_3 with 0.04 mM of TNBS during 30 min and separated by HPLC as described above. In this case to avoid any precipitation, 0.5 ml of ethanol was added when the reaction was finished.

L-T_3 Release by Liposomes

Multilamellar and large unilamellar vesicles were prepared in the presence of 1.0×10^{-12} M of (^{125}I) L-T_3 or (^{125}I) L-T_4. These concentrations are in the range of the free hormone in the human plasma (Najad et al., 1975; Gartner et al., 1980). The liposomal suspension was incubated during 5 min with 500 mg of anionic Dowex exchange resin (Dowex 1X8 Cl 200–400 Mesh) to separate the free hormone and hormone-bound liposomes (Farías et al., 1995). The beads bind the carboxyl group of thyroid hormones. Depending on the liposomal composition, this initial incubation with the resin removed from 10 to 35% of the total hormone. At different times, aliquots of 750 μl

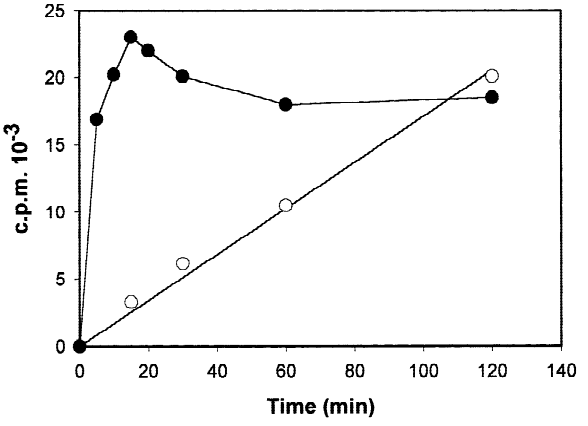

Fig. 1. Formation of L-T_3 sulfonamide derivative as a function of time in the liposome (○) and liposome-free (●) media. The experimental conditions and radioactivity measurements are given in Materials and Methods.

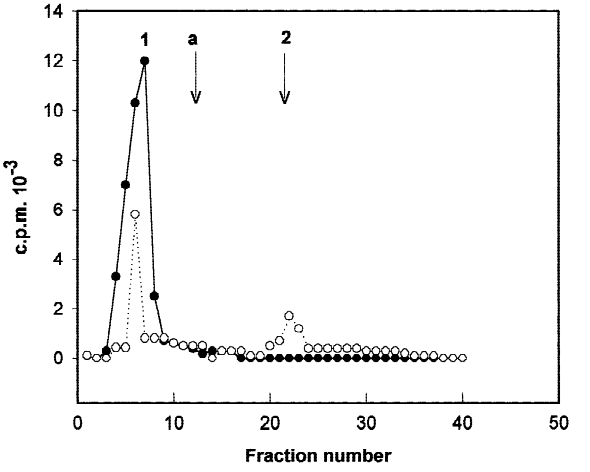

Fig. 2. HPLC separation of L-T_3 (1) and L-T_3 sulfonamide derivative (2) after incubation time of 0 hr (●) and 2 hr (○), respectively. The arrow indicates the solvent change (a). The experimental conditions and radioactivity measurements are given in Materials and Methods.

of the liposomes-resin suspension were taken and transferred to centrifuge tubes. After a short centrifugation (0.5 min at 2,000 rpm), the hormone-bound vesicles were recovered in the supernatant. To evaluate the amount of hormone remaining in the membranes, the radioactivity of the supernatant (aliquot of 200 μl) was determined. The Dowex resin can fix 95–98% of free hormones. The amount of hormone-bound vesicles was corrected by subtracting the remaining free L-T_3 values obtained without liposomes. Control of liposomal recovery in the supernatant after successive centrifugations was checked by determination of inorganic phosphate determination in another parallel experiment performed in HEPES buffer by the Ames methods (Ames, 1966). The resin did not alter the vesicle permeability as it was determined by using the Tb-dipicolinic release assay (Bentz, Duzgunez & Nir, 1983).

Results

Uptake of L-T_3 by Liposomes

Figure 1 shows the reaction between external (^{125}I) L-T_3 and TNBS preloaded PC-liposome suspension. The increase of radioactivity represents the amount of thyroid hormone able to react with the TNBS located inside of the vesicles. This reaction was linear at least for 120 min whereas in the absence of liposome it is completed before 10 min. Similar results were obtained using (^{125}I) L-T_4. Figure 2 shows the HPLC elution profile of the reaction between external (^{125}I) L-T_3 and TNBS preloaded PC-liposome suspension. The second peak is the sulfonamide compound. Similar results were obtained with DPPC and PC: chol (6:4) liposomes, but the amount of radioactivity in the L-T_3-sulfonamide derivative was smaller (*data not shown*).

Release of L-T_3 and L-T_4 from Lipid Vesicles

Transmembrane diffusion assays were performed with large unilamellar vesicles (400 nm). In this kind of vesicle, the outer-to-inner phospholipid monolayer ratio is 1:1, being similar to the biological membranes (Hope et al., 1985; New, 1990).

PC Liposomes

The transbilayer diffusion time was determined with the amount of hormone associated to the liposomes. The amount of L-T_3 remaining in the liposomes decreased and the kinetics data obtained can be fitted as a single exponential. The time at which the amount of hormone associated with the liposomes reaches the 50% of the initial values was considered as the half time ($t_{1/2}$). This time represents the rate constant of the thyroid hormone transposition from the inner to the outer monolayer of the liposomal membrane (Bloj & Zilvesmith, 1976; Zachowski, 1993). The $t_{1/2}$ values were 36 min for L-T_3 and 30 min for L-T_4 (Table). Similar diffusion times were found with multilamellar liposomes.

DMPC and DPPC Liposomes

The characteristic of diffusion time depends both on the molecules and on the host membrane (Zachowski, 1993). Experiments similar to those described above were carried out with DMPC and DPPC liposomes in which the transition temperatures from liquid crystalline to gel phase were around 24° and 40°C, respectively (New, 1990). The diffusion time depends on the lipid phase of the liposomal membrane (Table). L-T_3 transmembrane diffusion time increased from about 29 to 83 min when the lipid phase of DPPC liposomes changed from liquid crystalline to gel state (Fig. 3). Surprisingly,

Table. Transbilayer diffusion time of L-T_3 and L-T_4 in multilamellar or large unilamellar liposomes

Liposomes	$t_{1/2}$ (min)[a]	
	L-T_3	L-T_4
PC (37°C)[b]	36 ± 4	30 ± 3
DPPC (60°C)	29 ± 4	28 ± 5
DPPC (37°C)	83 ± 9	∞[c]
DMPC (37°C)	33 ± 3	35 ± 5
DMPC (4°C)	83 ± 11	∞
PC:Cholesterol (9:1)[d] (25°C)	34 ± 4	72 ± 8
PC:Cholesterol (6:4) (25°C)	108 ± 14	∞

(a) The values are means ± SD of at least three independent experiments.
(b) Assay temperature is indicated in brackets.
(c) Initial values of L-T_4 do not change during 3 hr of assay.
(d) The relation PC cholesterol is indicated in brackets.

L-T_4 could not cross the liposomal membrane when lipids were in the gel state (Fig. 3).

PC:Cholesterol Liposomes

Cholesterol is able to generate a liquid-ordered phase in PC membranes containing more than 25 mol % of cholesterol (Thewalt & Bloom, 1991). To study the influence of liquid-ordered lipid phase on passive diffusion of the thyroid hormones, PC-liposomes containing 10 and 40 moles % of cholesterol were used (Table). The increment of cholesterol in the PC membrane influenced the L-T_3 diffusion time only when the cholesterol rose to 40 moles %. L-T_4 increased the diffusion time in PC-:cholesterol (9:1) liposomes and could not move across PC:cholesterol (6:4) membranes. Additional studies in liquid-ordered phase using PC:cholesterol (7:3) and PC-:cholesterol (5:5) liposomes shows similar results (*data not shown*).

REVERSIBILITY OF THE DIFFUSION RATE

Figure 4 shows that the changes in the temperature from 20 to 60°C in large unilamellar DPPC liposomes induced a new rate of transposition corresponding to the liquid crystalline phase for both hormones. Additionally, in the case of L-T_4, a rapid or instantaneous release of the hormone was observed. When similar experiments were performed with DMPC liposomes, identical results were obtained (*data not shown*).

Discussion

L-T_3 TRANSPORT

Data obtained from model membranes do not suggest that the passive diffusion is an exclusive or universal

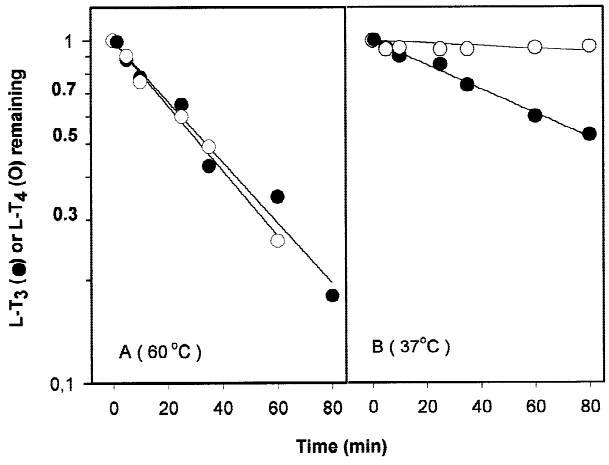

Fig. 3. L-T_3 (●) or L-T_4 (○) remaining in DPPC large unilamellar liposomes as a time function. The assay was performed at 60°C (*A*) and 37°C (*B*) as indicated in Materials and Methods. Remaining hormone values were calculated as a ratio of the values obtained at different times to the values obtained at the start of the experiment.

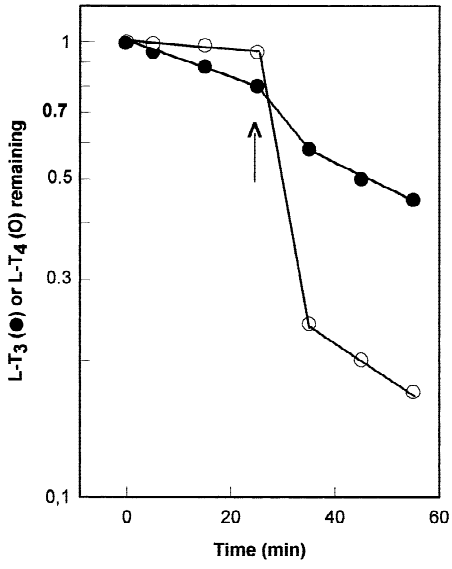

Fig. 4. Reversibility of diffusion rate. Changes from gel to liquid crystalline state in large unilamellar DPPC and L-T_3 (●) or L-T_4 (○) diffusion. The arrow indicates the temperature change form 20 to 60°C. Remaining hormone values were calculated as a ratio of the values obtained at different times to the values obtained at the start of the experiment.

mechanism of L-T_3 transport in biomembranes. Whether the passage of a hydrophobic molecule, such as L-T_3, into the interior of the cells needs to be facilitated by specialized proteins or not, critically depends on cellular requirements. L-T_3 has chemical properties and is sufficiently small to be soluble in the phospholipid regions of biological membranes. Our studies suggest that a diffusional process of L-T_3 through the biological

membranes takes place. After the partition process and transbilayer movement, the cytosolic L-T_3 binding proteins may well serve the purpose of keeping the hormone in the aqueous phase before L-T_3 reaches the nucleus. It was suggested that the cytosolic L-T_3 binding proteins might participate in the intracellular transport of L-T_3 toward the nucleus (Hashizume et al., 1989; Fanjul & Farías, 1991; Fanjul & Farías, 1993*a;* Fanjul & Farías, 1993*b*). Conventional transport experiments using cells do not distinguish between L-T_3 movement across the plasma membrane and another intracellular step such as the binding of L-T_3 to cytosolic or nuclear proteins or their corresponding metabolism.

L-T_4 Transport

Additionally, the present report shows the phenomenon of selective and differential transmembrane diffusion of L-T_4 with respect to L-T_3 in liposomes in the gel and liquid-ordered state (Table). In one gel (Farías et al., 1995) and liquid-ordered (Chehín et al., 1995) phase, lower incorporation of L-T_4 compared with L-T_3 was reported. Also, liposomes made with lipids extracted from natural membranes, such as those obtained from human red blood cells that contain about 40–45 moles % of cholesterol, showed a differential L-T_4 and L-T_3 incorporation (Chehín et al., 1995). It was suggested that the larger size of L-T_4 compared to L-T_3 prevents L-T_4 incorporation into model membranes in gel and liquid-ordered states (Farías et al., 1995; Chehín et al., 1995). The liquid-ordered phase is postulated to be the most relevant physical state for many biological membranes that contain substantial amounts of cholesterol (Thewalt & Bloom, 1991). The existence of some protein-based mechanisms for cells L-T_4 uptake in the membrane containing high cholesterol concentration deserves further investigations. These issues are of special importance since intracellular L-T_3 derives mainly from the cellular 5′ deiodination of L-T_4 (Tsai et al., 1994).

On the other hand, results presented in this paper showing the diffusion of underivative L-T_4 in a membrane model, could have (patho) physiological relevance because L-T_3 is the principal active thyroid hormone. The regulation of L-T_4 transport by lipid composition of biological membranes may play a role in the ultimate determination of thyroid hormone status in tissues. In this context, studies from our laboratory showed that the lipid composition of the biological membrane can regulate the response (activation or inhibition) of an enzyme system to thyroid hormones (Galo, Uñates & Farías, 1982).

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