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Differential Effect of Triiodothyronine and Thyroxine on the Liposomal Membrane in Liquid-Crystalline and Gel State

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Abstract. The effect of thyroid hormones on the degree of order or fluidity of dimyristoyl, dipalmitoyl or egg yolk phosphatidyl choline liposomes was evaluated by fluorescence spectroscopy methods. The freedom of molecular motion above the phase transition temperature was decreased, while below the transition, the mobility was actually increased by the incorporation of triiodothyronine to liposomes. While thyroxine decreases the fluidity in the liquid crystalline state, it cannot increase the fluidity in the gel state.

A differential effect of triiodothyronine and thyroxine on the release of the liposomal content was found, depending on the liquid crystalline or gel state of the liposomes. These facts were correlated with the differential incorporation of the hormones to liposomes above and below the phase transition temperature of dimyristoyl and dipalmitoyl phospholipid choline. In gel state, a low incorporation of thyroxine compared with triiodothyronine was found.

Key words: Thyroid hormones — Liposomes — Membrane fluidity

Introduction

Physiological plasma concentration of 3,5,3′ triiodo L-thyronine (L-T_3) in vivo and in vitro affects the cooperative behavior of membrane bound enzymes (De Mendoza et al., 1977; De Mendoza & Farías, 1978; De Mendoza, Moreno & Farías, 1978). This action was detected in vitro on acetylcholinesterase and (Na^+ + K^+)-ATPase from rat erythrocyte and (Ca^{2+})-ATPase from *Escherichia coli* membranes (De Mendoza et al., 1977). L-T_3 0.1 nM decreased the Hill coefficient values (n) of erythrocyte acetylcholinesterase and *E. coli* (Ca^{2+})-ATPase (De Mendoza & Farías, 1978) and increased the values of the erythrocyte (Na^+ + K^+)-ATPase system (De Mendoza et al., 1977; De Mendoza & Farías, 1978). A positive correlation between the membrane fluidity and the n values was found for the two former enzymes, while the correlation was negative for the latter (Bloj et al., 1973; Siñeriz et al., 1973; Farías et al., 1975). On the basis of these findings, we postulated that L-T_3 decreases the erythrocyte and *E. coli* membrane fluidity (Bloj et al., 1973; Siñeriz et al., 1973; Farías et al., 1975; De Mendoza & Farías, 1977, Farías, 1980). Thyroxine (L-T_4) was not able to modify the Hill coefficients of membrane-bound enzymes assayed (De Mendoza et al., 1977, 1978; De Mendoza & Farías, 1978). In the case of the erythrocyte system a protein membrane L-T_3 binding site was found (Botta et al., 1981) and a correlation between L-T_3 binding and the changes in the Hill coefficients of membrane cooperative enzymes was shown (Farías, 1980).

In the present report we studied by fluorescence spectroscopy methods the effect of L-T_3 on membrane fluidity of DMPC and DPPC liposomes above and below the main transition temperature. We clearly show here that L-T_3 is able to modulate the membrane fluidity in a protein-free membrane system. The effects of L-T_3 on membrane fluidity are in principle similar to the effects obtained with cholesterol (Presti, 1985; Yeagle, 1985; Demel & De Kruyff, 1991; Tewalt & Bloom, 1991). Additional studies performed with thyroid hormone analogues indicate a high structural specificity for the L-T_3 effect on membrane fluidity changes and release of the liposomal content.

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Materials and Methods

PC and PA from egg yolk were obtained as described elsewhere (Luthra & Sheltawary, 1972). Phospholipid preparations were dissolved in chloroform under nitrogen and stored at −20°C. DPH and TMA-DPH supplied by Molecular Probes (Junction City, OR) were solubilized in dimethylformamide and stored at −20°C. DMPC, DPPC and L-T_3 analogues were purchased from Sigma Chemical (St. Louis, MO). All lipids were chromatographically pure tested by thin layer chromatography on silica gel (^{125}I) L-T_3 (specific activity, 150 μCi/μg) and (^{125}I)L-T_4 (specific activity 150 μCi/μg) were obtained from Dupont-New England Nuclear.

Preparation of Liposomes

Phospholipids (35 μg lipid/ml) were dried under a nitrogen stream and suspended in 50 mM sodium phosphate (pH 7.4). Small unilamellar vesicles were obtained by sonicating the multilamellar vesicles to a clear solution with a probe-type or bath-type sonicator in a capped glass test tube under nitrogen. When DMPC or DPPC vesicles were prepared, the temperature was maintained 10°C above the phase transition temperature of each phospholipid. Large unilamellar vesicles (400 nm) of PC were prepared by extrusion through the pores of a Nucleopore polycarbonate membrane (Hope et al., 1985). Total phospholipid concentrations were determined by phosphate analysis according to Ames (1966).

Fluorescence Polarization Measurement

The fluorescent probes were introduced in the lipid bilayer at a molar ratio of 0.2%, adding DPH or TMA-DPH solution to the lipid suspension before drying the mixture, according to Shinitzky and Barenholz (1987). The steady-state fluorescence polarization (P) was measured in 1.5 ml of mM sodium phosphate buffer (pH 7.4) at a final phospholipid concentration of 50 μM. The degree of polarization: $P = (I_{II} - I_I)/(I_{II} + I_I)$ was determined using a SLM Instrument model 4800 fluorimeter (SLM Aminco, Urbana, IL) equipped with a thermostatic cuvette holder. The excitation wavelength was selected at 360 nm, and all the fluorescence was collected through a cutoff filter (Corning 3-73). The L-T_3 effect was recorded 1–2 min after the addition of 25 μl of 3 mM L-T_3 to liposome suspension. Before hormone addition, the liposomes were equilibrated at each temperature for 10 min. L-T_3 and their analogues were solubilized in 1 mM NaOH. No change in the pH and turbidity were noted after hormone addition.

Measurement of Liposomal Leakage

The release of liposomal content was measured using Tb-dipicolic assay, according to Bentz, Düzgunes and Nir (1983). The residual fluorescence of liposomes containing 15 mM $TbCl_3$ and 150 mM dipicolic acid was taken as 0% of release. The fluorescence obtained after disruption of the liposome with Triton X-100 (0.5%) was taken as 100%. Leakage experiment was started by injection of a small volume of L-T_3 or L-T_4 (5–20 μl) using a Hamilton syringe into a cuvette containing 1.5 ml. To decrease the light scattering, a C-371 cutoff filter was used. When the concentrations of L-T_3 were higher than 60 μM and the temperature was below 20°C, the solution became turbid.

Incorporation of (^{125}I)L-T_3 or (^{125}I) L-T_4 into Lipsomes

Increasing concentrations of liposomes were incubated with 50 μM (^{125}I) L-T_3 or (^{125}I) L-T_4 in 1.0 ml 50 mM sodium phosphate buffer pH 7.4 as indicated in the figures. After incubation, bound and free hormone were immediately separated by filtration through an anionic Dowex exchange resin (Dowex I × 8 Cl 200–400 mesh). The mixture of liposome and hormone was applied on top of the resin column (300 mg) placed into a centrifuge tube (S and S centrifugal microfilter) and centrifuged at 3,000 rpm for 1 min. The filtrated suspension was counted for radioactivity in an Espectromatic gamma counter. The partition coefficient at equilibrium, defined as the ratio of liposome-associated to external thyroid hormones, was 0.25 under our experimental conditions. The resin fixed 95–98% of the free hormones. The incorporation values were corrected for nonfixed L-T_3 or L-T_4. One hundred percent of the liposomes was recovered in the filtrate suspension, as evaluated by the recovery of Pi and DPH or TMA-DPH fluorophores previously incorporated in the liposomes. No changes in the suspension volume were noticed after the filtration process. Alternatively, the free and bound hormones were determined by the equilibrium dialysis method. A dialysis cell of Teflon with a membrane disc between its two halves was used. Dialysis studies were performed with membranes having a 6,000 molecular weight cutoff. The cells were submerged into a water bath for temperature control and rotated continuously. The resin and dialysis methods have the same results in the determination of free and bound L-T_3 or L-T_4.

Abbreviations

DPH, 1,6 diphenyl-1,3,5 hexatriene; TMA-DPH, 1-(4-trimethylamoniophenyl)-6 phenyl-1,3,5-hexatriene; DMPC, dimyristoyl-phosphatidylcholine; DPPC, dipalmitoylphosphatidyl choline; PC, egg phosphatidylcholine; PA egg phosphatidic acid; L-T_3, 3,5,3′ triiodo L-thyronine; D-T_3, 3,5,3′triiodo D-thyronine; L-T_4, L-thyroxine; D-T_4, D-thyroxine; DIT_2, 3,5 diiodothyronine.

Results

L-T_3 and Membrane Fluidity

DMPC and DPPC Vesicles

Fluorescence polarization of DPH can be used as an indicator for the relative fluidity of the phospholipid membrane (Aldrich & Vandekooi, 1969; Shinitzky & Barenholz, 1987; Florine-Casteel & Feigenson, 1988). DPH-polarization values and membrane fluidity are inversely correlated. Figure 1 shows the temperature profiles of DPH-fluorescence polarization of phospholipid vesicles, before and after L-T_3 addition. On the pure DMPC (Fig. 1*A*) and DPPC (Fig. 1*B*) liposomes, the main transition temperature appears around 23 and 40°C, respectively. An alternative way to present the L-T_3 effect is proposed in Fig. 2. Here the difference of polarization (Δ Polarization) between polarization values obtained in the absence and presence of L-T_3 is plotted against the assay temperature. As shown, L-T_3 decreases the fluidity of both DMPC and DPPC liposomes at temperatures above the transition temperature; meanwhile, at

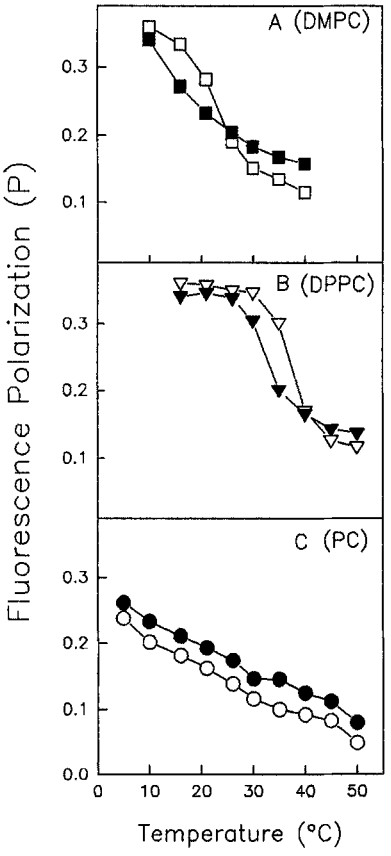

Fig. 1. Effect of L-T_3 on liposomal membrane fluidity. (A) Polarization of DPH bound to DMPC (□, ■) DPPC (▽, ▼) and PC (○, ●) liposomes (50 μM) in the absence (□, ▽, ○) and in the presence of 50 μM of L-T_3 (■, ▼, ●).

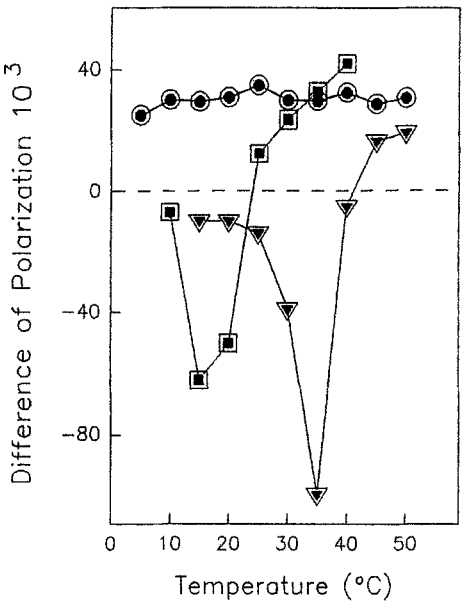

Fig. 2. Difference of polarization values of DMPC (■) DPPC (▼) and PC (●) liposomes in the presence and absence of 50 μM L-T_3. Similar results were obtained in three separate experiments.

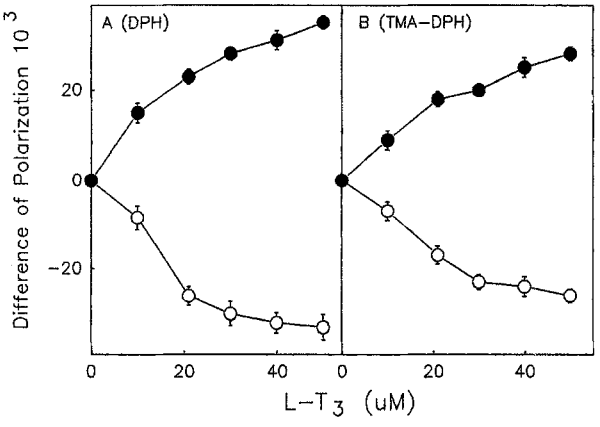

Fig. 3. Doses-response curves. Difference of polarization at 16°C (○) and 36°C. DMPC (50 μM) labeled with DPH (*A*) or TMA-DPH (*B*) was used. Each point is the mean of results of three separate experiments. Vertical bars indicate mean ± SD.

temperatures below the transition temperature the opposed effect was obtained. In these cases, the maximal L-T_3 effects were obtained at 16°C for DMPC and 36°C for DPPC. DPH is known to be located within the hydrophobic core of the liposomal membrane and provides information on the fluidity of this region. Cationic TMA-DPH is anchored in close proximity to the bilayer surface and so provides information on the bilayer lipid environment (New, 1990). The doses-response curves for L-T_3 on liposomal membrane fluidity of DMPC vesicles at 16 and 36°C are shown in Fig. 3. The effect of L-T_3 on 50 μM of DMPC liposome increased almost linearly with the hormone concentration. Under our experimental condition higher than 50 μM, L-T_3 concentration could not be used since the scatter of the sample interferes with the fluorescence polarization determinations. A similar behavior could be observed using DPH (Fig. 3*A*) or TMA-DPH (Fig. 3*B*) as probe.

PC Liposomes

The transition temperature of egg yolk PC liposomes is in the range of −15 to −7°C; thus, at temperatures from 5 to 50°C the lipids are in the fluid state. According to the above results, L-T_3 decreases the membrane fluidity of PC vesicles in this temperature range (Figs. 1*C* and 2). Small (15 nm) and large (400 nm) PC unilamellar vesicles showed similar membrane fluidity response to the incorporation of L-T_3 indicating that the L-T_3 effect was independent of the liposomal bilayer bilayer curvature. Also, L-T_3 decreases the membrane fluidity of PC:PA (8:2) liposomes (*not shown*). These results indicate that the presence of negative charges does not influence the L-T_3 effect.

Table. Membrane fluidity change values

Analogues						Δ Polarization × 10^3	
	3′	5′	3	5	Ala	36°C	16°C
L-T_3	1		1	1	L-	35 ± 4	−42 ± 3
D-T_3	1		1	1	D-	35 ± 5	−42 ± 4
L-T_4	1	1	1	1	L-	20 ± 3	11 ± 4
D-T_4	1	1	1	1	D-	20 ± 4	9 ± 3
DIT2			1	1	L-	3 ± 2	6 ± 4

The experiments were performed with 50 *u*M of L-T_3 and 50 *u*M of DMPC liposomes. Results are mean ± SD.

Thyroid Hormone Analogues and Membrane Fluidity

The Table shows the ability of different L-T_3 analogues to modify the Δ Polarization values of DPH introduced in DMPC liposomes at 16 and 36°C. At 36°C the effect was in the order L-T_3 = D-T_3 > L-T_4 = D-T_4 > DIT_2, indicating that the simultaneous presence of iodine in positions 3′ and 5′ (L-T_4, D-T_4) or its absence in the outer ring (DIT_2) of the thyronine molecule decrease the effect of L-T_3 analogues. The presence of iodine in position 3′ was an important requisite in order to obtain a high decreasing membrane fluidity (*compare* L-T_3 with DIT_2). At 16°C a very high specific L-T_3 effect appeared since only the L-T_3 was able to increase the membrane fluidity of DMPC liposomes. Similar results were obtained with DPPC liposomes (*not shown*). At both temperatures assayed, 36 and 16°C, L-T_3 and D-T_3 isomers showed identical behavior.

Leakage of Liposomal Content by L-T_3 and L-T_4

To confirm the differential L-T_3 and L-T_4 behavior on liposomal systems, an alternative method for the study of the membrane perturbation was used. Figure 4 shows that L-T_4 was unable to provoke the leakage of both DMPC and DPPC vesicles when they were in the gel state (8°C for DMPC, Fig. 4*E* and 20°C for DPPC, Fig. 4*F*). Above the transition temperature (Fig. 4*A,B*), both phospholipids were able to induce the release of the liposomal content. A similar effect was observed at 16°C for DMPC and 36°C for DPPC (Fig. 4*C,D*).

Incorporation of L-T_3 or L-T_4 to DMPC and DPPC Liposomes

The incorporation of L-T_3 or L-T_4 into the liposomes occurs in a very short time, reaching a plateau in 1 min (*not shown*). These incorporations were linear with the concentration of DMPC or DPPC liposomes (Fig. 5). Above the transition temperature of the DMPC and DPPC liposomes, the incorporation of L-T_4 and L-T_3

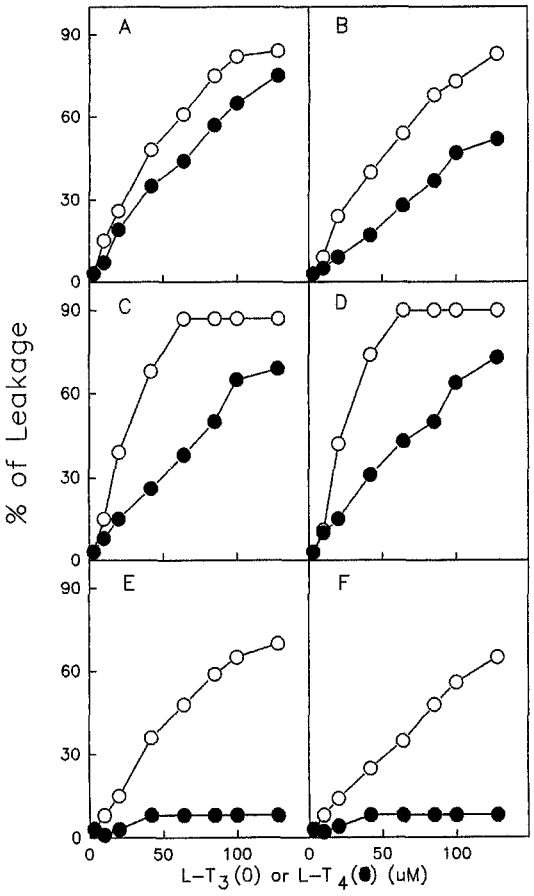

Fig. 4. Effect of L-T_3 and L-T_4 on leakage of liposomal content. The experiments were carried out at 36°C (*A*), 16°C (*C*) and 8°C (*E*) for DMPC liposomes and at 50°C (*B*), 36°C (*D*) and 20°C (*F*) for DPPC liposomes. In both cases 50 μM of phospholipid was used. The leakage was started with different concentrations of L-T_3 (○) or L-T_4 (●). Similar results were obtained in three independent assays.

was similar (Fig. 5*A,B*). In the gel phase (8°C for DMPC, Fig. 5*E* and 20°C for DPPC, Fig. 5*F*) the incorporation of L-T_4 was much lower than L-T_3. A difference in the incorporation between L-T_3 and L-T_4 is also noted at 16°C for DMPC and 36°C for DPPC (Fig. 5*C,D*). The percentage of incorporation of L-T_3 or L-T_4 to liposomes was independent of the hormone concentration at any temperature of assay (*not shown*). The experiments shows in Fig. 5 were performed with 50 μM of L-T_3. This concentration is the same as those used in the polarization and release assays. While this hormone concentration is dictated by the sensitivity of the membrane perturbation system studied, it is higher than the physiological concentrations. In plasma, the L-T_3 and L-T_4 concentration (bound +free) is about 1 and 100 nM, respectively (Najad et al., 1975; Norman & Litwarck, 1987). Using 1 nM of L-T_4, the influence of the physical state of the lipids was also demonstrated, since the hormone incorporation to DPPC liposomes increases from 0.03 to 0.08 pmol/100 nmol of DPPC by changing the

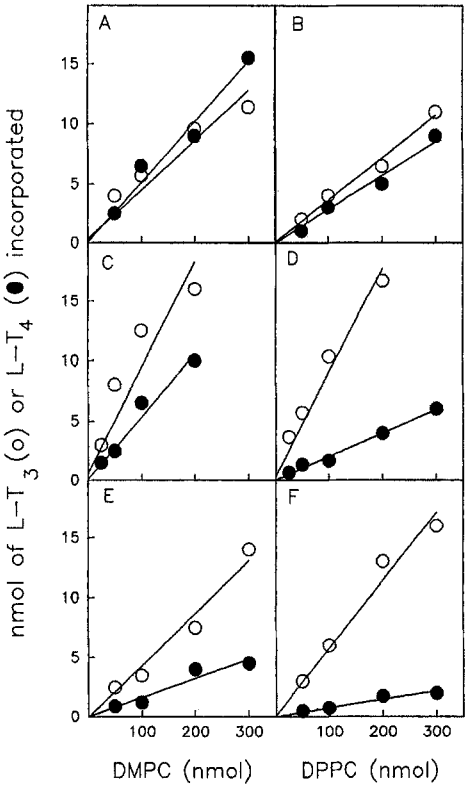

Fig. 5. Incorporation of L-T_3 or L-T_4 to liposomes. L-T_3 (○) and L-T_4 (●) bound as a function of DMPC (*A, C* and *E*) or DPPC (*B, D* and *F*) liposome concentration at 36°C (*A*), 16°C (*C*) and 8°C (*E*) for DMPC vesicles and 50°C (*B*), 36°C (*D*) and 20°C (*F*) for DPPC vesicles. In all cases 50 μM of L-T_3 or L-T_4 was used. Hormones bound to liposomes were determined as indicated in Materials and Methods. Similar results were obtained in three independent experiments.

assay temperature from 8°C (gel state) to 55°C (liquid-crystalline state). In the latter experiment, an aliquot of the liposomal-hormone suspension was separated at the start of the experiment for determination of L-T_4 incorporation at 8°C.

Discussion

The interaction of thyroid hormones with biological membranes was studied by several methods. Lai and Cheng (1982, 1984), using electronic spin resonance techniques, reported that the lateral diffusion of the spin-labeled L-T_3 and L-T_4 was similar to the lateral diffusion of spin-labeled fatty acid in DMPC liposomes. Studies of Lai et al. (1985) also showed the orientation of the nonpolar phenolic group of L-T_3 in the liposomal membranes. They suggested that the nonpolar phenolic group is close to the lipid core. In addition, studies performed with 12-doxylstearic acid spin-labeled liposomes (J. Pedersen, S. Ruffini, and R.N. Farías, *unpublished results*) observed that thyroid hormones can disturb the lipid core region of membrane models.

This work deals with the effects of thyroid hormones on physical properties of the membrane lipid bilayer. We describe the incorporation of thyroid hormones into PC, DMPC and DPPC vesicles.

L-T_3 incorporation mimics the cholesterol effect on the membrane fluidity; thus, this hormone decreases the membrane fluidity in the gel phase and increases it in the liquid-crystalline phase. On the other hand, L-T_4 incorporation decreases the fluidity above the transition temperature but it does not increase the fluidity in the gel phase (Figs. 1, 2).

Furthermore, L-T_3 incorporation induces similar percentage of release from phospholipid vesicles at temperatures below and above the transition temperatures (*compare* Fig. 3 with Fig. 4*A, C*). Meanwhile, L-T_4 does not induce release of DMPC and DPPC vesicles in the gel state, which appears well correlated with the low incorporation of the hormone to phospholipid vesicles (Fig. 5*E, F*).

At present, based on the large size of L-T_4 compared to L-T_3, we have an intuitive explanation for these observations. From the physicochemical point of view L-T_3 and L-T_4 are amphipathic molecules with a hydrophillic alanyl side chain and a hydrophobic diphenyl ether nucleus. Both aromatic rings are planar and close to being mutually perpendicular. It was suggested from three-dimensional X-ray diffraction studies (McDevitt, 1972) that 3′ iodine of β ring must be oriented proximal to the diiodotyrosine α ring rather than the distal as inferred from pharmacological studies (Jorgensen et al., 1962). The additional 5′ iodine in the β ring of diphenyl ether structure (Fig. 6) of L-T_4 would prevent the fitting into gel phase of hydrocarbon chains, which would be only capable of accepting the smaller L-T_3 molecule.

Current thinking is that these hormones act by binding to a soluble receptor that is a member of the steroid hormone receptor superfamily. The hormone-receptor complex then binds to DNA and affects gene expression (Green 1993). This paradigm is so widely accepted that it is difficult to see how the incorporation to membranes or the effects on physicochemical lipid bilayer properties can be more than just a tangential phenomenon of minor physiological importance. However, the thyroid hormones must cross the hydrophobic core of the plasma membrane of target cells to reach the deiodinase enzymes and the L-T_3 nuclear receptors before they can express their biological activity. The low incorporation of L-T_4 to membrane in the gel phase, and the opposite behavior of L-T_3 shown in this paper, could be of great importance to the interpretation of L-T_3 and L-T_4 membrane transport described previously (Osty et al., 1988; Osty et al., 1990).

To confirm our hypothesis, we are developing more experiments in our laboratory.

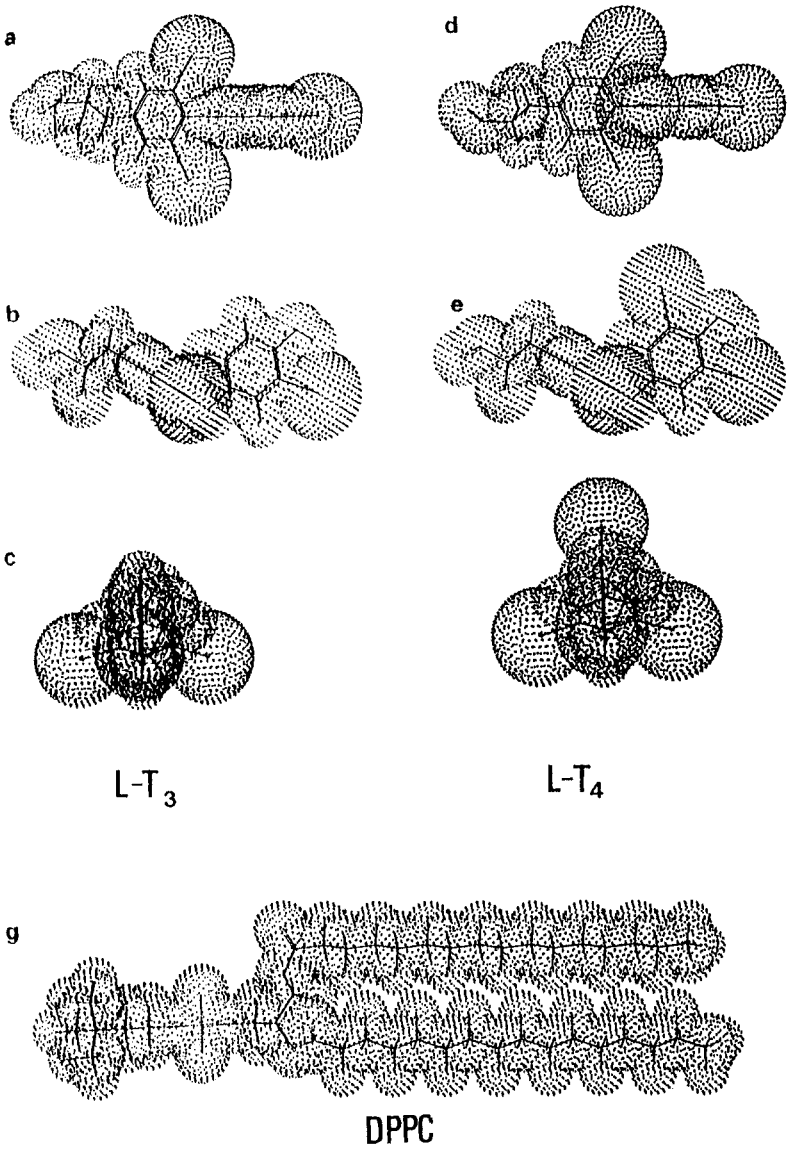

Fig. 6. Space-filling model of L-T_3 (*a,b,c*), L-T_4 (*d,e,f*) and DPPC (*g*). Different views of the thyroid hormone molecules: overviews (*a,d*); the same as *a,d* rotated by 90° (*b,e*) and endviews (*c,f*). For comparison, the overview of DPPC molecule (*g*).

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