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# ROTATIONAL AND LATERAL DIFFUSIONS OF L-THYROXINE IN PHOSPHOLIPID BILAYERS

CHING-SAN LAI [a] and SHEUE-YANN CHENG [b]

[a] *National Biomedical ESR Center, Department of Radiology, Medical College of Wisconsin, Milwaukee, WI 53226 and* [b] *Laboratory of Molecular Biology, National Cancer Institute, National Institues of Health, Bethesda, MD 20205 (U.S.A.)*

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**The rotational diffusion and lateral diffusion of 3-{[α-carboxy-4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenethyl]-carbamoyl}-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy-ethyl ester (spin-labeled L-thyroxine) in dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles were investigated by using electron spin resonance (ESR) techniques. Using the motional narrowing formalisms, the effective rotational correlation times of spin-labeled L-thyroxine in DMPC vesicles at 31°C were estimated as $3.1 \cdot 10^{-9}$ and $4.8 \cdot 10^{-9}$ s for the linear term and the quadratic term, respectively. The difference in calculated rotational correlation times of the linear and quadratic terms suggests an anisotropic rotational diffusion of spin-lableled L-thyroxine in the fluid DMPC vesicles. Moreover, at 31°C the rate of lateral diffusion of spin-labeled L-thyroxine in DMPC was approximately $5.2 \cdot 10^{-8}$ cm$^2$/ s as determined by the ESR line-broadening method. The value is similar to that of spin-labeled fatty acids. These results indicate that spin-labeled L-thyroxines diffuse freely in the DMPC matrix.**

## Introduction

Thyroid hormone regulates differentiation and development in a number of species and is important for the regulation of metabolic homeostasis in the adult [1]. In the past two decades, it was believed that the delivery of thyroid hormones into cells was by simple passive diffusion [2–4]. Recently, however, cell surface receptors for thyroid hormones have been detected in purified rat liver plasma membranes [5,6], rabbit adipocytes [7], and human erythrocyte ghosts [8]. Krenning et al. [9] and Rao et al. [10,11] have provided evidence that the uptake of 3,3',5-triiodothyronine ($T_3$) in isolated rat liver cells may be receptor-mediated. In addition, Cheng et al. [12,13] demonstrated that the entry of $T_3$ into cultured mouse fibroblasts occurs by receptor-mediated endocytosis.

At the present time, the characterization of the dynamic interactions of thyroid hormones with the plasma membranes is still lacking. Recently, electron spin resonance (ESR) is used to study the interaction of ligands with the receptor in the membrane [14]. In this communication, we used 3-{[α-carboxy-4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenethyl]-carbamoyl}-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy-ethyl ester (SL-$T_4$, see Fig. 1a), as a probe to study the interaction of L-thyroxine with the liposomes derived from L-α-dimyristoylphosphatidylcholine (DMPC).

## Materials and Methods

L-α-Dimyristoylphosphatidylcholine (DMPC) was obtained from Sigma. 5-Doxyl stearic acid

Abbreviations: ESR, electron spin resonance; DMPC, L-α-dimyristoylphosphatidylcholine; doxyl, the *N*-oxy-4',4'-dimethyloxazolidine derivatives of keto fatty acids; SL-$T_4$, 3-{[α-carboxy-4(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenethyl]-carbamoyl}-2,2,5,5-tetramethyl-3-pyrrolin-1-yloxy-ethyl ester.

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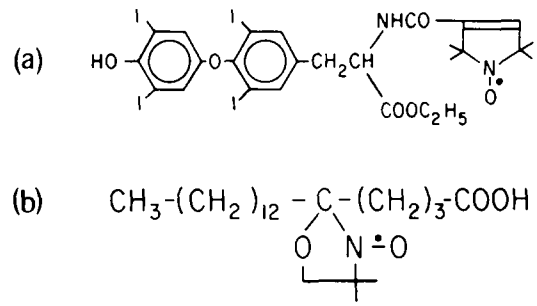


Fig. 1. Chemical formula of spin probes: (a) spin-labeled L-thyroxine (SL-$T_4$); (b) 5-doxyl stearic acid spin label.

spin label (Fig. 1b) was product of Syva. SL-$T_4$ was synthesized as previously described [15].

*Preparation of DMPC multilamellar vesicles*

An aliquot of ethanolic solution of SL-$T_4$ was added to DMPC dissolved in chloroform. The solvent was dried under a stream of nitrogen. The samples were then placed under vacuum overnight to remove residual solvent. Vesicles were prepared by the addition of 0.5 ml phosphate-buffered saline, pH 7.3 (OXOID Dulbecco 'A' solution without $Mg^{2+}$ and $Ca^{2+}$) vortexed for 10 s immediately after equilibrating at 35°C for 1 min. The sample was centrifuged at $300 \times g$ for 10 min and the vesicles were resuspended in 0.03 ml phosphate-buffered saline buffer (pH 7.3). The concentration of lipids was about 17 mM and SL-$T_4$ was incorporated into vesicles with mol% from 0.5 to 18. Based on the amount of free spins remaining in the supernatant after centrifugation it was estimated that at least 90% of SL-$T_4$ was incorporated into DMPC vesicles. The method for preparing 5-doxyl stearic acid spin label-lipid vesicles was the same as described above for SL-$T_4$.

*ESR mesurements*

The ESR measurements were performed on a Century Line Varian Spectrometer equipped with a variable temperature controller and a digital thermometer (Fluke 2100A model). The incident microwave power was 5 mW. The field sweep was 100 G and the modulation amplitude was 1.0 G. For determining the peak-to-peak linewidth of the central-field line $\Delta H$ (see Tables I and II) a 40 G field sweep was employed. The effective rotational correlation times for isotropic rotation of the spin probes were calculated using the linear and quadratic terms of the ESR motional narrowing formalisms [16–18] from the ESR spectra of SL-$T_4$ in DMPC vesicles at 31°C.

$$\tau_2 = 6.51 \cdot 10^{-10} \Delta H(0)\left[(h_0/h_{-1})^{1/2} - (h_0/h_{+1})^{1/2}\right] \times s \text{ (linear term)}$$

$$\tau_2 = 6.51 \cdot 10^{-10} \Delta H(0)\left[(h_0/h_{-1})^{1/2} + (h_0/h_{+1})^{1/2} - 2\right] \times s \text{ (quadratic term)}$$

where $\Delta H(0)$ is the peak-to-peak linewidth of the central field line and $h_0$, $h_{-1}$ and $h_{+1}$ are the peak-to-peak amplitudes of the first derivative resonances of the central, high- and low-field peaks, respectively.

## Results and Discussion

The temperature dependency of the ESR spectrum of SL-$T_4$ in DMPC multilamellar vesicles is shown in Fig. 2. The main phase transition of DMPC is about 23.7°C [19]. Above the phase-transition temperature the three-line pattern of the ESR spectrum appears to be symmetrical indicating a rapid motion of SL-$T_4$ in the fluid DMPC matrix. Below the phase-transition temperature, however, the line became broader as the temperature decreased. Fig. 3 shows that the maximum splitting values (in gauss) of the ESR spectra of SL-$T_4$ change as a function of temperature (see also the dotted lines of Fig. 2). The estimated phase-transition temperature of DMPC was about 22.6°C (Fig. 3) which is about 1°C lower than that obtained by differential scanning calorimetry method [19]. The results here suggest that the motion of SL-$T_4$ in DMPC vesicles reflects the structural organization of the membrane and that the maximum splitting value may be a sensitive parameter for determining the rotational motion of SL-$T_4$ in the membrane. Assuming isotropic rotational diffussion the effective rotational correlation times of the spin probe can be calculated using the linear and quadratic terms of the ESR motional narrowing formalisms [16–18]. When these two values are similar it is suggested that the spin probe undergoes isotropic rotational diffu-

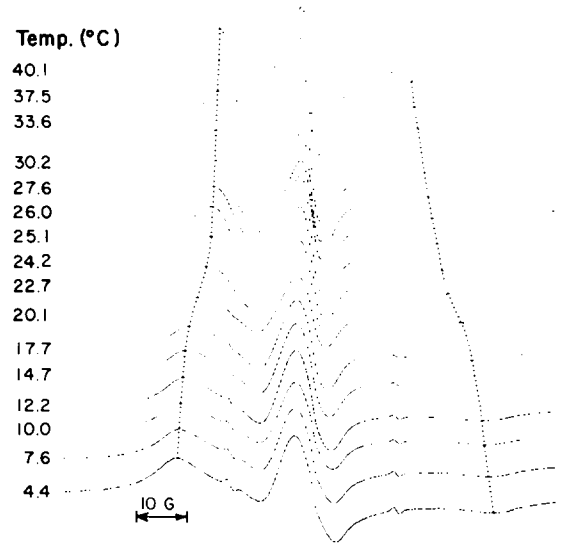


Fig. 2. Temperature dependency of the ESR spectrum of SL-$T_4$ in DMPC vesicles. The label-to-lipid ratio was 0.03. Each spectrum was taken at the temperature as indicated. The maximum splittings were connected by the dotted lines.

sion. When these two values are substantially dissimilar it is argued that the spin probe undergoes anisotropic rotational diffusion [20]. At 31°C the

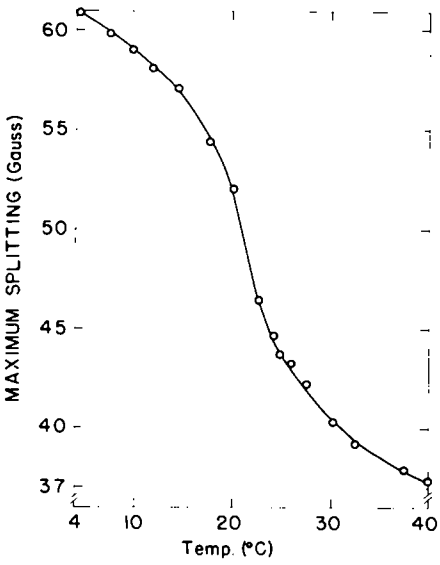


Fig. 3. Maximum splitting vlaues of the ESR spectrum of SL-$T_4$ in DMPC vesicles as a function of temperature. A 5 G modulation amplitude was employed to measure the maximum splitting value. Prior to measurement, the sample was equilibrated at each indicated temperature for 10 min.

effective rotational correlation times of SL-$T_4$ were $3.1 \cdot 10^{-9}$ s (the linear value) and $4.8 \cdot 10^{-9}$ s (the quadratic value), respectively. It is therefore plausible that SL-$T_4$ undergoes anisotropic rotational diffusion in the membrane. The average isotropic hyperfine constant $A_0$ is sensitive to solvent polarity [21]. At 31°C the $A_0$ values of the ESR spectrum of SL-$T_4$ in phosphate-buffered saline, DMPC vesicles and benzene were 16.0, 15.5 and 14.2 G, respectively. The polarity of SL-$T_4$ in DMPC vesicles is closer to phosphate buffered saline than to benzene. This suggests that the nitroxyl group of SL-$T_4$ is near the polar head group rather than the lipid core. In addition, the phenolic groups of SL-$T_4$ should be chemically more hydrophobic than the nitroxyl moiety. It appears that the orientation of SL-$T_4$ in DMPC vesicles is that the nitroxyl moiety is near the polar head group and phenolic groups are near the lipid core.

Fig. 4 shows the ESR spectra observed at 31°C and 14°C with SL-$T_4$ in DMPC vesicles. The ESR line became broader with increasing SL-$T_4$ to DMPC ratios. The line broadening can be due to two modes of interaction: the dipolar and the spin exchange. The dipolar interaction can be neglected for label-to-lipid ratios smaller than 0.2 at temperatures higher than the main transition temperature [22]. The spin exchange may therefore be responsible for observed line broadening in this study. The exchange broadening $\Delta H_{ex}$ is directly related to the exchange frequency $W_{ex}$ (the probability of spin exchange per second) which in turn is directly proportional to the rate of lateral diffusion $D_{diff}$ by the following equation [22]:

$$W_{ex} = \frac{4}{3} \frac{d_c D_{diff}}{F\lambda} \frac{C}{(1+C)}$$

Where $F$, $d_c$ and $\lambda$ are the area per lipid molecule (60 Å for $T > T_t$), the critical interaction distance (approx. 20 Å) and the length of one diffusional jump in the lipid lattice (8 Å). In the case of random distributions of the label, the plot of $W_{ex}$ against $C/(1+C)$ should yield a straight line. If the labels are clustered in the membrane, the plot of $W_{ex}$ against $C/(1+C)$ will no longer be linear [22].

Fig. 5 shows that at 31°C $W_{ex}$ is not proportional to $C/(1+C)$ for SL-$T_4$ in DMPC vesicles

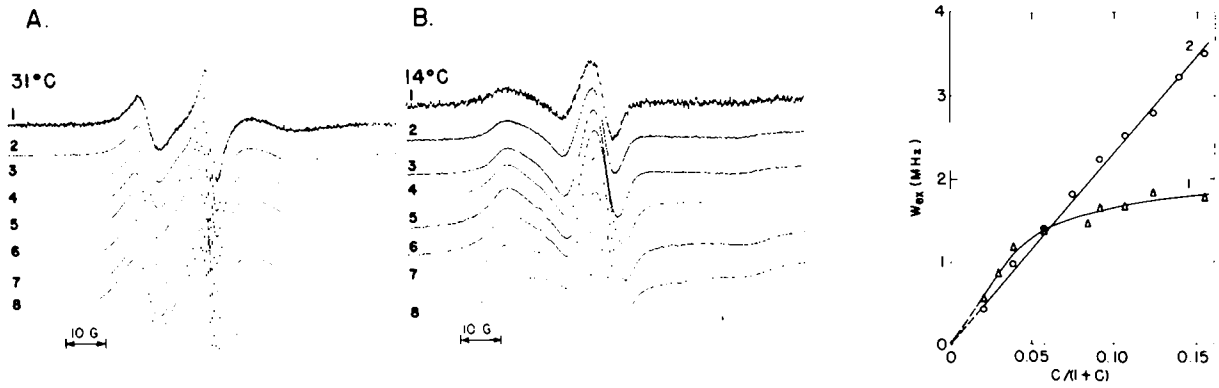


Fig. 4. Concentration dependency of the ESR spectrum of SL-$T_4$ in DMPC vesicles. The molar ratios of label to lipid were: (1) 0.001, (2) 0.005, (3) 0.01, (4) 0.03, (5) 0.060, (6) 0.09, (7) 0.120, and (8) 0.18. The measured temperatures were (A) 31°C and (B) 14°C.

Fig. 5. Exchange frequency $W_{ex}$ as a function of $C/(1+C)$: (1) SL-$T_4$ in DMPC vesicles; (2) 5-doxyl stearic acid spin label in DMPC vesicles. The data points for SL-$T_4$ and 5-doxyl stearic acid spin label were obtained from Tables I and II, respectively. The ESR measurement was carried out at $31 \pm 0.2$°C.

(curve 1 of Fig. 5 and Table I). The result suggests that at label-to-lipid ratio higher than 0.05, SL-$T_4$ is clustered in the fluid DMPC matrix. This cannot be due to the limitation of SL-$T_4$ incorporating into DMPC vesicles at high concentrations inasmuch as at 14°C the linewidth of the ESR spectra broadened continuously at high concentrations (see Fig. 4b). Also at pH 7.4 the partition coefficient of thyroxine between phospholipid and water was $1.2 \cdot 10^4$ [23]. On the other hand, curve 2 of Fig. 5 shows that at 31°C $W_{ex}$ is proportional to $C/(1+C)$ for 5-doxyl stearic acid spin label in DMPC vesicles (also see Table II). This result suggests that the spin-labeled fatty acids are randomly distributed within the fluid DMPC lipid matrix which is consistent with the results obtained by Hauser and Guyer using microelectrophoresis technique [24]. With the initial linear

TABLE I

CONCENTRATION DEPENDENCY OF EXCHANGE FREQUENCY, $W_{ex}$, OF THE SPIN-LABELED THYROXINE (SL-$T_4$) IN DMPC VESICLES

DMPC vesicles were prepared as described in Materials and Methods. The ESR measurement was carried out at 31°C ± 0.2°C. $\Delta H$ and $\Delta H_0$ are the total linewidth and natural linewidth of the central-field line, respectively. $\Delta H_{ex} = \Delta H - \Delta H_0$ and $W_{ex}$ (MHz) = 1.4 × $\Delta H_{ex}$. The linewidth due to the dipolar interaction ($\Delta H_d$) is negligible, $C < 20$ [22].

| Label-to-lipid ratio, $C$ (mol%) | $C/1+C$ | $\Delta H$ (G) | $\Delta H_0$ (G) | $\Delta H_{ex}$ (G) | $W_{ex}$ (MHz) |
|---|---|---|---|---|---|
| 0.5 | 0.005 | 3.0 | 3.0 | - | - |
| 2 | 0.020 | 3.4 | 3.0 | 0.4 | 0.56 |
| 3 | 0.029 | 3.63 | 3.0 | 0.63 | 0.88 |
| 4 | 0.038 | 3.84 | 3.0 | 0.84 | 1.18 |
| 6 | 0.057 | 3.98 | 3.0 | 0.98 | 1.37 |
| 9 | 0.083 | 4.03 | 3.0 | 1.03 | 1.44 |
| 10 | 0.091 | 4.20 | 3.0 | 1.20 | 1.68 |
| 12 | 0.107 | 4.20 | 3.0 | 1.20 | 1.68 |
| 14 | 0.123 | 4.30 | 3.0 | 1.30 | 1.82 |
| 18 | 0.152 | 4.28 | 3.0 | 1.28 | 1.79 |

TABLE II

CONCENTRATION DEPENDENCY OF EXCHANGE FREQUENCY, $W_{ex}$, OF THE 5-DOXYL STEARIC ACID IN DMPC VESICLES

DMPC vesicles were prepared as described in Materials and Methods. The ESR measurement was carried out at 31°C ± 0.2°C. $\Delta H$ and $\Delta H_0$ are the total linewidth and natural linewidth of the central field line, respectively. $\Delta H_{ex} = \Delta H - \Delta H_0$ and $W_{ex}$ (MHz) = $1.4 \times \Delta H_{ex}$. The linewidth due to the dipolar interaction ($\Delta H_d$) is negligible at $C < 20$ [22].

| Label-to-lipid ratio, $C$ (mol%) | $C/1+C$ | $\Delta H$ (G) | $\Delta H_0$ (G) | $\Delta H_{ex}$ (G) | $W_{ex}$ (MHz) |
|---|---|---|---|---|---|
| 1 | 0.001 | 2.5 | 2.5 | - | - |
| 2 | 0.020 | 2.8 | 2.5 | 0.3 | 0.42 |
| 4 | 0.038 | 3.2 | 2.5 | 0.7 | 0.98 |
| 6 | 0.057 | 3.5 | 2.5 | 1.0 | 1.40 |
| 8 | 0.074 | 3.8 | 2.5 | 1.3 | 1.82 |
| 10 | 0.091 | 4.1 | 2.5 | 1.6 | 2.24 |
| 12 | 0.107 | 4.3 | 2.5 | 1.8 | 2.52 |
| 14 | 0.129 | 4.5 | 2.5 | 2.0 | 2.80 |
| 16 | 0.138 | 4.8 | 2.5 | 2.3 | 3.22 |
| 18 | 0.153 | 5.0 | 2.5 | 2.5 | 3.50 |

portion of curve 1 in Fig. 5, the rate of lateral diffusion of SL-$T_4$ was estimated as $5.2 \cdot 10^{-8}$ $cm^2/s$ at 31°C in DMPC membranes whereas the rate of lateral diffusion of fatty acid spin labels was about $4.2 \cdot 10^{-8}$ $cm^2/s$. The lateral diffusion of fatty acid spin labels in *Escherichia coli* membranes at 40°C was determined as $3.25 \cdot 10^{-8}$ $cm^2/s$ [22]. The finding that SL-$T_4$ has a lateral diffusion coefficient $5.2 \cdot 10^{-8}$ $cm^2/s$ in liposomes which lack thyroid hormone receptors is significant. Earlier, using fluorescence photobleaching recovery techniques, Maxfield et al. [13] reported that the lateral diffusion coefficient of rhodamine-labeled 3,3',5-triiodothyronine bound to protein receptors on 3T3 Swiss fibroblasts was $2.8 \cdot 10^{-10}$ $cm^2/s$.

When the spin label to lipid ratio exceeds 5 : 100, SL-$T_4$ aggregates in the fluid DMPC membranes (see curve 1 of Fig. 5). However, this may not occur under physiological conditions, since the free circulating hormones are in the picomolar range. The ESR approach will be a useful tool for characterizing the dynamic interactions of thyroid hormones whith the receptor in the plasma membrane.

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## References

1 Werner, S.C. (1978) The Thyroid, 4th Edn., Harper&Row, New York
2 Ingbar, S.H. and Freinkel, N. (1960) Recent Prog. Horm. Res. 16, 353–403
3 Tata, J.R. (1964) in The Thyroid Gland (Pitt-Rivers, R. and Trotter, W.B., eds.), Vol. 1, pp. 163–168, Butterworth, London
4 Lein, A. and Dowben, R.M. (1961) Am. J. Physiol. 200, 1029–1031
5 Pliam, N.B. and Goldfine, I.D. (1977) Biochem. Biophys. Res. Commun. 79, 166–172
6 Gharbi, J. and Torresani, J. (1979) Biochem. Biophys. Res. Commun. 88, 170–177
7 Parl, F., Korcek, L., Siegel, J.S. and Tabachnick, M. (1977) FEBS Lett. 83, 145–147
8 Holm, A.-C. and Jacquemin, C. (1979) Biochem. Biophys. Res. Comunn. 89, 1006–1017
9 Krenning, E.P., Docter, R., Bernard, H.F., Visser, T.J. and Hennemann, G. (1978) FEBS Lett. 91, 113–116
10 Eckel, J., Rao, G.S., Rao, M.L. and Breuer, H. (1979) Biochem. J. 182, 473–491
11 Rao, G.S., Eckel, J., Rao, M.L. and Breuer, H. (1976) Biochem. Biophys. Res. Commun. 73, 98–104
12 Cheng, S.-Y., Maxfield, F.R., Robbins, J., Willingham, M.C. and Pastan, I.H. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 3425–3429
13 Maxfield, F.R., Willingham, M.C., Pastan, I., Dragsten, P. and Cheng, S.-Y. (1981) Science 211, 63–65

14 Ellena, J.F. and McNamee, M.G. (1980) FEBS Lett. 110, 301–304
15 Cheng, S.-Y., Rakhit, G., Erard, F., Robbins, J. and Chignell, C.F. (1981) J. Biol. Chem. 256, 831–836
16 Kivelson, D. (1960) J. Chem. Phys. 33, 1094–1106
17 Freed, J.H. and Fraenkel, G.K. (1963) J. Chem. Phys. 39, 326–348
18 McConnell, H.M. (1956) J. Chem. Phys. 25, 709–711
19 Hinz, H.-J. and Sturtevant, J.M. (1972) J. Biol. Chem. 247, 6071–6075
20 Cannon, B., Polnaszek, C.F., Butler, K.W., Eriksson, L.E.G., and Smith, I.C.P. (1975) Arch. Biochem. Biophys. 167, 505–518
21 Griffith, O.H. and Jost, P.C. (1976) in Spin Labeling (Berliner, L.J., ed.), Vol. 1, pp. 453–523, Academic Press, New York
22 Sackmann, E., Trauble, H., Galla, H.-J., and Overath, P. (1973) Biochemistry 12, 5360–5369
23 Hillier, A.P. (1970) J. Physiol. 211, 585–597
24 Hauser, H. and Guyer, W. (1979) Biochim. Biophys. Acta 553, 359–363