Rotational Relaxation of 1,6-Diphenylhexatriene in Membrane Lipids of Cells Acclimated to High and Low Growth Temperatures†

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ABSTRACT: Measurement of the time-resolved fluorescence depolarization of 1,6-diphenylhexatriene (DPH) in artificial bilayers of microsomal membrane lipids from *Tetrahymena* gives detailed information concerning the molecular motion of this probe and fluid properties of the membrane lipids which are obscured with steady-state methods. The rotational motion of DPH in these lipids from cells acclimated to 15 and 39.5 °C growth temperatures was anisotropic, which agrees with recent time-resolved studies of this probe in synthetic phospholipid systems. Evaluation of DPH polarization data obtained from these lipid fractions at their respective growth temperatures showed differences in physical properties which suggest that "viscosity", per se, of the microsomal lipids is not as strictly regulated as it is in prokaryotic systems. Rotational relaxation of DPH in 39.5 °C microsomal lipids measured at 15 °C is more complex than that of either lipid fraction measured at its actual growth temperature, suggesting that the probe has partitioned into two dissimilar environments within the bilayer. Similar effects are observed in the microsomes of 39.5 °C cells by freeze-fracture electron microscopy following rapid cooling to 15 °C. Under these conditions, two distinct regions are observed on the fracture faces, suggesting a correlation between lipid phase changes and alterations in membrane structure.

The preceding paper (Martin & Thompson, 1978) described the use of fluorescence polarization methods to study changes in the fluid properties of *Tetrahymena* membrane lipids during temperature acclimation. Steady-state polarization measurements of lipid properties using 1,6-diphenyl-1,3,5-hexatriene (DPH) as a probe correlate well with freeze-fracture electron microscopy observations of their parent membranes, and thus underscore the importance of alterations of lipid composition in the regulation of membrane fluidity. Neither of these methods permits a precise description of the membrane fluid properties under actual growth conditions, however. Therefore, in order to examine the nature of the lipid environment within cell membranes from both normally growing and temperature-acclimating cells, we have initiated a series of experiments using time-resolved fluorescence polarization methods.

Recent time-resolved studies of DPH polarization in synthetic phospholipid bilayers have demonstrated the utility of such methods in describing the rotational motion of chromophores embedded within membranes (Kawato et al., 1977; Dale et al., 1977). Since the relaxational modes of the probe molecule are determined by the physical state of its immediate environment, a detailed picture of the membrane interior can be drawn from such observations. This paper describes the rotational relaxation of DPH in microsomal membrane lipids of cells acclimated to high and low growth temperatures and the alterations in the thermotropic properties of these lipids produced by a drastic shift in environmental temperature.

Materials and Methods

The isolation of membranes, extraction of membrane lipids, and the preparation of lipid vesicles for polarization measurements have been described in the preceding paper (Martin & Thompson, 1978). The final lipid:probe ratio in these experiments was 500:1.

Nanosecond Time-Resolved Measurements. Fluorescence lifetimes were measured with a single-photon counting device constructed on similar principles to one described by Easter et al. (1976) which allowed semisimultaneous determination of the excitation and the orthogonally polarized decay functions to compensate for drifts in the timing and intensity of the excitation pulse. Timing of the data collection periods was controlled by a microprocessor interfaced with the polarizer switching apparatus and the multichannel analyzer data storage device. The major differences between our device and that of Easter et al. (1976) lay in the manner in which the excitation pulse was recorded. Our apparatus sampled the excitation pulse via a shuttered quartz fiber optic which routed the lamp pulse to the photomultiplier tube independently of the light path through the cuvette. This arrangement allowed the use of movable polarizers on the emission side of the apparatus and permitted measurements of both fluorescence decay functions from the same sample. In order to ensure that the excitation pulse transmitted by the fiber optic was identical with that transmitted through the cuvette, repeated comparisons were made with the apparatus alternately sampling excitation pulses through the fiber optic and through the cuvette path of the instrument. Superimposition of the two excitation functions collected in this manner revealed no differences in the shape of the excitation pulse. Timing delays due to differences between the two excitation pathlengths were also determined by this procedure and the excitation pulse data was shifted the appropriate number of channels in order to align it with the fluorescence decay curves. A detailed description of the device will be given elsewhere (Craig B., Martin, C. E., & Walser, R. A. W., manuscript in preparation).

The excitation source was a thyratron-gated air flash lamp run at 12 kHz. The excitation bandwidth was selected by a 3400-Å narrow bandpass filter and was polarized by doubled polacoat UVWRMR filters. Fluorescence emission was measured through 4800-Å bandpass filters and through polarizers with transmission axes oriented alternately in parallel and perpendicular directions to that of the excitation beam.

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TABLE I: Deconvolved Fits of $S(t) = \sum_{i=1}^{2} s_{0_i} e^{-t/\tau_i}$ and $D(t) = \sum_{i=1}^{2} d_{0_i} e^{-t/\theta_i}$ with $A_{calcd} = \int_0^\infty D(t)dt / \int_0^\infty S(t)dt$.[a]

	a	b	c
S_{0_1}	0.3379	0.3337	0.4289
S_{0_2}			0.3559
τ_1 (ns)	7.67	8.19	4.95
τ_2 (ns)			10.17
d_{0_1}	0.0594	0.06561	0.160
d_{0_2}	0.007077	0.01961	0.04819
θ_1 (ns)	2.16	2.72	2.31
θ_2 (ns)	7.67	8.19	7.70
A_{calcd}	0.071	0.124	0.133
A_{ss}	0.082	0.125	0.167

[a] Abbreviations are: (a) 39.5 °C microsomes at 39.5 °C; (b) 15 °C microsomes at 15 °C; (c) 39.5 °C microsomes at 15 °C. [b] Anisotropy calculated by integration of time-resolved data. [c] Steady-state data from Martin & Thompson (1978).

Each polarized decay component was measured for 30 s and the excitation pulse was measured for 5 s in each measurement cycle. Data collection periods ranged from 10 to 16 h for each sample. Scattering correction was applied by subtracting appropriately scaled blank sample decay measurements from the fluorescence decay curves. Measurements of a completely depolarized sample of DPH in chloroform with the excitation polarizers oriented through 90° showed no statistically significant differences in the detection of the polarized decay components by the apparatus; therefore no correction to steady-state polarization values was applied to the data sets.

Analysis of Fluorescence Decay Data. The underlying principles of nanosecond fluorescence polarization spectroscopy have been discussed at length by a number of authors; two representative reviews of the theory and methodology behind these experiments are those of Yguerabide (1973) and Cantor & Tao (1971).

The decay components $I_\parallel(t)$ and $I_\perp(t)$ which are measured by photon counting are complex functions which are dependent on both the rotational motion and the fluorescence lifetime, τ, of the probe molecule. The dependence on τ can be separated from the rotational components by defining two functions designated as the total emission, $S(t)$, and the emission anisotropy, $R(t)$. The former yields information on the decay characteristics of the chromophore and is independent of rotational motion, while the latter is dependent only on the rotational behavior of the molecule. In order to determine the solutions of these functions, the experimental decay curves, $I_\parallel(t)$ and $I_\perp(t)$, were combined to give the sum and difference functions:

$$S(t) = I_\parallel(t) + 2I_\perp(t) \quad (1)$$

and

$$D(t) = I_\parallel(t) - I_\perp(t) \quad (2)$$

which were then fitted to a single or double exponential decay function. The fit was accomplished by means of a nonlinear least-squares search. For the data sets requiring only a single exponential lifetime for $S(t)$, the lifetimes ϕ_1 and ϕ_2 of the anisotropy decay, $R(t)$, were obtained from the lifetimes, θ_1 and θ_2 of the difference curve, $D(t)$, by means of the relation

$$1/\theta_i = 1/\phi_i + 1/\tau \quad (3)$$

which follows from the definition

$$R(t) = D(t)/S(t) \quad (4)$$

In those cases where τ and one of the θ_i terms were equal within the limits of validity of the fit, the least-sqaures search was modified to hold $\theta_2 = \tau$ constant and to fit θ_1 and the preexponential factors only, yielding an infinite correlation time, ϕ_2 (Dale et al., 1977; Kawato et al., 1977).

For the data which required a double exponential fit for $S(t)$, the anisotropy decay, $R(t)$, was analyzed according to two models:

$$\text{let } r_i(t) = r_{0i} e^{-t/\phi_i} \quad (5)$$

$$S_i(t) = s_{oi} e^{-t/\tau_i} \quad (6)$$

where

$$S(t) = \sum_{i=1}^{2} s_i(t) \quad (7)$$

has already been determined as described above. Then if the biexponential character of $S(t)$ is due to microheterogeneity (i.e., two distinct probe environments), we have

$$D(t) = \sum_{i=1}^{2} r_i(t) s_i(t) \quad (8)$$

whereas if it is due to rotational anisotropy with a single probe environment, the difference function is given by (Dale et al., 1977)

$$D(t) = \left(\sum_{i=1}^{2} r_i(t)\right)\left(\sum_{i=1}^{2} s_i(t)\right) \quad (9)$$

These fits were also accomplished via convolution coupled with a nonlinear least-squares search. The parameters obtained by direct fits of exponentials to $S(t)$ and $D(t)$ are presented in Table I, together with the steady-state anisotropies calculated by integration of the fitted functions. In all cases, these are in good agreement with the measured steady-state anisotropies (Martin & Thompson, 1978).

Results

Relaxation of DPH in Lipids at Growth Temperatures. The total fluorescence decay function $S(t)$ measured at the temperatures where the cells were grown (15 °C in one case and 39.5 °C in the other) was adequately fit by a single exponential function as evidenced both by the statistical criteria of goodness of fit and by the fact that inclusion of a second exponential into the decay function resulted in an insignificantly small preexponential coefficient and a negligible change in the parameters of the principal exponential. These results are in agreement with the findings of Kawato et al. (1977) for DPH in dipalmitoylphosphatidylcholine (DPPC) liposomes, but differs from the behavior of the same probe in highly sonicated egg lecithin vesicles (Dale et al., 1977).

The corresponding difference functions, $D(t)$, for the two lipid preparations were each analyzed as a sum of two exponentials, one of which was found to agree closely with the lifetime of $S(t)$. The relaxation time calculated from this value by eq 3 is infinite, which is in agreement with earlier studies. Holding this value constant resulted in excellent fits of our data with the inclusion of only one other exponential component. This procedure is consistent with the fitting methods used by other laboratories in which a constant was introduced into the deconvolution for an infinite lifetime parameter. From this fit, the parameters of the anisotropy decay

$$r(t) = (r_0 - r_\infty)e^{-t/\phi_1} + r_\infty \quad (10)$$

were calculated and are displayed in Table II.

The biexponential character of $D(t)$ points out the difficulty

TABLE II: Deconvolved Fits of the Anisotropy $R(t) = (r_0 - r_\infty)e^{-t/\theta_1} + r_\infty$ for Fluorescence Measured at the Growth Temperature of the Cells.

	39.5 °C	15 °C
r_0	0.1967	0.2554
r_∞	0.0209	0.0588
ϕ_1	3.0 ns	4.2 ns
D_w	0.074	0.044
θ_{max}	63	53

TABLE III: Deconvolved Fits of the Anisotropy Functions Corresponding to Two Models for the DPH Probe at 15 °C in Microsome Lipids from Cells Grown at 39.5 °C.

	microheterogeneity (eq 8)	constrained rotation (eq 9)
r_1	0.3940	0.1928
r_2	0.1354	0.0843
ϕ_1	4.3 ns	3.2 ns
ϕ_2	37.7 ns	64.3 ns

of extracting a single parameter, such as viscosity, from steady-state polarization data which will adequately describe the local environment of the probe. Therefore, in order to describe the environment in more meaningful terms, it is necessary to analyze the molecular motion of the probe and, by inference, the nature of its surroundings, in terms of a suitable model. The two-exponential character of the difference function is consistent with either of two assumptions: that there are two distinct probe environments (microheterogeneity); or that the probe's rotation is constrained but in a single type of local environment (rotational anisotropy).

The previous studies of synthetic phospholipids and egg lecithin systems support the latter assumption (Kawato et al., 1977; Dale et al., 1977; Chen et al., 1977) and the nature of the decay data presented here is also consistent with this model. These conclusions are based on the existence of a monoexponential decay function $S(t)$ of the probe, as well as the occurrence of an infinite rotational correlation time in the calculated anisotropy decay. Since the fluorescence lifetimes of polyenes are sensitive to their environments (Birks & Birch, 1975), it seems unlikely that $S(t)$ could be fitted properly with a single exponential function if there were two probe environments, one of which was so "viscous" as to yield an infinite relaxation time while the other was orders of magnitude lower by comparison.

Although rotational anisotropy represents the more complex of the two models, these data can be analyzed in terms of a theoretical model constructed for this case (Kinosita et al., 1977) in which the rod-shaped probe is confined within a cone described by an angle θ_{max} around the normal to the membrane surface, with the long axis (which coincides with the emission moment μ) fluctuating uniformly within the cone with an average wobbling diffusion coefficient $\langle D_\omega \rangle$. Then $\langle D_\omega \rangle$ and θ_{max} are given by

$$\langle D_\omega \rangle = \langle \sigma \rangle / \phi_1 \tag{11}$$

and

$$r_\infty / r_0 = [\tfrac{1}{2} \cos \theta_{max}(1 + \cos \theta_{max})]^2 \tag{12}$$

where $\langle \sigma \rangle$ is a function of r_∞/r_0 (Kinosita et al., 1977).

The values of $\langle D_\omega \rangle$ and the cone angle, θ_{max}, obtained for 39.5 °C and 15 °C lipids are given in Table II. The 15 °C lipids have both $\langle D_\omega \rangle$ and θ_{max} smaller than the 39.5 °C lipids, which indicates that the motion of the probe is more tightly restricted in the 15 °C lipids.

It is possible, within the limits of validity of the cone model, to calculate a "viscosity within the cone" parameter with which we can compare the lipids of the cells at their actual growth temperature. Although this is a theoretical construction and does not represent an absolute determination of viscosity, such a comparison is relevant to the questions of whether, and to what extent, the observed changes in chemical composition of these two lipid preparations serve to offset the change in fluidity due to temperature. An effective "viscosity in the cone", η, is related to the wobbling diffusion constant by the equation

$$\langle D_\omega \rangle = KT/\eta \tag{13}$$

(Kinosita et al., 1977) where κ is a composite of Boltzmann's constant and molecular volume and shape factors which are assumed to be constant within the range of measurements. Calculation of η from these data yields a "viscosity" of 4200κ for the 39.5 °C lipids and a value of 650κ for the lipids from 15 °C cells. Thus it appears that the lipid composition at the lower growth temperature produces significantly higher "viscosity" in vivo than does its 39.5 °C counterpart.

Fluorescence Measurements Taken at 15 °C for Lipids from 39.5 °C Cells. In order to examine the behavior of membrane lipids under conditions which have been shown to produce changes in the freeze-fracture EM morphology of microsomal membranes, we measured the fluorescence polarization of 39.5 °C microsomal membranes at 15 °C. Following a rapid shift of the cells to the lower temperature, the fracture faces of the endoplasmic reticulum contain broad expanses of particle-free areas. These areas have been postulated to be composed of membrane lipids which have undergone a liquid-crystalline → gel phase separation (Wunderlich et al., 1975).

The fluorescence decay data for 39.5 °C lipids measured under these conditions also present a more complex picture than the corresponding data for measurements taken at the growth temperature of the cells. Both the sum and the difference functions are clearly biexponential if not multiexponential. Figures 1 and 2 compare single and double exponential fits to a set of data from these lipids measured at 15 °C. This added complexity may, in principle, be due either to additional constraints on probe rotation or to microheterogeneity of the probe environment.

Deconvolved fits for both models were obtained as described in the Materials and Methods section. The parameters of the separate fits to $S(t)$ and $D(t)$ are given in Table I, and the parameters derived for the anisotropy decay, as defined in eq 5-9, are given in Table III.

Several lines of evidence point to the microheterogeneity model as being reasonable in this case. The appearance of two distinct regions in freeze-fracture electron micrographs suggests the possibility of two dissimilar lipid environments forming under these conditions (Martin et al., 1976). Furthermore, the relatively shorter anisotropic lifetime of 3.2 ns obtained under the assumption of constrained rotation alone is suspect given the lifetimes of 3.0 and 4.2 ns measured in cells at the actual growth temperatures (Table II). In light of the high steady-state polarization values observed under these conditions as compared with both fractions measured at their growth temperatures (Table I), the 39.5 °C lipids should restrict the rotation of the probe more severely when cooled to 15 °C. If rotational anisotropy was the sole characteristic of DPH motion in this case, the expected anisotropic lifetime ϕ_1

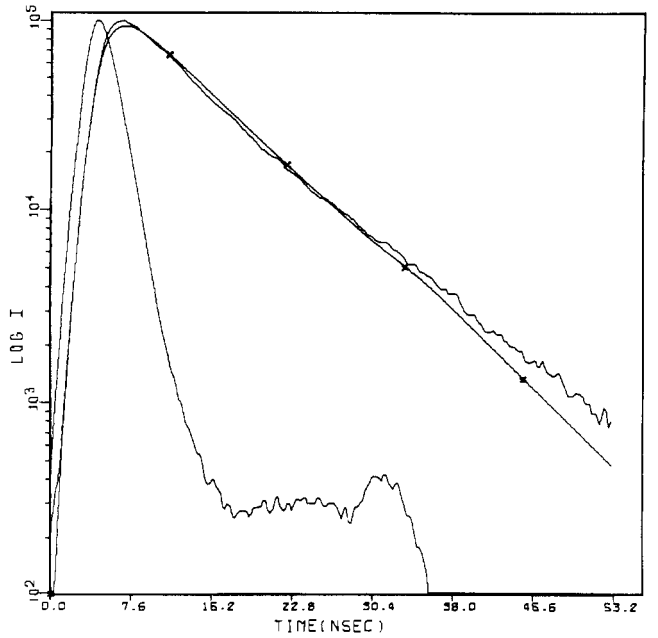

FIGURE 1: Single exponential fit of DPH total emission intensity ($I_{\parallel}(t) + 2I_{\perp}(t)$) in 39.5 °C microsomal lipids measured at 15 °C. The Y axis is the log of fluorescence intensity. The initial peak is the excitation pulse and the smooth curve is the convolved theoretical fit. Fitted values are $\tau = 8.0$ ns and $s_0 = 0.40$.

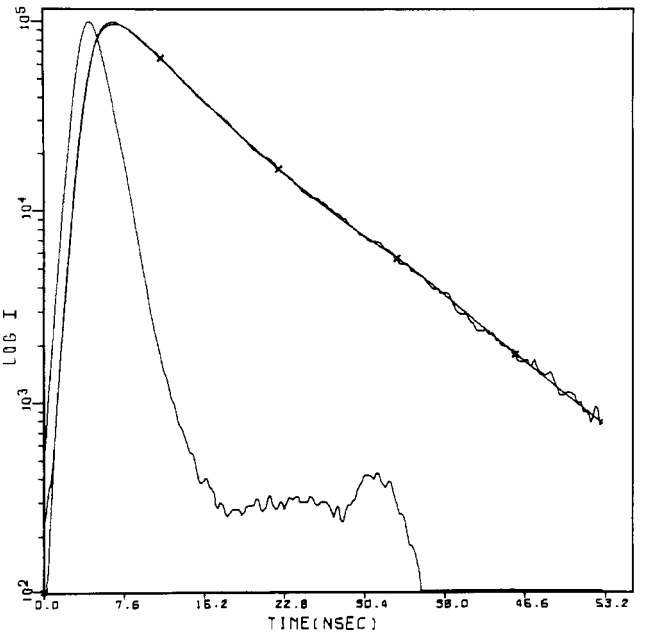

FIGURE 2: Double exponential fit of DPH total emission intensity ($I_{\parallel}(t) + 2I_{\perp}(t)$) in 39.5 °C microsomal lipids measured at 15 °C. See legend of Figure 1 for details. Fitted exponentials are: $\tau_1 = 4.9$ ns; $\tau_2 = 10.2$ ns; $s_1 = 0.24$; $s_2 = 0.20$.

would be significantly larger than the 3.0 ns measured at the growth temperature. However, since an apparent phase separation occurs when the membranes are cooled from 39.5 °C to this temperature, we cannot rule out the possibility that one of the phases actually has lipid mobility comparable to the uniform phase at 39.5 °C.

A more definitive comparison of the temperature altered lipids with those measured at their growth temperatures would require a more ambitious analysis of the data in which each of the two postulated local environments of the probe would be analyzed on the model of anisotropic rotation confined within a cone. In such an analysis, eq 5 would be replaced by

$$r_i(t) = (r_{0i} - r_{\infty i})e^{-t/\phi_i} + r_{\infty i} \qquad (14)$$

analogous to eq 10. The resulting expression for $D(t)$, given by eq 8, would then involve six parameters, namely, $r_{01}, r_{02}, r_{\infty 1}, r_{\infty 2}$, ϕ_1, and ϕ_2. $\langle D_w \rangle$ and θ_{max} would be calculated as in eq 11 and 12. Indeed, it is quite probable that the long lifetime (64.3 ns) of Table III is a composite of the correct ϕ_2 and the infinite lifetime (constant) terms found in lipids at their growth temperatures and in synthetic phospholipid systems. It is also unlikely that a rotational constraint leading to an infinite lifetime at 39.5 °C would be removed upon cooling to 15 °C, unless, of course the lipid bilayer structure was disrupted and randomized. Although the latter possibility can be ruled out, the quality of photon counting data is not sufficiently good to permit a meaningful deconvolved fit of the six-parameter function described above.

Discussion

The fluorescence polarization studies undertaken in this and the preceding paper (Martin & Thompson, 1978) point out the dramatic effects of temperature changes on membrane lipid properties. It is clear from these studies that the adaptive changes we have observed in membrane lipid compositions following shifts in growth temperatures (Martin et al., 1976; Kasai et al., 1976) occur in a manner which compensates for the temperature-induced changes in membrane lipid mobility.

Our experiments agree closely with those of Kawato et al. (1977), Chen et al. (1977), and Dale et al. (1977) in establishing that the motion of DPH in phospholipid membranes is asymmetric. As these authors have pointed out, this precludes describing the interior of the bilayer in terms of a single parameter, such as viscosity, from steady-state data alone. The use of time-resolved polarization measurements in conjunction with steady-state observations, however, promises to provide a powerful method for studying the properties of biological membranes.

The initial experiments that we have reported here were undertaken in order to gain further insight into the actual fluid properties of *Tetrahymena* membranes which are under regulatory control. In prokaryotic systems it appears that the membrane "viscosity" is adjusted so as to remain at a constant level with respect to the growth temperature (Sinensky, 1974; Esser & Souza, 1974; Huang et al., 1974). Sinensky has referred to this process as "homeoviscous adaptation". It seems clear from the freeze-fracture observations of Kitajima & Thompson (1977) that the fluid properties of *Tetrahymena* membranes are also regulated in a very precise manner. This is evidenced by the findings that membrane particle redistribution occurs consistently within a given membrane at a fixed number of degrees below the growth temperature of the cell. The time-resolved fluorescence data, however, suggest that in *Tetrahymena* temperature acclimation can produce significant differences in physical properties analogous to those described as "viscosity" in the prokaryotic cells. Our data further indicate that lipid mobility is greater (or the "viscosity" is lower) in the 39.5 °C microsomal lipids than in their 15 °C counterparts when measured at their respective growth temperatures. Thus it seems likely that in *Tetrahymena* microsomes there are physical properties other than "viscosity" which are more directly related to the observed (in vivo) phase separation temperatures and which are under actual regulatory control.

It is not entirely clear from our present data how extensive the differences in lipid mobility are between the two membrane

fractions at their actual growth temperatures. It is readily apparent, however, that these differences are small compared to the changes produced in 39.5 °C lipids when measured at 15 °C. We presently interpret the multiexponential decay associated with the latter observation as indicative of probe segregation into phase separated lipid environments, a case which is supported by the microscopic observation of two distinct phases of microsomal membrane structure, in vivo, under the same conditions. These observations, along with the previously determined morphological alterations which occur as a consequence of rapid cooling, again serve to emphasize the extent of the compensatory changes in membrane properties which are brought about through temperature acclimation.

The results obtained from this study suggest that the detailed information gained through time-resolved fluorescence methods holds considerable promise for studying the dynamic properties of biological membranes. In order to extend our observations to purified intact membranes and membranes in vivo, we are currently developing picosecond laser methods which will circumvent the difficulties raised by the long data collection times required by current photon counting techniques.

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