From: MEMBRANE RECEPTORS, DYNAMICS, AND ENERGETICS
Edited by K. W. A. Wirtz
(Plenum Publishing Corporation, 1987)

TIME-RESOLVED FLUORESCENCE DEPOLARIZATION STUDIES OF PARINAROYL PHOSPHATIDYLCHOLINE IN TRITON X-100 MICELLES AND RAT SKELETAL MUSCLE MEMBRANES

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INTRODUCTION

Fluorescence anisotropy under constant illumination is a technique that provides information on the local order of cylindrical probes like 1,6-diphenyl-1,3,5-hexatriene (DPH) or cis/trans-parinaric acid in membrane vesicles, which as such can be considered as a macroscopically isotropic system. It was shown that the steady-state fluorescence anisotropy, at a time scale much longer than the average fluorescence lifetime, is connected to the second-rank order parameter for rod-shaped probe molecules in membranes [1,2].

Time-dependent fluorescence anisotropy measurements of cylindrical probes (mainly DPH) have been widely used in order to obtain dynamic and orientational order information on artificial and biological lipid bilayers [3-15] and on the interaction between proteins and membranes, e.g. cytochrome oxidase [16], M13 virus coat protein [17,18], apolipoprotein C-I [19] and blood clotting factor Va [20].

Considerable progress has been made in a theoretical description of fluorescence depolarization of cylindrical and other probes in membrane bilayers. The concepts of order parameter (second and fourth rank) and correlation function are needed to describe the orientational distribution and dynamics of the probe in the membrane. Several models have been advanced such as the cone model, in which the probe symmetry axis wobbles within a cone of certain semiangle [21-23] and the Gaussian model, which has been applied either to strong collision or diffusion problems [24,25]. A general expression of the fluorescence anisotropy decay on the basis of the Smoluchowski equation for hindered rotational diffusion has been derived showing general applicability to any particular distribution model [26]. Experimental verification was achieved for DPH in dimyristoyl-phosphatidylcholine (DMPC) or dipalmitoylphosphatidylcholine (DPPC) vesicles and for DPH in DMPC vesicles with α-lactalbumin attached at acidic pH [27]. A comprehensive and unified formalism encompassing several physical situations has been developed to describe time-dependent fluorescence depolarization in macromolecules and membranes [28].

Probes like DPH (and possibly parinaric acid) have the disadvantage that they have a nonnegligable orientational distribution in a plane perpendicular to the membrane normal [27]. Fluorescent parinaroyl phospholi-

pids are very appropriate probes for artificial and biological membrane structures, because the parinaric acyl chain of the lipid is uniquely oriented along the fatty acids of the membranes. The fluorescent lipid probe can be considered as a minimal perturbing agent. A DPH-phosphatidyl choline probe has also been designed [29]. The spectral properties of parinaric acid and its incorporation into artificial bilayers have been reported [30,31].

In this paper we describe the fluorescence anisotropy results obtained with parinaroyl phosphatidylcholine incorporated into micelles and rat skeletal muscle membranes (sarcolemma and sarcoplasmic reticulum). The lipid used was sn-2-cis-parinaroyl-phosphatidylcholine (PnA-PC) This probe was also used in vesicles and microsomal membranes by other workers [32-34].

The fluorescent lipid can be incorporated into the outer monolayer of the biological membranes without any disturbance by incubating the membrane preparations with phospholipid transfer proteins loaded with the particular lipid [35].

The interpretation of steady-state fluorescence anisotropy of probes in natural membranes has been reviewed by Zannoni et al. [25]. Schroeder [36] has reviewed fluorescence applications of a variety of probes incorporated into different membranes. The latter author also described the incorporation of parinaric acid into plasma membranes from rat liver and results from time-resolved fluorescence and steady-state fluorescence anisotropy [37]. The application of time-resolved fluorescence of lipid probes in biological membranes has been reported previously [4,12].

METHODOLOGY

The fluorescent lipid was synthesized as described previously [38]. Time-resolved fluorescence measurements were carried out using the laser excitation source and time-correlated single photon counting as described in detail elsewhere [39-41]. Data analysis has been described in detail by Vos et al. [42]. In the latter paper advantage of the utilization of a reference compound, in order to remove the color effect of the photomultiplier response, has been outlined. For parinaroyl fluorescence POPOP (p-bis[2-(5-phenyloxazolyl)]benzene) is a suitable reference compound (τ=1.3 ns). The excitation wavelength was 305 nm, the emission was viewed through a K45 band-pass filter (Balzers). All experiments were carried out at 20°C.

We will briefly summarize the main points of the data analysis of anisotropy decay. The two intensity components, $i_{//}(t)$ and $i_{\perp}(t)$, were analyzed according to the total fluorescence s(t) to yield the fluorescence lifetime components τ_i with amplitudes α_i:

$$s(t) = i_{//}(t) + 2i_{\perp}(t) = \sum_i \alpha_i \exp(-t/\tau_i).$$

For anisotropy decay $i_{//}(t)$ and $i_{\perp}(t)$ were fitted simultaneously to yield the parameters describing anisotropy decay r(t):

$$i_{//}(t) = s(t)/3\ [1 + 2r(t)]$$

$$i_{\perp}(t) = s(t)/3\ [1 - r(t)]$$

Details of this procedure and its associated advantage (preservation of Poissonian statistics and usual fitting criteria) have been outlined [27,42,43].

Anisotropy decay data were fitted according to various models: For Triton X-100 micelles:

$$r(t) = \beta_{int}exp(-t/\phi_{int}) + \beta_{mic}exp(-t/\phi_{mic})$$

where the subscript 'int' refers to internal motion and the subscript 'mic' to overall micellar rotation. The amplitude of the internal motion, expressed in the angle Θ_o, is given by:

$$\beta_{mic}/(\beta_{int} + \beta_{mic}) = [1/2cos\Theta_o(1 + cos\Theta_o)]^2$$

For the natural membranes we restrict ourselves to the cone model:

$$r(t) = (r_o - r_\infty)exp(-t/\phi) + r_\infty$$

where r_∞ is the residual anisotropy at the end of the fluorescence experiment (about 50 ns), ϕ is the relaxation time in the cone model.
Other models consisting of 1,2 or 3 exponentials and a constant anisotropy have been tested by Ameloot et al. [27].
The second-rank order parameter <P2> can be obtained from:

$$r_\infty/r_0 = \langle P_2 \rangle^2$$

The cone angle Θ_C, describing the range of acyl motion in the cone model is determined from:

$$\langle P_2 \rangle = 1/2cos\Theta_C(1 + cos\Theta_C)$$

The wobbling diffusion constant D_W, conceived as the rate of probe motion in the cone model, is calculated from:

$$D_W\phi(r_o - r_\infty)/r_o = f(cos\Theta_C) = f(x)$$

$$f(x) = -x^2(1+x^2)\left[ln\{(1+x)/2\}+(1-x)/2\right]/\{2(1-x)\}+(1-x)(6+8x-x^2-12x^3-7x^4)/24$$

Also the diffusion constant $D_\perp$ for rotation around an axis perpendicular to the probe symmetry axis can be evaluated from

$$D_\perp = (r_o - r_\infty)/(6r_o\phi)$$

$D_\perp$ is a model-independent parameter and is equal to the derivative of r(t) with respect to time at t=o.
It should be noted that $D_{//}$ cannot be obtained, since such rotation would not depolarize the fluorescence.

In the biological membranes the anisotropy shows an initial rapid decay followed by a subsequent gradual increase. The anisotropy decay can then be approximated to arise from two populations A and B characterized by different average lifetimes, correlation times and order:

$$r(t) = [r_A(t) + r_B(t)]/[s_A(t) + s_B(t)]$$

For example population A:

$$s_A(t) = exp(-t/\tau_A)$$

$$r_A(t) = s_A(t)[(r_o - r_\infty)exp(-t/\phi) + r_\infty]_A$$

where the subscript A under the square brackets denotes the parameters r_o, r_∞ and ϕ belonging to population A.

RESULTS AND DISCUSSION

We will present fluorescence anisotropy decay examples of PnA-PC in three different environments. The probe, dispersed in micelles, is located in a medium that is comparable to an apolar solvent as regard to polarity and fluidity. In biological membranes the parinaroyl lipid is embedded in lipid regions with or without cholesterol or in the vicinity of membrane proteins. In lipid extracts of biological membranes the probe is only in a lipid environment with the possible presence of cholesterol. The results are presented in three tables: Table 1 for fluorescence lifetimes, Table 2 for correlation times and order parameters and Table 3 for a limited population analysis of intact membranes. In the paper by Wirtz et al. some time-resolved fluorescence results are presented of PnA-PC and PnA-PI in egg PC vesicles and membranes from the electric organ of fish (Torpedo marmorata).

Triton X-100 Micelles

We have carried out this experiment in order to assess the average fluorescence lifetime of a parinaroyl lipid in an apolar environment. The fluorescence lifetimes are collected in Table 1. The lifetimes are shorter than those found in membranes or liposomes (see below). This phenomenon might be related to the lack of rigidity of the micellar environment. The correlation between rigidity and lifetimes was also noted by others [3,11,31]. On the other hand, the presence of water molecules in the vicinity of the probe, thus a polarity effect, cannot be ruled out.

The initial anisotropy amounted to 0.31, which is the same value as found for the parinaroyl lipids bound to transfer proteins [44]. Because of the small micellar size the anisotropy decayed to zero within the time scale of the experiment. In Figure 1 the initial fluorescence anisotropy decay of PnA-PC and the calculated one are shown. The best fit consisted of a biexponential function. The shorter correlation time of 2.1 ns reflects internal motion and the longer one of 7.8 ns micellar rotation. The latter correlation time is similar to the correlation times found by others using different probes and techniques [45,46]. The internal motion as observed here was not found earlier. From the preexponential factors, the amplitude of the motion can be estimated (see preceding section). The angle of internal motion amounted to 32°.

Sarcolemma and Sarcoplasmic Reticulum Membranes

The average fluorescence lifetime of the lipid probe is not drastically different in the membrane preparations (Table 1).

In both lipid extracts the anisotropy decay could be well represented by an exponential and a residual anisotropy. A typical example is shown in Figure 2.

In the intact membranes, both from sarcolemma and sarcoplasmic reticulum, the striking observation is that the time-dependent fluorescence anisotropy decays initially, but increases after having reached a minimum (Figure 3). The latter behavior has been noted also by Wolber and Hudson [18] and assigned to partitioning of probe molecules between membrane regions of different fluidity and ordening. While Wolber and Hudson simulated several examples, we have fitted the anisotropy data by an analysis in two populations. The shorter fluorescence lifetime component of the biexponential decay was linked to one class with a particular residual anisotropy and correlation time and the longer fluorescence lifetime to another class with different parameters.

Table 1. Fluorescence lifetimes of parinaroyl phosphatidylcholine in different media.

Medium	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$\bar{\tau}$ (ns)
Triton X-100	0.51	3.0	0.49	6.4	4.7
Sarcolemma	0.73	7.1	0.27	16.0	9.5
Sarcolemma lipid extract	0.57	5.0	0.43	11.4	7.7
Sarcoplasmic reticulum	0.78	4.8	0.22	12.2	6.4
Sarcoplasmic r. lipid extract	0.52	4.4	0.48	9.3	6.7

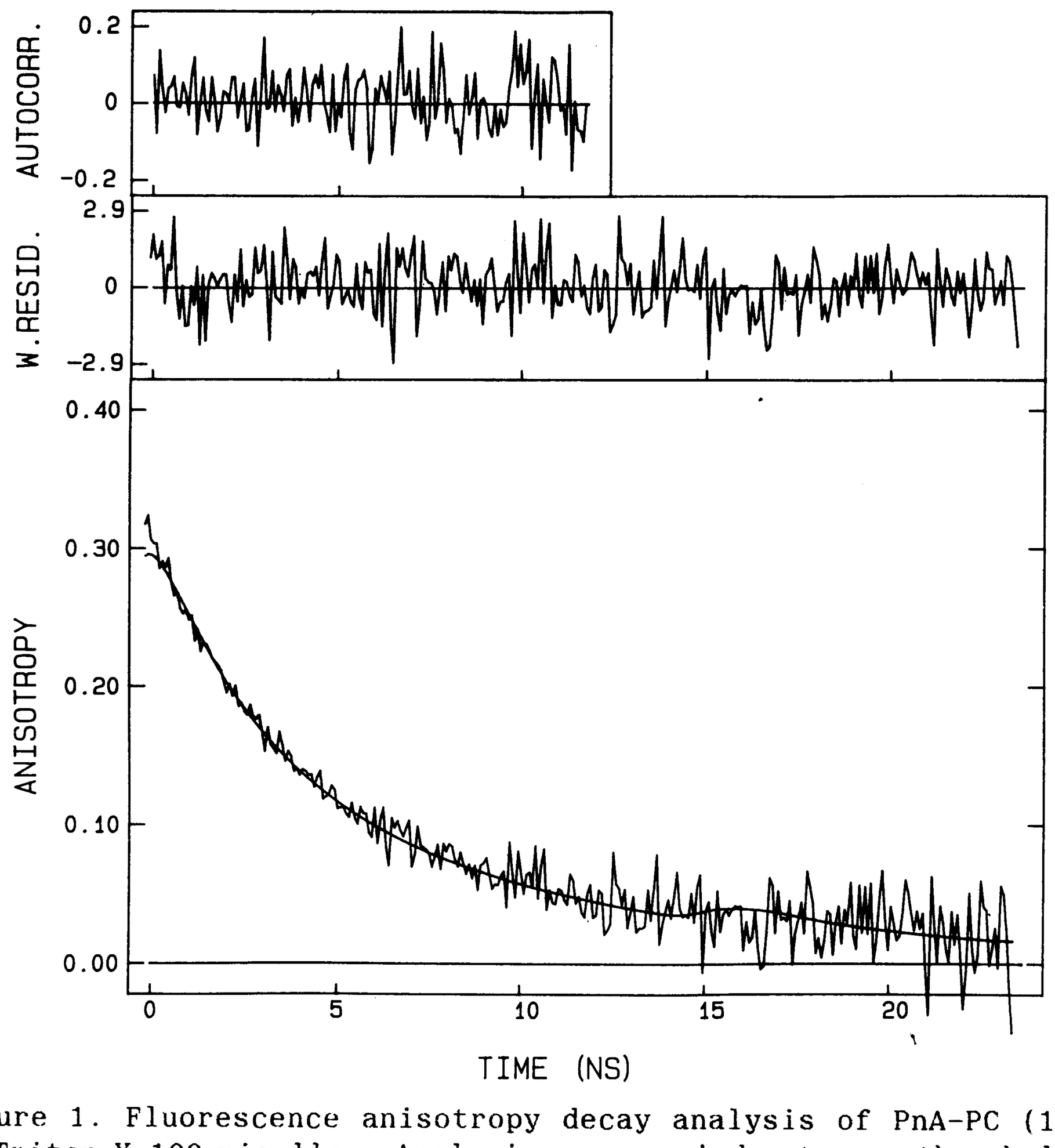

Figure 1. Fluorescence anisotropy decay analysis of PnA-PC (13μM) in Triton X-100 micelles. Analysis was carried out over the whole decay curves (1024 channels with 79 ps time spacing) Shown is a replot over a limited time range of the experimental anisotropy (noisy curve). The initial anisotropy is 0.31 and the fitted curve (smooth line) is a biexponential:0.12exp(-t/2.1) + 0.19exp(-t/7.8) with t in ns. The quality of the fit is indicated by the weighted residuals and autocorrelation function on top of the curves. Fitting criteria: χ^2=1.08 and Durbin-Watson parameter DW=2.1

Table 2. Fluorescence anisotropy decay parameters of parinaroyl phosphatidylcholine in lipid extracts of sarcolemma and sarcoplasmic reticulum membranes.

Membrane	ϕ (ns)	r_∞	$\langle P_2 \rangle$	Θ_C (o)	D_W (GHz)	$D_\perp$ (GHz)
Sarcolemma	1.4	0.091	0.56	48	0.114	0.084
Sarcoplasmic reticulum	1.9	0.068	0.51	51	0.085	0.064

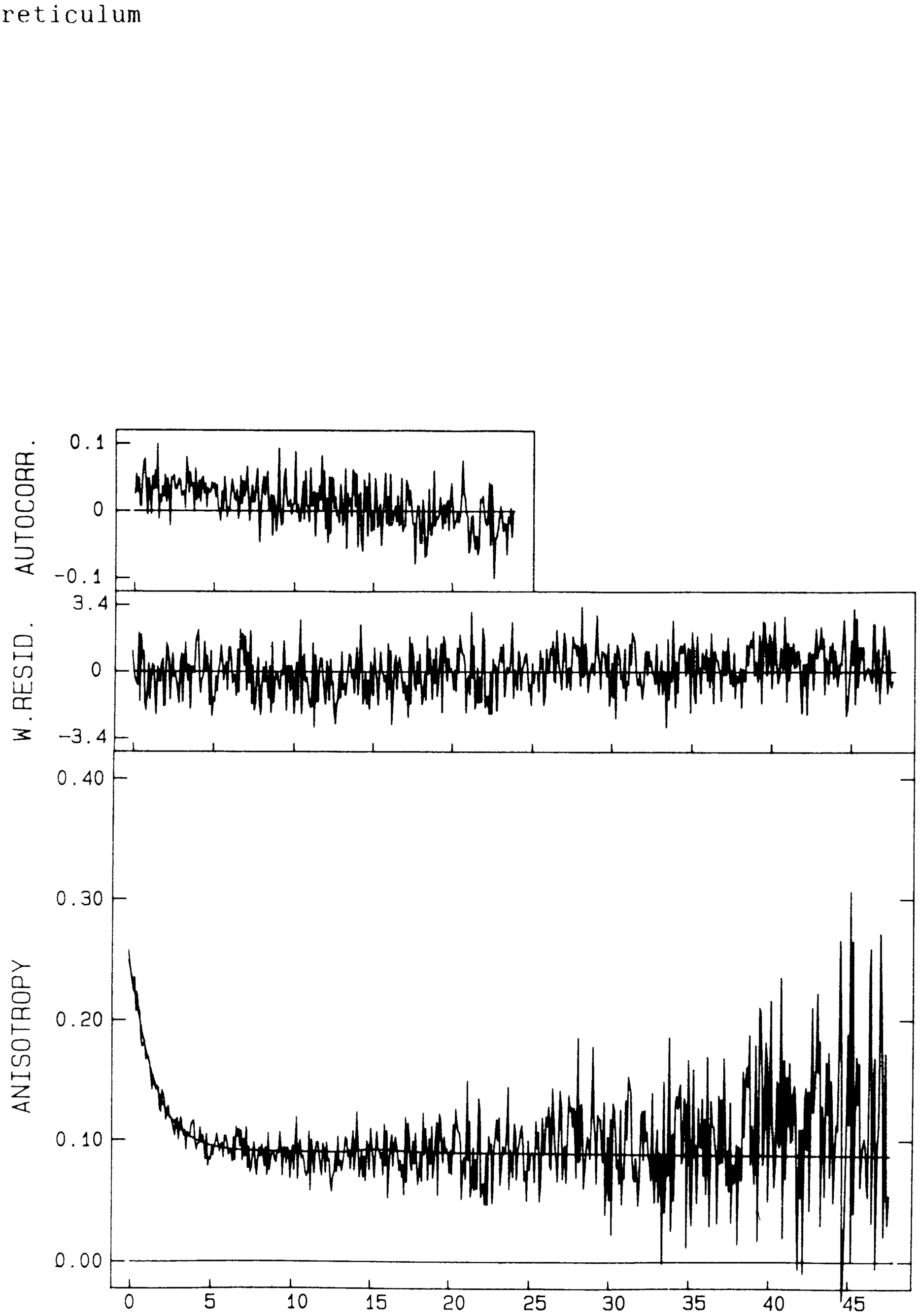

Figure 2. Fluorescence anisotropy decay analysis of PnA-PC (1.3μM) in liposomes extracted from sarcolemmal rat skeletal muscle membranes. Details as in Figure 1. The noisy curve is the experimental anisotropy and the smooth curve a fit according to the function $0.20\exp(-t/1.4) + 0.091$. Fitting criteria: χ^2=1.35 and DW=2.1

Table 3. Population analysis of the fluorescence anisotropy decay of parinaroyl phosphatidylcholine in intact sarcolemmal and sarcoplasmic reticulum membranes.

	Shorter lifetime[a)]			Longer lifetime[a)]		
Membrane	ϕ (ns)	r_∞	$\langle P_2 \rangle$	ϕ (ns)	r_∞	$\langle P_2 \rangle$
Sarcolemma	1.2	0.035	0.48	3.1	0.040	0.54
Sarcoplasmic reticulum	1.0	0.028	0.55	2.2	0.021	0.33

a) Listed in Table 1

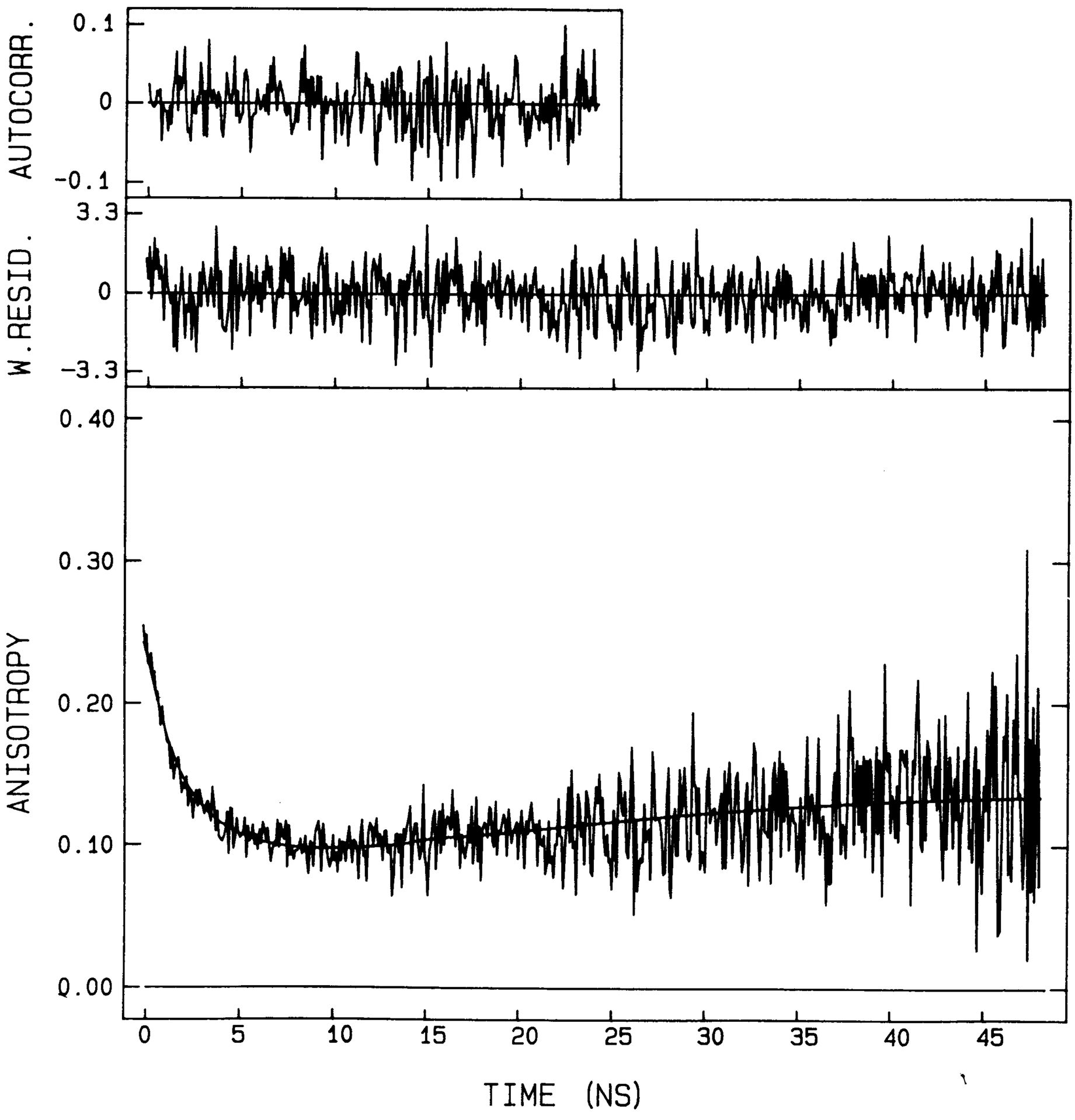

Figure 3. Fluorescence anisotropy decay analysis of PnA-PC (1.3μM) in intact sarcolemmal membranes from rat skeletal muscle. The noisy curve indicates experimental data, while the fitted decay function is represented by the smooth line. The fluorescence anisotropy clearly does not decay to a constant value, but rises after passage through a minimum. The figure shows the results obtained with a model in which two fluorescence lifetimes were coupled to two separate restricted rotational motions. The parameters are given in Table 3. Fitting criteria: χ^2=1.38 and DW=1.8.

This approach is a simplification since the probe fluorescence decay is already nonexponential in more homogeneous media like apolar solvents [47] and micelles (Table 1). The average fluorescence lifetime is probably composed of more components. Results of these analyses for the two membranes are given in Table 3. Based on the anisotropy results both coexisting phases must be considered as fluid, the one belonging to the shorter lifetime is more fluid than the other. Schroeder [37] used the fluorescence lifetimes of parinaric acid to assign the coexisting phases in rat liver membranes as fluid and solid. Our anisotropy data are in contrast with the distinction between fluid and solid phases, since neither probe exists in a gel type of environment at 20°C. Ellena et al. [48] have confirmed the latter conclusion by ^{31}P NMR spectroscopy of rod outer segments and sarcoplasmic reticulum membranes showing that at least 90% of the phospholipids are in the liquid-crystalline phase at 22°C. At most 10% of the phospholipids could be immobilized to the Ca^{2+}-ATPase in sarcoplasmic reticulum membranes.

Although slightly increasing anisotropy of DPH-probes in sarcoplasmic reticulum can be noticed in their figure, Stubbs et al. [12] analyzed the data in terms of a single wobbling in cone. A single wobbling-in-cone model gave poor fits for our experimental data. Because of the large number of parameters involved in the population analysis of a single anisotropy decay curve, the correlation between the various parameters is very high and firm conclusions about possible differences are not possible yet.

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